THE PHYSIOLOGY OF NITROGEN FIXATION IN TROPICAL GRAIN LEGUMES

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I. INTRODUCTION

Among the many factors that contribute to improving crop yields, increasing the availability of nitrogen at critical stages of plant growth is of great importance. For legume crops, biological nitrogen fixation through symbiosis with *Rhizobium* has an important role, especially in the tropics, in providing nitrogen for adequate vegetative growth and reproductive development while maintaining low production costs. In the tropics, grain legumes constitute the main source of protein, especially for populations of low income, and also represent important cash crops. Some species, such as cowpeas and groundnuts, are important summer crops, whereas lentils and chickpeas are grown during the cold season or in the highlands (Table 1).

Growth and seed yields of grain legumes depending on biological nitrogen fixation are a result of interactions between plant cultivars and *Rhizobium* strains which affect the intake, distribution, and utilization of both carbon and nitrogen within the plants. Therefore, seed yields can only be expected to increase with a comprehensive understanding of the physiology of the plant and the identification of the major factors limiting the rates and the duration of the processes related to biological nitrogen fixation because only then can the whole plant/ *Rhizobium* symbiosis be effectively manipulated and optimized.

II. CARBON UTILIZATION FOR NODULE FUNCTIONING

Biological nitrogen fixation requires high inputs of energy which, in symbiotic systems, are supplied by products derived from photosynthesis. The relationship between carbon and nitrogen metabolism has been extensively reviewed.[1-7]

A. Photosynthate Availability and Nitrogen Fixation

The importance of photosynthates in nitrogen fixation is related to their use for the generation of reducing power and ATP for the nitrogenase system, growth and maintenance of nodule cells, and supply of carbon skeletons, ATP, and reducing power for ammonia assimilation.[7] It must also be considered that in addition to the direct role of photosynthates in the metabolism of nitrogen fixation the consumption of carbohydrates by nodulated roots, which may spend up to 30 to 50% of the daily net photosynthates of the plant during periods of active growth and nitrogen assimilation, is very high and variations in this requirement can reflect directly in seed production (see Section III.B).[3]

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Table 1

MAJOR TROPICAL GRAIN LEGUMES AND THE ASSOCIATED *RHIZOBIUM* SPECIES

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Legumes species	Common names	Associated *Rhizobium* species
Arachis hypogea	Peanut, groundnut	*Bradyrhizobium* sp.
Cajanus cajan	Pigeon pea	*Bradyrhizobium* sp.
Cicer arietinum	Chickpea	*Rhizobium loti* and *Bradyrhizobium* sp.
Glycine max	Soybean	*B. japonicum*
Lens culinaris	Lentil	*R. leguminosarum* biovar. *viceae*
Phaseolus lunatus	Lima bean	*Bradyrhizobium* sp.
P. vulgaris	Drybean, common bean	*R. leguminosarum* biovar. *phaseoli*
Psophocarpus tetragonolobus	Winged bean	*Bradyrhiszobium* sp.
Vigna angularis	Adzuki bean	*Bradyrhizobium* sp.
V. mungo	Black gram	*Bradyrhizobium* sp.
V. radiata	Mung bean, green gram	*Bradyrhizobium* sp.
V. unguiculata	Cowpea	*Bradyrhizobium* sp.

The importance of carbohydrates was recognized long ago,[8,9] and many experiments have since been performed manipulating nodulated plants in order to investigate the relationship between photosynthesis and nitrogen fixation (Table 2). The following manipulations of the plant to increase the availability of photosynthates to the nodules have been shown to increase nitrogen fixation: enrichment of CO_2 around the plant canopy,[10-12] decrease in oxygen partial pressure in the aerial part to reduce photorespiration,[13] grafting of a second shoot onto a root stock,[14] a decrease in planting density,[15] an increase in light intensity,[16,17] and many other manipulations that can result in an increase in the availability of carbon to the nodules (Table 2).

On the other hand, shading or decreases in light intensity to complete darkness lead to decreases in the nitrogen fixation of *Phaseolus* beans,[18] pigeon peas,[19] and soybeans.[20-24] Reductions in total plant photosynthesis by removal of some leaves also decreases nitrogen fixation in soybeans and cowpeas,[16,22,24,25] whereas increases in the carbon flux to the nodules, by removing competitive sinks such as pods, increases the nitrogen fixation of soybeans (Table 2). Since in many of these experiments the principal short-term effect of altered carbon supply was on specific activity, it was concluded that the carbon supply limits the activity of nitrogenase.

However, the idea that the main limitation to the process of nitrogen fixation is carbon availability to the nodules began to be questioned after some experiments with *Phaseolus* beans[18] and soybeans[26,27] which demonstrated that the increase in nitrogenase activity following increases in photosynthate availability was related to greater nodule mass and not to increased nodule efficiency, indicating that nodule functioning was not limited by carbon.

In experiments where symbiotically grown soybeans were either fed with increased levels of mineral nitrogen or submitted to an atmosphere containing increased levels of CO_2, the results obtained showed that nodule development and function were not limited by photosynthate products in seedlings[28,29] or mature soybean plants[27] but by available nitrogen. The effect of grafting a second shoot to a root stock was later shown to be linked to physiological effects, possibly hormones, and it was found that increases in nitrogenase activity of soybeans only occurred following graftings of young scions and never with older shoots, even when they still had green leaves.[30] Considering the results obtained in experiments where the effect of light intensity was studied, it can be seen that in the majority of these studies (e.g., References 18, 22, and 31) the level of irradiance was either drastically reduced or the plants were grown in greenhouses where the light intensity was already lower than natural conditions. Light intensity required for photosynthesis is lower in C_3 than in C_4 plants and on

Table 2

EFFECTS OF PHOTOSYNTHATE SOURCE-SINK MANIPULATIONS ON NITROGEN FIXATION IN TROPICAL GRAIN LEGUMES

Treatment	Plant	Effect on N_2 fixation			Observations	Ref.
		Increase	No effect	Decrease		
Increase in pCO_2	Soybeans	X^a	X^b		[b]Seedling	10[a], 15[a], 26;[a], 28[a], 29[b]
		X^a	X^b		[a]Use of straw	11[a], 12[a], 26[a], 27[b]
	Mung beans		X			390
Depriving shoots of atmospheric CO_2	Soybeans			X		391
Carbon supply to the nodules	Soybeans	X				392
Decrease in photo-respiration by the decrease in pO_2	Soybeans	X				13
Stem ringing	Soybeans			X	50% Decrease in nitrogenase activity after 2 hr	22
	Phaseolus beans			X	Decrease after 24 hr	49
Increased planting density	Soybeans			X	Decrease in nitrogen fixed per plant	15
	Phaseolus beans	X^a		X^b	[a]Prostrated cultivar; [b]climbing cultivar	393
Grafting of a second shoot on a root stock	Soybeans	X^a	X^b		[a]Grafting of a young scion; [b]Grafting of a shoot with the same age	30
		X				14
Use of chemical inhibitor of photosynthesis	Phaseolus beans			X		394
Long days	Soybeans	X				145, 395
	Cowpeas	X				395
Decreasing light	Soybeans	$X^{a,b}$			[a]Shading in bright summer day; [b]18% shading	33[a], 34[b]
				X	Maintained nitrogenase activity for 72 hr after darkness + 7 days until activity zero was reached,	92
				X	but increased nodule efficiency	20
				X	from 20% shading until complete darkness	16, 21, 22, 24, 31, 34
	Phaseolus beans	X^a		X^b	[a]20—30% shading; [b]40% shading	36[a], 44[a], 44[b]
	Cowpea			X	Complete darkness	351
	Pigeon peas		X^a	X^b		19[b], 396[a]
Increased light intensity	Soybeans	$X^{a,b}$			[a]Supplemental light to lower leaves	16[a], 17[b], 22[b]

Table 2 (continued)
**EFFECTS OF PHOTOSYNTHATE SOURCE-SINK MANIPULATIONS ON
NITROGEN FIXATION IN TROPICAL GRAIN LEGUMES**

Treatment	Plant	Effect on N_2 fixation			Observations	Ref.
		Increase	No effect	Decrease		
			X[a]	X[b]	Mature plants; [b]seedlings	397
	Phaseolus beans	X				18
	Pigeon peas		X		No effect on seed nitrogen, but decreased nodule activity	396
Flower bud removal	Soybeans	.		X		145
Flower removal	Soybeans			X		17, 145
	Phaseolus beans			X		49
Partial or total pod removal	Soybeans	X				22
			X			16, 24, 165
				X		57, 145, 146
	Pigeon peas		X		No effect on the nitrogen accumulated, but increased nodule efficiency	396
Male sterile plant	Soybeans	X[a]	X[b]			55[b], 146[b], 398[a]
Partial leaf removal	Soybeans			X		16, 22, 24
	Phaseolus beans			X		49
	Cowpeas			X		25
	Pigeon peas		X		No effect on seed nitrogen, but decreased nodule activity	396
Detached nodules	Soybeans			X		148
			X		Activity maintained constant for 8 hr	399

Note: Superscripts relate effects on N_2 fixation and observations with appropriate reference.

bright days may not be a limiting factor, even in temperate regions.[32] Furthermore, photosynthesis in C_3 plants does not increase linearly with light intensity, and the rate of net CO_2 exchange varies considerably according to the level of irradiance.[32]

These conflicting results may just reflect different plant-growth conditions. Early reports[33] have already shown that shading soybean plants on very bright summer days could lead to an increase in nitrogen fixation and it was concluded that a very high carbon-to-nitrogen ratio (C:N) could be as detrimental to symbiosis as a low ratio. In the same way, reducing the light intensity by 20% on a bright sunny day after 3 days without precipitation did not inhibit nitrogen fixation.[34] The beneficial effect of shading under these conditions was attributed to the prevention of photoinhibition of photosynthesis caused by a disruption of the photosynthetic apparatus by an excess of energy[35] which indirectly affected the nitrogen fixation.

Shading effects are important under tropical conditions because some grain legumes such as *Phaseolus* beans and cowpeas are usually grown in association with other plant species (maize, sugar cane, sorghum, and cassava) and therefore become shaded. In experiments performed in Colombia[36] and Brazil,[37] both nodulation and nitrogenase activity were greater in associated cultures than in monocultures. The beneficial effect of shading in this system

was related to smaller variations in soil temperature and moisture,[38] which are probably two of the most serious problems limiting nitrogen fixation under tropical conditions.[39] High temperatures and low humidity can directly inhibit the process of nitrogen fixation,[40-42] or they can adversely affect plant photosynthesis due to stomata closure.[43]

Recent results from the present authors' institute[44] also demonstrated that when *Phaseolus* bean plants were lightly shaded (75% full light) there was an increase, in relation to unshaded plants, in nitrogenase activity, total nitrogen transported in the xylem sap, and total nitrogen accumulated in the shoot tissue (Table 3). The stimulatory effect was caused by a decrease in maximum diurnal temperature in the shaded pots (Figure 1). However, when the plants were heavily shaded (40% of shading), the beneficial effect of decreasing rhizosphere temperature was not enough to compensate for the lower photosynthetic activity and as a result, nitrogen fixation was decreased (Figure 1). It is also interesting to note that the specific activity of nodules of soybeans[20] and *Phaseolus* beans[44] was increased by shading under tropical conditions, thus indicating that the efficiency in carbon utilization by the nodules was probably greater than in plants grown at full light.

Although there are many reports on the prejudicial effect of leaf removal on nitrogen fixation (which simulates attack by pests and diseases), the effects are not all negative (Table 2) since plants may improve the photosynthetic efficiency by increasing the rate of CO_2 exchange[45] and also the leaf area of the remaining leaves.[46] Partial leaf removal in soybeans,[47] pigeon peas,[48] and *Phaseolus* beans[49] did not result in proportional decreases in seed yield and nitrogen fixation. Additionally, it has also been shown that partial leaf removal delays the senescence of the remaining leaves,[50,51] which may be related to the delay in nodule senescence observed after leaf removal,[49] possibly induced by phytohormones.

Increased planting density will lead to the shading of lower leaves supplying the nodules[52] and consequently should lead to a decrease in nitrogen-fixation rates. However, although in soybeans the nitrogen fixation per plant decreased when the number of plants per hectare increased,[15] in field conditions in Colombia, *Phaseolus* bean nodules competed well for available carbohydrates at the higher plant densities, and nitrogen fixation was not altered.[53] Planting density on its own, however, should be used with caution when comparing species or genotypes within species. Leaf-area index is probably the most important characteristic for such comparisons, although it is seldom measured in field studies.

If the chemical basis for the relationship between photosynthesis and nitrogen fixation is carbohydrate content, then carbon availability in nodules should be related to nitrogen fixation. However, experiments have shown that this relationship depends on the particular carbohydrate analyzed. In *Phaseolus* beans, there was a strong positive correlation between the total carbohydrate content of nodules and the nitrogenase activity for all harvests prior to flowering.[54] However, in soybeans, the total sugar plus starch content of nodules,[4] as well as the total carbohydrate content of nodulated roots,[55] was not related to the nitrogenase activity.

Under carbon-limited conditions, significant correlations were found between nitrogen-fixation rates and sucrose or pinitol concentrations in nodules of soybeans.[4,31,56] Indeterminate cultivars of soybeans[57,58] and *Phaseolus* beans[59] showed higher rates of nitrogen fixation than determinate cultivars. This has been attributed, at least in the case of *Phaseolus* bean plants, to differences in the composition of carbohydrates in nodules. Although the plant roots of bush- and climbing-bean cultivars are similar in total carbohydrate contents, the climbing cultivars appear to transfer a greater proportion of their nonstructural carbohydrates to the nodules, sometimes as much as 100-fold, which may lead to the much greater rates of nitrogenase activity observed in these cultivars.[59] In soybeans, on the other hand, a comparison of the data obtained in ^{14}C-labeling experiments shows that indeterminate soybean cultivars usually translocate to the nodules from 0.4 to 2% of the total photosynthate produced,[60] whereas a determinate cultivar transported from 4 to 5% of the total photosynthate

Table 3
EFFECT OF SHADING ON NODULATION, NITROGENASE ACTIVITY, NITROGEN TRANSPORT, AND TOTAL SHOOT NITROGEN OF *PHASEOLUS* BEANS CV. NEGRO ARGEL INOCULATED WITH THE STRAIN CO5; MEANS OF EIGHT REPLICATES[44]

Treatments	Nodule dry weight (mg · plant⁻¹)	Nitrogenase activity		N transport in the xylem sap		Total N in shoot (mg · plant⁻¹)
		Total (μmol · plant⁻¹ · h⁻¹)	Specific (μmol · g nod⁻¹ · hr⁻¹)	Total N (nmol N · plant⁻¹ · min⁻¹)	% of N as ureides	
25 Days After Emergence						
Full light	119a	7.9b	66.7c	—	—	19.8b
Shading of 25%	115a	9.1b	78.4b	—	—	24.5a
Shading of 40%	122a	17.4a	146.4a	—	—	23.3ab
35 Days After Emergence						
Full light	283a	10.5a	37.2c	94.8b	78.8b	38.2b
Shading of 25%	244ab	10.9a	47.0b	149.0a	84.8a	50.3a
Shading of 40%	160b	11.5a	71.1a	89.8b	81.3a	35.0b

Note: Numbers followed by the same letter are not significantly different at $p = 0.05$.
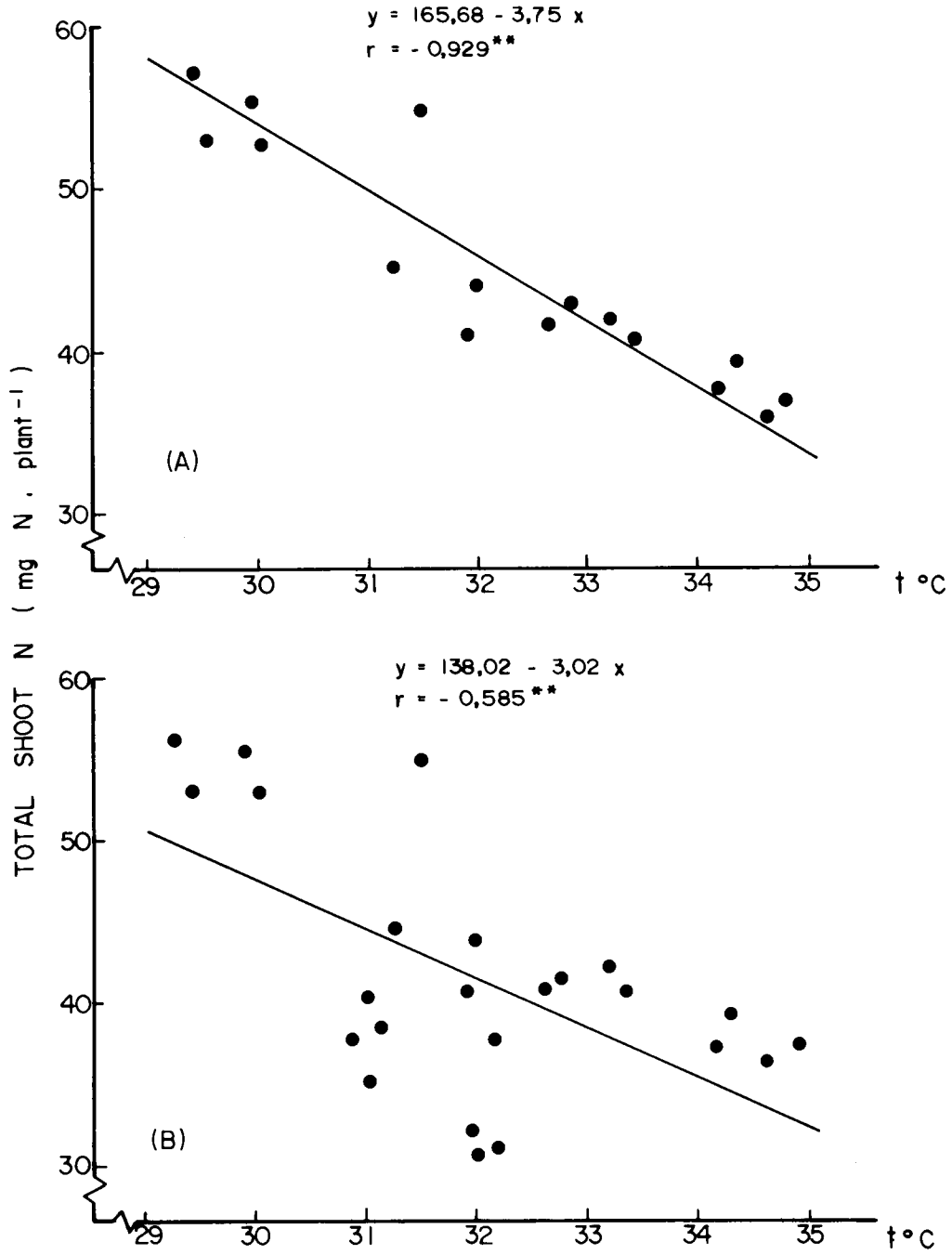
FIGURE 1.   Relationship between (x) mean maximum diurnal root temperatures during the growth cycle of *Phaseolus* bean cv. Negro Argel/*Rhizobium* strain CO-5 and (y) total shoot nitrogen (at 35 days after germination). (A) data related to pots submitted to full light or 25% shading; (B) data related to pots submitted to full light, 25, or 40% shading. (Modified from Hungria, M., Thomas, R. J., and Döbereiner, J., *Pesqui. Agropecu. Bras.*, 20, 1143, 1985.)

during vegetative and reproductive developments.[61] As nodule dry weight is not greater in indeterminate cultivars, it was concluded that determinate cultivars should have higher specific nitrogenase activity unless the growth habit of the plant could be responsible for big changes in the carbon composition or carbon-utilization efficiency of the nodules. This area requires further investigation.

B. Carbon Metabolism in Nodules

Although a large number of reports have investigated the relationship between the carbon and nitrogen metabolisms of nodules and/or quantified the flux of photosynthates to nodules, very little is known about the metabolism of carbon in the various compartments of the nodules.

Sucrose is the major photosynthetic product transported by the phloem,[62-64] but the initial processes of sucrose metabolism in nodules are not yet clear. Sucrose, glucose, and fructose have all been found in nodules of soybeans,[65,66] lupins,[67] *Phaseolus* beans,[68] white clover,[69] and *Vicia faba*,[66,70] as well as some "unusual" compounds such as cyclitols (6-carbon-ring compounds) reported in soybean nodules.[63,64,71] While it is generally assumed that *Rhizobium* bacteroids have limited capability for direct utilization of carbohydrates (see reviews of Stowers[72] and Streeter and Salminen[64]), recent work reported rapid reconstitution of hexoses, from labeled precursors, in soybean bacteroids.[73]

Although many potential carbon substrates for nodule metabolism are available in the host-cell cytosol, it is now generally agreed that the bacteroids are able to use only some of these compounds as carbon and energy sources, namely, organic acids, aldehydes, or alcohols.[72,74] Organic acids support the highest rates of respiration and are the most effective substrates for nitrogen fixation.[63,74-76] They can be metabolized even at the low flux and concentration of oxygen maintained by leghemoglobin.[66,77] But the role of the various carbon sources for nodule fuction has still not been clarified,[78] and recently a role has been suggested for glutamate as a carbon substrate for bacteroids.[79]

Infected cells contain the enzymes of the Embden-Meyerhof-Parnas (EMP) pathway and the pentose phosphate (PP) pathway (see review of Stowers[72]), but the presence of the PP pathway in *Rhizobium japonicum* bacteroids is still questionable.[64] There is some indication that the metabolism in the nodule cytosol can proceed via the EMP and PP pathways and the evidence suggests that a complete tricarboxylic acid (TCA) cycle is required for effective nitrogen fixation in soybean.[80]

Nodule cytosol also contains enzymes of anaerobic carbon metabolism (see reviews of LaRue et al.[81] and Stowers[72]), which may be an important source of energy for nitrogen fixation because of the very low partial pressure of oxygen found in legume nodules.[82]

Although technically difficult because of the diversity of nodule tissue, precise elucidation of the sources and mechanisms of carbon utilization by the nodule tissues is required, as this may lead to genetic manipulations which envisage increases in carbon availability for nitrogen fixation.

C. Stored Carbon Compounds

Although experiments have shown that photosynthates are rapidly metabolized on arrival in nodules[70] and that nodule respiration is heavily dependent on recently assimilated carbon,[66] the presence of stored carbohydrate can also affect the correlation between carbohydrate contents of nodules and nitrogen fixation.

Soybean bacteroids accumulate large amounts of poly-β-hydroxy-butyrate (PHB),[83-85] but although it may represent 50% of the bacteroid volume,[86] the role of this compound in bacteroid metabolism has not yet been determined. It has been suggested that this compound may be important under light-limited conditions or conditions in which photosynthesis is otherwise restricted.[76] However, in a study on the loss of nitrogen-fixing activity of soybean

bacteroids following dark periods of up to 16 days or after the detachment of nodules from the parent roots, it was shown that the decline in activity was not correlated with the disappearance of PHB.[84] In both situations, no significant reduction in the concentration of PHB reserves occurred during the period in which nitrogenase activity was declining, even though enzymes for metabolism of PHB were present in the bacteroids. The reported values for nitrogenase activity in these experiments were, however, very low (2 μmol C_2H_4/g.h), making it difficult to detect the small depletions of the polymer in the presence of such a large quantity in the bacteroids. Other experiments have shown a decline in PHB with increased respiration and nitrogen fixation of lupin, pea, and V. faba bacteroids.[87,88]

Bacteroids of other legume species such as Phaseolus beans, cowpeas, and pigeon peas, however, contain very little PHB. Starch is another form of stored carbon in nodules which can be used for nodule activity under conditions of limited photosynthate supply or during the dark periods.[89,90] In Phaseolus beans, 5 days after the irradiance level was reduced, nodule starch disappeared completely.[18] The starch accumulated in Phaseolus bean nodules is probably the best explanation for the retarded response of such plants to stem ringing (nitrogenase activity began to fall only 24 hr after the treatment was imposed.)[49,91]

Other forms of storage compounds have also been reported[76] and, additionally, nodules may also use storage compounds from the plant. In intact soybean plants maintained in continuous dark conditions but at a favorable temperature, nitrogenase activity was sustained for 72 hrs and analysis of the shoot starch suggested that shoot tissues may serve as a primary source of carbohydrate for nodule activity under stress conditions.[92]

It seems, then, reasonable to assume that nodules rely on both the recently imported photosynthates and on stored carbon compounds,[71] and it is possible that some compounds are preferentially used to generate energy and reducing power for nitrogen fixation.

D. Anapleurotic Carbon Assimilation in Nodules

Besides photosynthates and stored compounds, the anapleurotic reactions occurring in the nodules may play an important role in the carbon economy of symbiotic systems. The idea that nodules are able to fix CO_2 was put forward in 1960 when it was observed that nodulated legumes grown in nutrient solution without CO_2 yielded significantly less than plants aerated with air plus approximately 4% CO_2.[93]

A definitive relationship between the phosphoenol pyruvate (PEP) carboxylase and nitrogen-fixation activity was first established in V. faba, where the PEP carboxylase activity was found to be 50 times higher in nodules in relation to roots.[94] High activities of PEP carboxylase were also found in nodule extracts of many temperate[95-97] and tropical legumes.[97-101]

In the amide-producing nodules of temperate legumes (Section V.A), the oxaloacetate produced by PEP carboxylase activity is metabolized into glutamate, aspartate, and asparagine as shown by supplying detached nodules of V. faba with an atmosphere containing $^{14}CO_2$.[94] Although some of the reported PEP carboxylase activities are able to cope with all CO_2 produced in the nodules,[95] usually only about 30% of respiratory CO_2 is fixed.[95,102] This low efficiency suggests that either CO_2 is not converted into bicarbonate at a sufficiently fast rate or that PEP carboxylase activity is limited by other factors such as low pH or low availability of PEP.[103] Some reported values of PEP carboxylase are also very low and cannot possibly recycle all produced CO_2 (see review of Minchin et al.[5]).

In tropical grain legumes which assimilate fixed nitrogen via ureides, the role of PEP carboxylase is still little known since oxaloacetate is not used for ureide biosynthesis,[7] but studies with labeled carbon indicated an indirect role of CO_2 fixation in the amino acid synthesis in soybeans.[99] The CO_2 fixation rates were, however, too high to be related only to the supply of carbon skeletons to amino acid synthesis. It was then postulated that in ureide-producing legumes, the CO_2 fixation constitutes an important source of respiratory

substrates,[99] especially under oxygen-limited conditions, where the malate produced via PEP carboxylase may become an important source of energy.[103] The anapleurotic recovery of metabolic CO_2 via PEP carboxylase in ureide-producing nodules may differ from that of amide-producing nodules in leading to glycine production which may then be used in the purine synthesis.[101,104]

Another possible role for PEP carboxylase is related to pH control of nodulated roots in conjunction with malate dehydrogenase and malic enzyme as occurs in other plant cells.[105] When leguminous plants are fixing nitrogen, an excess of cation uptake occurs,[106] and the plants must adjust their root metabolism to accomodate charge balance in upward-flowing xylem sap.[103] The continuous production of ammonia suggests that the pH control is very important, and about 75% of excess cations are neutralized by malate,[107] which can be produced by PEP carboxylase in roots or nodules and transported to the shoot in the xylem to maintain charge balance. Although the PEP is considered the main acceptor of CO_2 for dark fixation in nodules, the role of alternative acceptors cannot be discounted. Malonate has been suggested as an alternative acceptor of CO_2, explaining the metabolism of malonate and the role of malic enzyme in legume nodules.[108] Further and more detailed investigation on the anapleurotic carbon-assimilation processes in nodules is required for a better understanding of their role in nodule functioning.

E. Diurnal Variation in Nodule Functioning

Diurnal changes in nitrogenase activity have usually been attributed to diurnal changes in light intensity and, consequently, photosynthate supply.[5,15,109] However, as discussed previously, differences between species and even cultivars in the carbohydrate composition of nodules, as well as the differences in carbon storages in the nodules and the utilization of carbohydrates from other parts of the plant, can make this picture very confused. For example, experiments with lupins have failed to show any marked diurnal change in the rate of nitrogenase activity, apparently due to the fact that large carbohydrate reserves are accumulated in nodules and/or tap roots during the photoperiod and then utilized during the subsequent dark period.[110] In addition, differences in plant-growth conditions, such as low light intensity and adverse temperature and humidity, as well as plant-growth stage, can help to explain the diversity of diurnal patterns of nitrogenase activity found in many legume species (see also the review of Minchin et al.[5]).

Recent experiments have suggested that the diurnal changes in nitrogenase activity may be linked to fluctuations in temperature rather than in light intensity[17,27,111] because high temperatures not only can alter the activity of enzymes, but also can increase the carbon usage by nodules by altering the respiration rates. Moreover, data on diurnal patterns of nitrogen fixation have been collected from experiments using acetylene reduction, which reflects the overall activity of nitrogenase and does not necessarily reflect the rate of nitrogen incorporation into the plants, as has been demonstrated in cowpeas.[112] In the latter case, the actual rates of nitrogen fixation showed no diurnal variation, whereas the diurnal variation of nitrogenase activity was due to changes in hydrogen evolution, which is discussed at length in Section IV.

Recent studies on the existence of a gaseous diffusion barrier related to the oxygen-protection mechanisms in legume nodules[113] have raised the question as to whether nodule activity is limited by carbon substrate supply or by the oxygen supply. Long-term experiments on the manipulation of external oxygen supply to nodules have produced data which corroborate the role of carbon supply in limiting nodule activity. Short-term experiments, however, have indicated that nodule control over the oxygen-diffusion barrier may not change fast enough to cope with short-term increases in carbon supply, therefore, oxygen becomes a limiting factor during daytime when intense photosynthetic activity leads to production of surplus carbon.[114]

F. Photosynthate Availability and Nodule Ontogeny

The seasonal pattern of nitrogen fixation has always been regarded as a problem of carbon nutrition of the nodules.[22,115-117] Photosynthesis is not essential for nodule initiation, especially in large-seeded legumes, for example, both soybeans and *Phaseolus* beans are capable of forming nodules in conditions of complete darkness at the expense of the cotyledonary reserves.[118]

Seedlings, however, have as a priority the development of the photosynthetic apparatus, and nodulated plants need to synchronize the onset of photosynthesis and nitrogen fixation with the exhaustion of carbon and nitrogen reserves of the cotyledons.[119] In field peas, there is synchronous development of nodules and leaves, and nitrogen fixation begins before the carbon store in the cotyledons is depleted.[120] However, in soybeans[121,122] and *Phaseolus* beans,[91,101,123] a period of nitrogen stress has often been observed in plants grown in nitrogen-deficient conditions, before nodules begin to fix nitrogen. This fact seems to be a problem of epigeal legume species, where cotyledon nitrogen is used for nodule growth and the first leaves have a much greater area and contain a larger proportion of nitrogen reserves from the seeds.[119]

Preliminary results obtained at the present authors' institute[124] have, however, shown that nitrogenase activity could be detected in *Phaseolus* bean nodules (an epigeal plant) as early as 10 days after emergence, and the percentage of nitrogen in the nodules increased from 3.2 to 6.5% at 18 days after emergence, while simultaneously the plants exhibited the classical symptoms of nitrogen deficiency. The plants became green 2 days later and the percentage of nitrogen in nodules fell to 4.5%,[124] thus indicating that the problem of nitrogen stress may be linked to the transport of fixed nitrogen, possibly because the xylem vessels between nodules and the host plant were not yet connected. These results agree with a report published by Joshi in 1920.[125]

The establishment of the first nodules is likely to have a complicated mechanism of regulation and cannot be regarded solely as a problem of carbon and nitrogen nutrition because early nodulation can suppress later infection.[126,127] Early nodulation also uses up reserves which thus restricts shoot and root growth,[119] and, to prevent these limitations, the plant must possess control mechanisms, e.g., the production of nodulation inhibitors such as those found in the cotyledons of peas[128,129] and *V. faba*.[130]

On the other hand, cotyledons of soybeans[118,131] and *Phaseolus* beans[118,132,133] have some products that stimulate nodulation, which may be either hormonal or nutritional in nature. Addition of extracts of cotyledons markedly increased the number of nodules of *Phaseolus* plants.[134] There is some evidence that cytokinins[135] and auxins[136] may stimulate nodulation, whereas gibberelins and abscisic acid[137,138] may inhibit it. However, the nature of the compounds which control nodulation is still undetermined.

In *Phaseolus* beans, some *Rhizobium* strains recently isolated are capable of fixing nitrogen much earlier than the commonly used ones and promote nitrogen gains to the shoot earlier during the plant-growth cycle.[139] These strains seem to affect the time at which export of fixed nitrogen from nodules begins, thus eliminating the period of nitrogen deficiency which can restrict the initial growth of legumes in nitrogen-deficient soils (Figure 2).

After the initial phase of plant growth and nodule establishment, both photosynthesis and nitrogen fixation increase greatly. Thereafter, the correlation between them is not strictly constant throughout growth. In fact, relatively more nitrogen than carbon is assimilated during the vegetative and flowering stage and less nitrogen than carbon during pod-fill stage.[3,5,115]

Some factor(s) associated with the presence of flowers appears to promote nitrogen fixation during the early part of the reproductive period. The stimulus of flowering suggests a hormonal effect and may explain why indeterminate cultivars have a longer duration of maximum nitrogen-fixing activity.[57-59] The presumption that the competition between nodules
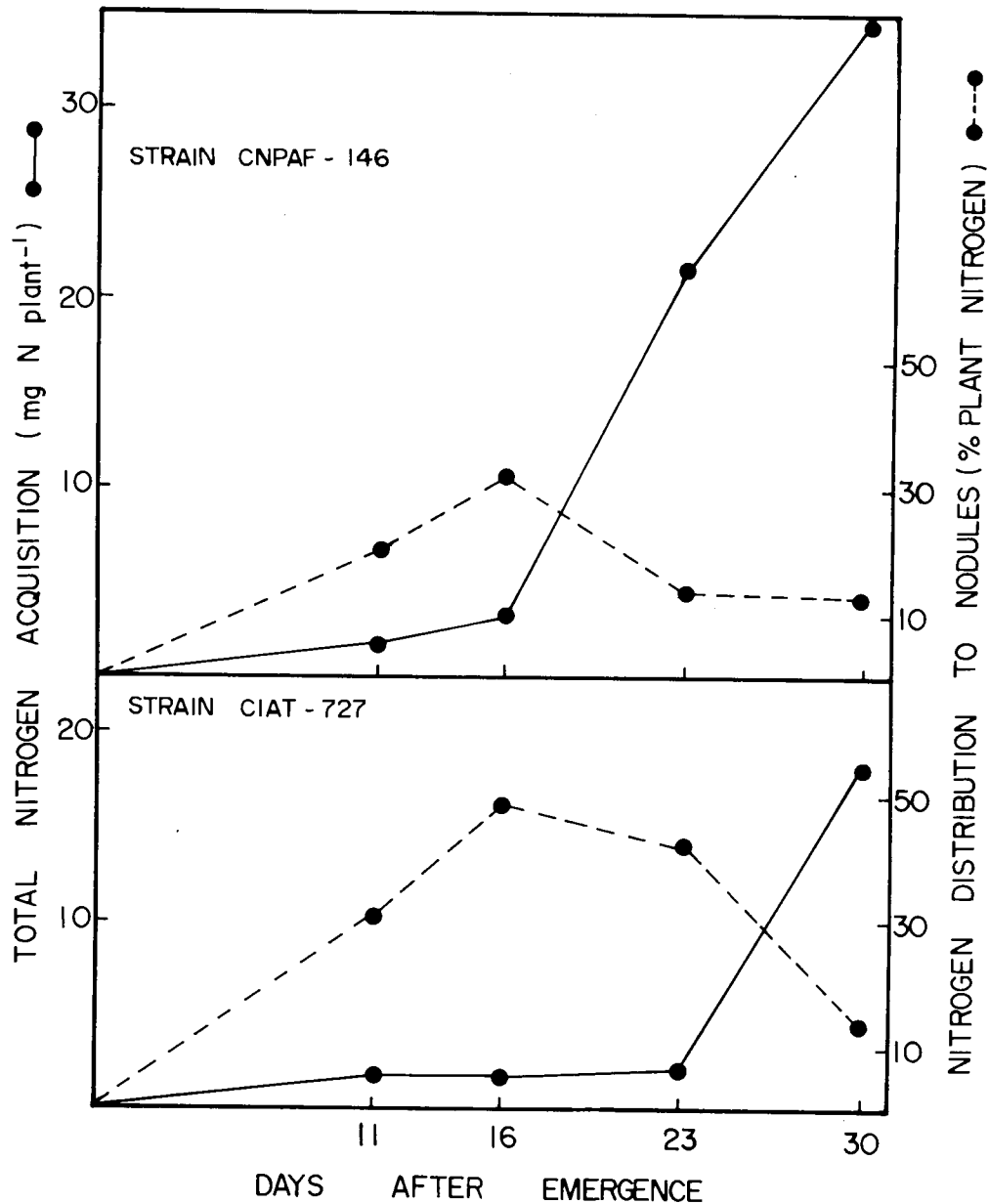
FIGURE 2. Effect of *Rhizobium* strain on nitrogen acquisition (nitrogen in leaves + stem + roots + cotyledons − nitrogen in seeds) and on nitrogen distribution to nodules of *Phaseolus* beans at the initial stage of growth under nitrogen-free culture conditions. (From Barradas, C. A. and Hungria, M., presented at 17th Reunião Brasileira de Fertilidade do Solo, Londrina, Brazil, 1986 [Abstr. 45].)

and developing fruits for the available carbon would decrease the nitrogenase activity came from the observation that there is a decline in nitrogenase activity during the seed-filling stage.[22,23,55,140] When the reproductive load was reduced by removal of some flowers or pods, the nitrogenase activity was then prolonged and nodule senescence delayed.[22,24,141-143] In contrast, other experiments have demonstrated that removal of flowers or pods to retain the plants in a vegetative stage, or even the use of male sterile plants, has not prolonged nitrogenase activity and sometimes even caused a faster decline in nitro-

gen fixation and accelerated nodule senescence.[30,49,55,144-146] It has also been observed that the fall in nitrogenase activity can occur later than the seed-filling stage, and so the decrease in nitrogen fixation cannot be attributed only to a competition for the available carbon.[57,146-148]

In relation to the mechanism controlling plant photosynthesis, there is a large body of evidence which supports the theory of assimilate control (see review of Neales and Incoll[149]). In soybeans[150,151] and *Phaseolus* beans,[152] high source/sink ratios were found to be associated with low rates of photosynthesis. Applying the theory of assimilate control to nitrogen fixation would imply that when flowers or pods are removed the decrease in the sink demand for nitrogen will consequently decrease nitrogenase activity, which, as mentioned before, has already been observed. However, later in seed development it is important to consider the influence of pod size on competition for assimilates[153,154] and to point out that perhaps the theory that competition for carbon is responsible for the decrease in nitrogenase activity should be reviewed.

Nodule longevity is closely associated with the growth habit of the host plant and, in general, annual herbaceous plants have nodules with a short life span.[155] The effects of different *Rhizobium* strains on nodule senescence still need to be determined. Premature nodule senescence has been demonstrated in ineffective *Rhizobium* strains.[156] In *Phaseolus* beans, preliminary data showed that both the plant cultivar and the *Rhizobium* strain affected the rate of nodule senescence.[157] Furthermore, some symbiotic combinations with not necessarily inefficient strains can produce nodules that are hard and green since formation[91,158] and show no nitrogenase activity. These nodules usually represent 20 to 30% of the total number of nodules in *Phaseolus* beans grown in Africa.[159] The waste of carbon and nitrogen in such nodules, which probably have the same sink capacity for nutrients as the active pink ones — hence, they reach the same size — is a problem to be further investigated.

Factors such as continuously active meristematic areas, an extensively developed vascular system, the prevalence of large numbers of noninfected cells, and the presence of well-defined protective cortical sclerenchyma were all associated with the longevity of nodules.[155] However, when the process of nodule senescence begins, it is irreversible. The visual changes in effective nodules consist of a color change from red to green and a wrinkling of the surface followed by the appearance of a soft texture and brown color.[155] In nodules produced by ineffective strains, however, the lack of leghemoglobin makes the senescence difficult to evaluate. Endogenously, the loss of nitrogenase activity and leghemoglobin, as well as cell "disorganization", are always observed during the onset of nodule senescence. The reasons for the changes in senesced nodules are still not clear (see review of Sutton[160]).

The relationship between the lack of photosynthesis and nodule senescence, however, was suggested long ago,[161] and a decrease in nodule starch content with senescence was observed.[162] Effective nodules senesce and turn green after shading,[163] and the effect can be partially overcome by carbon supply.[155] The lack of photosynthates could also lead to a defective vascular system and so accelerate the nodule senescence.[164]

At first, it was postulated that with the decline of photosynthesis during the plant-growth cycle there is an acceleration of nodule senescence[1,16,117] due to the presence of active sinks within the plants.[61,141,142,164] However, when the plants were maintained in the vegetative stage, with greater carbon availability to the nodules, the senescence process was not delayed.[49,165] Furthermore, when the potential for nitrogen fixation is sufficient to sustain high yields as occurred in *V. faba* grown under temperate conditions, shading can even delay nodule senescence.[166] In addition, the beginning of nodule senescence in cowpeas[117] and *Phaseolus* beans[91] was shown to occur long before the fruits had commenced to act as significant sinks for photosynthates.

In soybeans, the decline in the carbohydrate content of nodules does not precede the period of decay of the nodules, and senesced nodules still retain substantial quantities of

carbohydrates,[167] as was also demonstrated in some experiments that investigated the allocation of ^{14}C to the nodules during the growth cycle.[168] It is interesting to observe that even a moderate increase in the level of PHB in the bacteroids has been observed just prior to, or at the same time as, nodule senescence.[140]

Plant-growth conditions may also regulate nodule senescence by changing the sink activity of nodules. This is suggested by the results obtained with soybeans, where ^{14}C-labeled carbon in nodules at the pod-filling stage represented 6.2% of total plant carbon, whereas in water-stressed plants this percentage fell to 1,[168] with a decrease in the energy supply to nitrogen fixation. There is no doubt about the influence of temperature and humidity on nodule senescence (see revision of Sutton[160]), and under tropical conditions these two environmental factors are very important.

For example, temperature was found to play an important role in determining the duration of nodule activity of chickpeas.[169] Growth in a hot environment not only retarded and reduced early nodulation and decreased nitrogenase activity, but also drastically hastened nodule senescence resulting in reduced seed yields,[169,170] and although the addition of low doses of mineral nitrogen stimulated nodulation, nodule senescence was accelerated even more. Similar studies with cowpeas have shown that the detrimental effect of a hot environment in the duration of nodule activity involved hastened nodule senescence, limited production of secondary nodule population,[171] and resulted in very inefficient carbon utilization for nodule functioning.[172]

Another situation possibility related to carbon availability is that of *Phaseolus* beans grown in greenhouses where nitrogen-fixing activity begins earlier, reaches maximum activity earlier, and maintains it for a longer period than plants grown in the field[158] (Figure 3). However, in soybeans, the concentration of carbohydrates in tap-root nodules from field-grown plants[167] was very similar to that reported for roots of greenhouse-grown plants.[71]

Other factors may control the process of nodule senescence. One possibility is that during reproductive development a signal is transmitted to the nodules, which results in a loss of their effectiveness.[173] This "physiological factor" would then initiate the senescence process. In fact, plant selection for delayed leaf senescence was found to retain active nitrogen fixation of soybeans through to the pod-filling stage,[174] and some phytohormones have already been described as playing a role in nodule senescense.[175]

The ultimate objective of the research into physiology of grain legumes should be to increase seed yields. It is apparent from the above discussion that this may be achieved by selecting cultivar/strain combinations for delayed nodule senescence so that active nitrogen fixation is maintained throughout the critical stage of grain filling, as well as selection for combinations with increased nodule efficiency in terms of carbon utilization.

III. CARBON COST OF NODULE FUNCTIONING

A. Theoretical Cost of Nitrogen Fixation and Assimilation

A theoretical cost of 32 ATP has been estimated for the process of nitrogen fixation[176] considering that 25% of the total electron flux to nitrogenase is deviated to the obligatory ATP-dependent hydrogen evolution.[177] Part of the energy lost with this process may, however, be recovered by the hydrogen-uptake hydrogenase system, yielding a maximum of 3 ATP per mole of hydrogen,[178] thus reducing the overall cost of nitrogen fixation to 29 ATP per mole of nitrogen fixed.[176] Electron allocation to protons is, however, variable and affected by many factors (see Section IV.B). Estimates covering a series of hypothetical situations, combining variable electron allocation with increasing hydrogenase uptake activities, demonstrated that the energy saving due to an active hydrogenase system could be as high as 30%.[6]

Ammonia assimilation into asparagine requires another 6 to 7 ATP,[176] giving a cost of
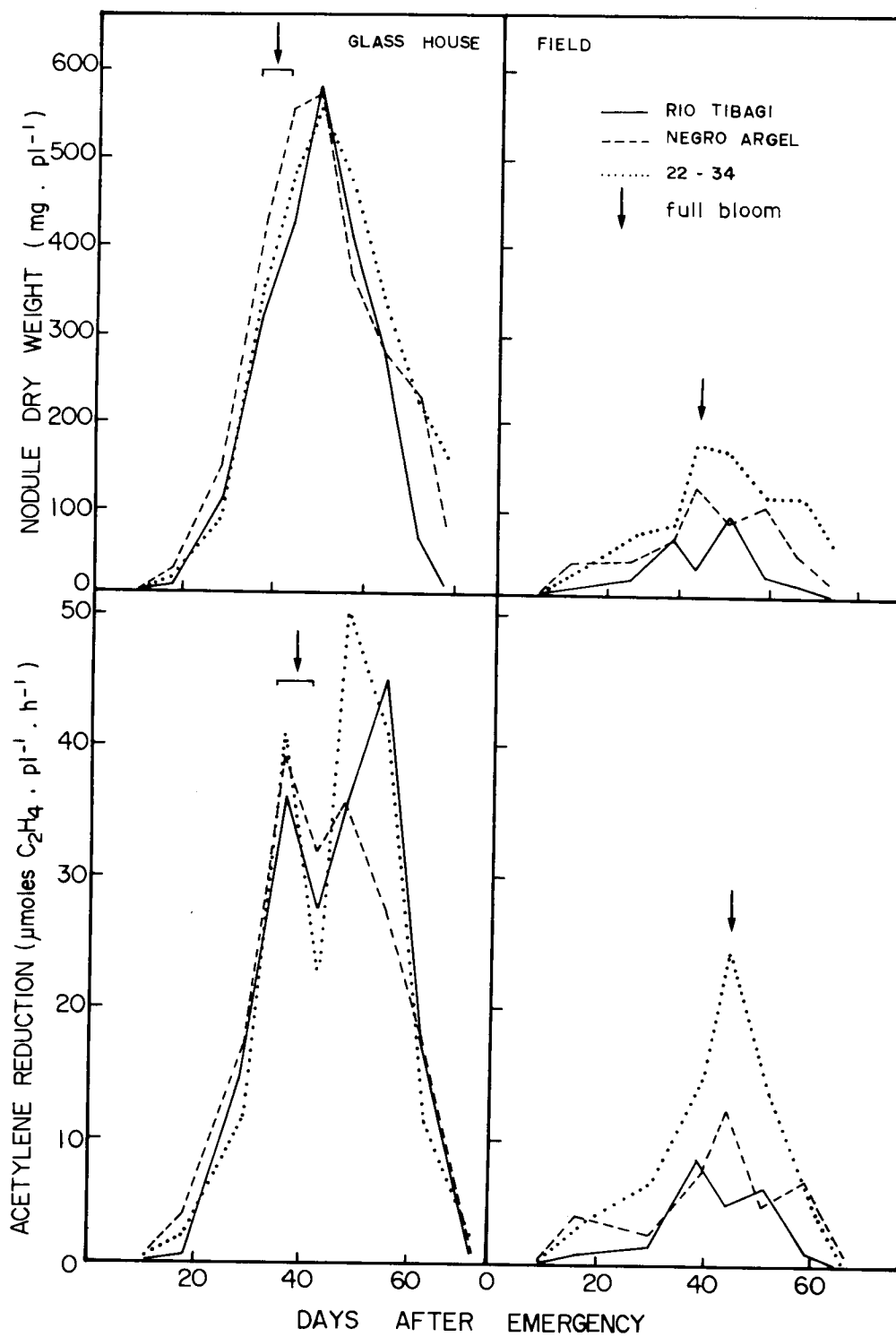
FIGURE 3.   Nodule dry weight and nitrogenase activity of three *Phaseolus* bean cultivars grown simultaneously in the field or the greenhouse.[158]

35 ATP per mole of nitrogen fixed and assimilated into asparagine. If ureides are produced, the theoretical estimates suggest a cost between 5 and 10 ATP per mole of ureide, however, with a potential to generate reductants,[176,179] thus reducing the overall cost to a minimum of 2 ATP equivalents.[176] As 4 mol of ammonia is assimilated into 1 mol of ureide, the minimum cost for fixation and assimilation of nitrogen into ureides may be estimated in 30 ATP per mole of nitrogen fixed and assimilated as ureides. Production of ureides thus represents an energy economy of 14% to the nodules. This costing, however, does not take into account the investment in the many enzymes required or in the production and maintenance of peroxisomes (see Section V.A).

Costs with the nitrogen transport, however, are not considered in those estimates and it is still unknown if there are differences in the energetic cost between the transport of ureides and amides.[6,176,180] In the same way, the costs of ureide assimilation into in-plant tissues are also unknown. The loading of amino acids into the phloem is an ATP-dependent process,[181] but we know nothing about the energy requirement involved in ureide remobilization.

B. Experimental Cost of Nitrogen Fixation and Assimilation

The high theoretical cost of nitrogen fixation has motivated various studies of the carbon requirements for nodule function. Since the earliest attempt made by Bond,[182] several values for the respiratory cost of nitrogen fixation have been reported for nodulated grain legumes and have been reviewed.[5,180,183-185] The difficulty of estimating the respiration of intact nodules without either including root respiration or damaging the plants or nodules or even interfering with other metabolic processes is a matter of concern (see reviews of Minchin et al.[5] and Pate et al.[6]) and has led to the use of a wide range of techniques, most of them failing in separating the maintenance component of nodule respiration.[5] Therefore, comparisons between plant species are difficult to make whenever diverse methods are used. Furthermore, methods of plant culture and husbandry in these studies have varied considerably and the importance of this towards nodule efficiency cannot be overlooked. In cowpeas, an adverse temperature regime (warm days and cool nights) was found to produce a three-fold increase in the respiratory cost of nitrogen fixation,[172] which no doubt affected the carbon economy of the whole plant. This legume has been intensively studied and, in general, the reported respiratory costs of nitrogen fixation in cowpeas are low;[5] some values are even lower than those theoretically calculated.[186-188]

The high PEP carboxylase activity, found in nodules of many legumes, including cowpeas, may, at least in part, explain the low CO_2 output observed in cowpea nodules,[100] thus representing an extra source of error during the estimates of the respiratory cost of nitrogen fixation. This error, however, is not taken into account by most of the techniques so far employed.

The presence of an active hydrogenase system in bacteroids is also related to the high respiratory efficiencies of cowpeas.[186] These, however, were not reflected by improved seed yields of cultivar Caloona, although root growth was increased,[186] perhaps because of the very low harvest index (0.19) recorded for this cultivar[187] bred for use as forage. More studies using high-yield cultivars are required to elucidate further the advantage of a hydrogen-recycling system towards the whole plant carbon economy benefiting seed yields.

Effects of *Rhizobium* strains on the respiratory cost of nitrogen fixation have been reported for cultivar TVu 1503.[188] The effects were small but more evident during the period of pod-fill stage and were reflected in significantly higher seed yields of plants associated with the most efficient strain.

A nondestructive technique based on the induced decline of respiration and nitrogenase activity has been used to compare the respiratory efficiency of several legume species and *Rhizobium* strains.[189] Again, the reported values for cowpeas are the lowest, and strain effects were observed in peas and *Phaseolus* beans. Unless ^{15}N-labeled nitrogen is used, this

technique does not take into account hydrogen production by nitrogenase, which is inhibited by acetylene, but it has the advantage of separating the component of respiration related to nitrogen fixation from that of nodule maintenance.

The overall carbon cost involved in obtaining nitrogen through biological fixation includes cost of nodule growth and maintenance, ammonia assimilation, and use of carbon skeletons for the transport of fixed nitrogen. For soybean cultivar Clark inoculated with a *Rhizobium* strain not capable of recycling evolved hydrogen, the costs of each of those components for an overall expenditure of 4.8 g of carbon per gram of nitrogen fixed were estimated as representing 25% (growth and maintenance) and 14% (ammonia assimilation and transport), and the remaining 61% was estimated to be consumed for nitrogen fixation and hydrogen evolution of vegetative plants, measured after 30 days from sowing.[102] Nodule functioning has, however, a dynamic nature and continuously varies with changes of the environmental and physiological conditions. The reconstruction of the carbon flow to and within nodules for an extended period of plant growth is complex, and data for some of those components of nodule carbon expenditure are available for a very limited number of legume species. The daily import of carbon by the nodules varies from 11.6 to 95.3 mg carbon (Table 4), representing from 13 to 28% of the daily gain of carbon by the shoot (compare with the 8 to 17% consumed by mycorrhizae).[190] Most of the acquired carbon (35 to 64%) is lost in respiration, whereas the export of nitrogen consumes 21 to 52% of the total carbon available to the nodules (Table 4).

Despite the large differences observed between reported values, comparisons are difficult to make because of the very large differences in nodule activity. Among cowpeas, for example, cultivar K2809 obtained two to five times more nitrogen per day than cultivar Caloona when inoculated with the same strain (Table 4), despite the fact that the former was raised in an environmental condition known to be adverse to nodule-dependent plants.[172] When cowpea plants with high rates of nitrogen fixation are compared (Table 4), it is possible to observe the advantage of transporting fixed nitrogen as ureides, which required from 24 to 45% of the available carbon to cowpea nodules (overall mean value of 33%), compared with a value of 52% expended by lupin nodules (Table 4). The same, however, is not observed when plants with low rates of nitrogen fixation are compared (Table 4) because they are likely to be limited by other growth factors. The data also show that variations due to cultivar strain and growth conditions are as large as those observed between different species (Table 4). In some systems, carbon lost in respiration can account for up to 64% of the nodule carbon income, which can be aggravated by adverse temperature conditions.[172,191]

The high energy cost of nitrogen fixation leading to a diversion of photosynthates that otherwise would be used for plant growth has motivated various comparative studies between the energy needs for nitrogen fixation and nitrate assimilation.[172,188,192-195] At high levels of nitrate, the ratio of carbon respired by below-ground organs per unit of nitrogen assimilated is smaller for non-nodulated plants.[172,188,193] At this nitrogen level, cowpeas and soybeans assimilate nitrate mainly in the shoot,[172,193] without any compensatory decrease in photosynthesis,[193,196a] therefore benefiting from an excess of ATP and reductants generated during photosynthesis. Energy would, however, be required for synthesis and maintenance of the enzymes that have a high rate of turnover,[51] for amino acid loading into the phloem,[181] and also for pH regulation during nitrogen assimilation,[196b] which is variable, depending on the location of the nitrate-reducing site in the plant and on the mode of excess OH^- neutralization employed.[196b] Non-nodulated roots also have the burden of a very active alternative oxidative phosphorylation pathway which produces only 1 mol of ATP per mole of oxygen consumed, which is much less active in nodulated roots, but its significance to the overall carbon economy of legume plants remains to be elucidated.[197]

At low levels of nitrate, assimilation proceeds mainly in the roots,[195,198] requiring energy

Table 4
PARTITIONING OF TRANSLOCATED CARBON IN NODULES DURING THE PERIOD OF INCREASING N$_2$ FIXATION

System	Total N$_2$ fixed (mg/plant · day)	Total C imported (mg/plant · day)	Daily C intake to nodules (%)			Ref.
			Respiration	N export	Dry matter	
Cajanus cajan						
cv. UPAS-120	3.6	18.0 (10)[a]	50	30	15	400
Lupinus albus						
cv. unnamed	10.6	46.7 (13)	40	52	9	401
cv. Ultra (strain Wu425)	2.8	18.4	55	27	18	100
Pisum sativum						
cv. Meteor	3.0	12.4 (28)	36	48	16	194
Vigna unguiculata						
cv. Caloona	8.4	25.0 (13)	36	43	21	187
cv. Caloona (strain CB756)	3.7	11.6	49	29	22	100
cv. TVu1403 (strain CB1024/R5028)	22.6	72.5 (17)	35	45	20	188
cv. K2809 (strain CB756)	20.0	95.3 (20)	63	21	16	172
cv. Caloona (strain 176A27)	11.5	56.2	64	24	12	186

[a] Figures in parentheses are percentage of net daily carbon gain per plant.

and reductants derived from respiration. Under such conditions, conflicting results have been reported, with ratios of carbon respired per unit of nitrate-nitrogen assimilated being sometimes identical,[199] bigger,[195,200] or even smaller[194] than those reported for nitrogen fixation and assimilation in the nodules because plants may compensate, with increased photosynthetic efficiency, for the additional carbon utilization of the symbiotic association.[201] Despite this, non-nodulated plants do not have the burden of nodule production and maintenance, therefore, more carbon becomes available for growth. This advantage is specially noticeable early in plant growth.[172] However, the allocation of greater proportions of carbon into supporting structures where it cannot be easily retrieved,[172,188] coupled with reduced nitrate assimilation during early reproductive growth,[202] and the accelerated leaf senescence of nitrate-grown plants[170] reduce their superiority relative to nodulated plants, benefiting more the vegetative than the reproductive growth of grain legumes such as cowpeas,[172] chickpeas,[169,170] and *Phaseolus* beans.[203]

IV. NODULE EFFICIENCY AND THE METABOLISM OF HYDROGEN

A. Hydrogen Metabolism in Nodules

Concomitant to nitrogen fixation, nodules evolve hydrogen in a process dependent on oxygen.[204] Studies have shown that a reduction of protons to form hydrogen occurs even at "infinite" pressures of nitrogen[205] and uses electrons and ATP which otherwise would be used for the reduction of nitrogen and is considered to be responsible for a considerable loss of energy.[206]

Whereas hydrogen evolution involves the nitrogenase site directly, nodules were also shown to take up hydrogen if this gas were present in the atmosphere around the nodules.[207] The enzyme responsible for oxidizing the hydrogen, the uptake hydrogenase, was reported in pea nodules as early as 1941[208] and is located in the bacteorids,[209] linked to the bacteroid membrane.[178,210]

A number of roles have been proposed for the uptake hydrogenase: (1) an auxiliary mechanism for respiratory protection, which removes oxygen from the immediate environment of nitrogenase;[211-213] (2) prevention of the inhibition of nitrogenase activity by the evolved hydrogen[211] which, due to the compartmentalization of nodule structure, may reach concentrations that are inhibitory to nitrogenase activity;[214] and (3) improvement of nodule efficiency by oxidizing the hydrogen otherwise lost, resulting in ATP production[178,211-213] and reducing power,[215] representing a mechanism of conservation of carbon substrates.

The relative efficiency (RE) of nitrogenase, in terms of electron allocation, estimated by the proportion of electrons lost in hydrogen evolution over the total electrons available to nitrogenase[206] has been reported for many nodulated legumes (Table 5), from which we may conclude that for many symbiotic systems more than 50% of the electrons can be lost through hydrogen evolution. It is considered that the obligatory ATP-dependent hydrogen evolution consumes a minimum of 25% of the total electron flux (see review of Eisbrenner and Evans[177]), although there is no direct experimental evidence to prove that a minimum of 1 mol of hydrogen is produced per mole of nitrogen fixed. As in most of the experiments listed in Table 5, the hydrogenase activity was not measured, and it is difficult to know whether values of RE higher than 0.75 would necessarily be linked to the activity of the uptake hydrogenase or if they just reflect a better allocation of the electrons to nitrogen reduction.

Very small values of RE have sometimes been reported and may reflect errors induced by the methodology. Nitrogenase activity of nodules of many legume species decreases shortly after exposure to acetylene,[216] therefore, all the data on RE obtained with long incubation under acetylene in closed systems should be reevaluated. This seems especially important because this decline in nitrogenase activity was found to vary with plant species

Table 5
RELATIVE EFFICIENCY OF NITROGENASE IN SOME SYMBIOTIC SYSTEMS

| Legume species | Type of strains used | Relative efficiency[a] | | |
		Range	Mean	Ref.
Glycine max	Wild-type strains		0.52	206
	Group of HUP + strains	0.96—1.00	0.98	219
	Group of HUP − strains	0.41—0.80	0.60	219
	Group of HUP + strains	0.97—1.00	0.98	242
	Group of HUP − strains	066—0.73	0.70	242
	HUP + original	0.99—1.00	1.00	242
	HUP − mutant strains	0.79—0.84	0.82	242
	HUP +		1.00	243
	HUP −	0.46—0.64	0.55	243
	Group of HUP + strains	0.94—1.00	0.97	239
	Group of HUP − strains	0.56—0.80	0.68	239
	HUP +		1.00	239
	HUP − mutant strains		0.70	239
	Wild-type strains	0.38—0.59	0.48	217
	Wild-type strains	0.30—0.98	0.64	246
Phaseolus vulgaris	Wild-type strains at various growth stages	0.65—0.68	0.66	402
	Wild-type strains	0.38—0.96	0.67	225
P. aureus	Wild-type strains		0.82	206
Vigna unguiculata	Wild-type strains		0.99	206
	Wild-type strains	0.65—1.00	0.82	218
Cajanus cajan	Group of HUP + strains	0.87—0.97	0.92	236
	Wild HUP − strains		0.71	236
	Group of HUP + strains	0.79—1.00	0.90	237
	Group of HUP − strains	0.55—0.61	0.58	237

[a] $$\text{Relative efficiency} = 1 - \frac{H_2(\text{air})}{H_2(\text{Ar})} \text{ or } 1 - \frac{H_2(\text{air})}{C_2H_2}.$$

and cultivar, *Rhizobium* strain, plant age, and environmental conditions,[216] reflecting differences in the resistance of the nodule membranes to oxygen diffusion.[113] No doubt many values were found to be negative and have not been reported. In many experiments, however, the RE values based on acetylene reduction agree well with results obtained with $^{15}N_2$, for example, such as those obtained with clover and soybeans by van Kessel and Burris.[217]

Initially, it was believed that the hydrogen metabolism was controlled only by the *Rhizobium* strain,[207,218] and many studies seemed to corroborate this idea (e.g., Carter et al.[219] and Dadarwal et al.[220]). Dixon was the first to suggest that hydrogen evolution could also be controlled by the host plant because different host plants induced various levels of uptake hydrogenase activity in *Rhizobium* bacteroids.[211] The first conclusive results about the effect of the host plant in the expression of hydrogenase came with the observation that two strains of *Rhizobium* sp. when inoculated on *Vigna radiata* evolved hydrogen from their nodules, but when associated with two other *Vigna* species, *V. unguiculata* and *V. mungo*, formed nodules which did not evolve hydrogen.[221] Similar results were also obtained with strains of nodulating soybeans and cowpeas.[222] Further data on soybeans, cowpeas, and many other legume species corroborated the influence of the host plant on the hydrogen metabolism of nodules.[223,224]

In *Phaseolus* beans, although the hydrogenase activity was not evaluated, the cultivars greatly affected nodule RE,[225] and the inoculation with six strains of *Rhizobium* gave a mean RE of 0.85 in the cultivar Negro Argel and 0.52 in the cultivar Rio Tibagi (Table 6). However, it has yet to be established if the host plant has genetic control over the expression of the hydrogenase in the bacteroids or if it is some endogenous factor, such as the available

Table 6
RHIZOBIUM STRAIN AND PHASEOLUS BEAN CULTIVAR EFFECTS ON NITROGENASE ACTIVITY, H₂ EVOLUTION, RELATIVE EFFICIENCY (RE) OF NITROGENASE, XYLEM SAP EXUDATION, RATE OF NITROGEN TRANSPORT, AND PERCENTAGE OF UREIDE-NITROGEN IN THE XYLEM SAP AND NODULE EFFICIENCY AT 35 DAYS AFTER EMERGENCE[225]

Treatment	C_2H_2 reduction (μmol · g nodule^{-1} · h^{-1})	H_2 evolution	RE	Xylem sap exudation ($\mu\ell$ · plant^{-1} · min^{-1})	Rate of N transport in the xylem sap (nmol N · plant^{-1} · min^{-1})	Ureide-N[a] in the xylem sap (%)	Total N in shoots (mg N · plant^{-1})	Nodule efficiency (mg N · g nod^{-1})
Rhizobium Strain (means of 20 Replicates)								
CO5	25.5a	3.1b	0.88a	1.13a	47.3a	90.6a	183.4a	382.8a
FP2	19.8b	3.4b	0.83a	0.89b	35.1b	85.3a	102.7b	140.9b
SEMIA 487	19.3b	4.0b	0.79a	1.02a	32.7b	87.1a	95.7b	150.5b
127 K-17	14.9c	5.7a	0.62b	0.79c	23.3c	78.3b	77.6c	122.4b
CIAT 727	9.0d	5.1a	0.44c	0.43d	12.8d	56.2c	22.0d	78.3c
CIAT 255	6.7e	3.8b	0.43c	0.31e	5.2e	51.1c	6.1e	45.6c
Bean Cultivar (means of 24 Replicates)								
Negro Argel	16.3b	2.5c	0.85a	1.19a	41.3a	80.2a	116.8a	216.0c
Carioca	18.6a	4.8b	0.74b	1.03a	40.5a	79.5a	92.0b	244.1a
Venezuela 350	15.3b	4.0b	0.74b	0.92a	25.2b	73.8b	80.5c	126.5b
Costa Rica	19.2a	6.1a	0.68b	0.61b	15.3c	71.4b	55.4d	125.4b
Rio Tibagi	9.8c	4.7c	0.52c	0.59b	15.5c	68.9c	61.4d	144.9b

Note: Numbers followed by the same letter are not significantly different at $p = 0.01$ (Tukey's test).

[a]   Ureide N · (total N)$^{-1}$·100.

carbon substrates, that determines the hydrogenase activity since it is known that hydrogenase derepression and activity can be greatly affected by many factors (Table 7), including carbon availability.

B. Factors Affecting Hydrogen Evolution

Some environmental factors can affect net hydrogen evolution, due either to changes in the shift of electrons to nitrogenase or to changes in uptake hydrogenase activity (Table 7). In soybeans, a very large decrease in the RE was observed when plants inoculated with a strain defective for uptake hydrogenase (HUP—) were grown at 25°C compared with those maintained at 15°C.[226] The results suggested that low temperatures directly affected the electron allocation of nitrogenase, and not hydrogenase, activity itself. Similar effects of high temperatures increasing hydrogen evolution were also observed in cowpeas[227] and soybeans grown at 25°C as compared with 10°C.[228] If temperature can have such an effect on electron allocation, under tropical conditions where the temperature often reaches 40°C, the loss of ATP through hydrogen evolution is likely to represent a significant burden on nodulated plants. However, the adaptability of tropical species and strains to high temperatures in relation to the allocation of electrons has yet to be investigated.

C. Uptake Hydrogenase Activity and Nodule Functioning

As acetylene inhibits hydrogen evolution, the addition of hydrogen to nodules formed by HUP+ strains during incubation with acetylene should result in increased acetylene-reduction activity, confirming the role of hydrogenase in generating ATP. In fact, addition of 72 μM of hydrogen to suspensions of HUP+ bacteroids of *R. japonicum* promoted a three- to fivefold increase in the rates of acetylene reduction but did not affect acetylene reduction of HUP— strains.[229] However, when hydrogen was supplied to intact nodules, different responses were obtained. Incubation of nodules formed with HUP+ strains of *Rhizobium* sp. under an atmosphere containing 1% hydrogen had no effect on the rate of $^{15}N_2$ fixation.[221] Similar lacks of response were also observed in terms of acetylene-reduction activity when nodules of *R. leguminosarum* previously deprived of substrate by stem excision were incubated under the same conditions.[230] On the other hand, the incubation of cowpea nodules formed by an HUP+ strain in an atmosphere of 10% hydrogen increased the acetylene-reduction activity by 22% (average of six host plants).[220]

Unfortunately, one can only speculate about the cause of these conflicting results obtained with nodulated plants. The first possibility is that the hydrogenase activity does not contribute significantly to increased nitrogenase activity due to the relatively small energy saving, as proposed by some authors.[221] In fact, in some HUP+ strains, the oxidation of hydrogen is not coupled with the ATP formation and, consequently, no carbon economy is achieved[213,230] and no stimulatory effect of hydrogen addition to nitrogenase activity is likely to be observed. The second possibility is that the hydrogen concentration furnished in the experiments was too low because with a tenfold increase in the hydrogen addition, increases in the acetylene reduction were observed.[220] The third possibility is related to the inhibitory effect of acetylene on nitrogenase activity,[216] changing the permeability of the membranes and, as a result, preventing an effect of hydrogen in the system.

Furthermore, there are strains with the HUP+ characteristic, but the hydrogenase of these strains is not capable of recycling all the hydrogen evolved in nodules of various legume species.[231-233] The low efficiency of the hydrogenase in these species may be linked to a limitation in the quantity of active hydrogenase or to a deficiency in some intermediate component of the hydrogen oxidation.[231]

D. Nodule RE and Plant Growth

Interpretation of the results about the effects of inoculation with HUP+ and HUP— strains must be examined with care because, first, the hydrogenase characteristic seems to be additive

Table 7
ENVIRONMENTAL OR ENDOGENOUS FACTORS AFFECTING THE SHIFT OF ELECTRONS TO NITROGENASE AND THE HYDROGEN EVOLUTION

Factor	Free-living organism or nodulated species	Effect	Ref.
Mo-Fe to Fe protein ratio	*Azotobacter chroococcum*	Increasing Mo-Fe to Fe protein ratio increased H_2 to N_2 ratio	403
Temperature	*Pisum sativum*	Increasing temperature, increased H_2 evolution	404
	Glycine max		177, 228
	Vigna unguiculata		227
N_2 partial pressure	*P. sativum*	Did not affect H_2 evolution	404
O_2 partial pressure	*Rhizobium japonicum*	O_2 did not inhibit hydrogenase activity;	405
		O_2 inhibited hydrogenase synthesis	406
	P. sativum	Increasing O_2 decreased RE	404
	Glycine max	Increasing O_2 increased RE	407
ATP to ADP ratio	*A. chroococcum*	Increasing ATP to ADP ratio; decreased H_2 to N_2 ratio	403
$SO_4^=$	*A. chroococcum*	$SO_4^=$ -limited conditions decreased the shift of electrons to hydrogen	403
CO_2 concentration	*R. japonicum*	No effect on hydrogenase activity	405
	P. sativum	10% CO_2 around nodules slightly increased RE	404
NH_4^+	*R. japonicum*	Did not affect hydrogenase expression	405
	G. max	No effect with one strain and inhibited H_2 production in other strain	407
NO_3	*R. japonicum*	Did not affect hydrogenase expression	405
	Phaseolus vulgaris	RE decreased	248
Bacteroids × intact nodules	*Pisum sativum*	Bacteroids had higher RE	404
Level of irradiance	*P. sativum*	Low levels of irradiance increased RE	230, 247
C availability	*R. leguminosarum*	C-limited conditions increased hydrogenase activity	215
	R. japonicum		405, 406,
	R. japonicum	C inhibited hydrogenase synthesis	408
H_2	*R. japonicum*	Induced hydrogenase activity	408
		H_2 was not necessary for hydrogenase synthesis	409
Plant age	*P. sativum*	RE increased during ontogeny	410
	G. max	RE did not change with plant age	218
	V. unguiculata		218

since various degrees of HUP+ exist,[234,235] and second, because strains should be checked for the coupling between the hydrogenase activity and ATP formation.[213,233]

The effects of hydrogen metabolism on plant growth can be separated into two classes: absent or significant effects. The relatively small energy saving promoted by the uptake hydrogenase was suggested to explain the absence of differences on plant dry weight of *Trifolium subterraneum* inoculated with HUP+ and HUP− strains.[221] Differences were not found in total dry-matter production, seed yield, and nitrogen fixed between treatments in cowpeas inoculated with a high and a low hydrogen-evolving strain.[186] There were, however, significant increases in the carbon and nitrogen content of roots but not in the nodule mass of plants associated with the low hydrogen-evolving strain. This increase in root mass was related to a lower loss of CO_2 in nodule respiration, representing a 36% greater economy of carbon during the period of maximum hydrogen evolution. Rainbird and co-workers suggested that a determinate quantity of photosynthates is made available to the root system and that extra energy costs, such as the hydrogen evolution, will only result in a decrease in root growth.[186] Improved root systems could result in increased plant growth under field conditions where water or nutrients might be in short supply. It should, however, be considered that variable amounts of photosynthates are destined for the root system. Changes in the environmental conditions, such as periods of water stress, are known to alter the availability of carbohydrates to the nodules.[168] Furthermore, generally more carbon is made available to the underground parts of nodulated cowpeas as compared with plants supplied with nitrate.[172,195]

No differences in plant nitrogen content, dry weight, and nitrogen accumulation were reported for pea plants inoculated with strains forming nodules with high or low RE.[232] In a later experiment, it was, however, discovered that in many HUP+ isolates, the hydrogen oxidation was not coupled with ATP formation,[213] but even the inoculation of HUP+ strains, which have their hydrogenase coupled to an ATP-generating system, resulted in no differences in shoot dry weight and total nitrogen accumulation of plants.[230] In *Cajanus cajan* the results were also inconclusive. Inoculation with HUP+ strain P 132 promoted significantly greater total shoot nitrogen than other treatments, but inoculation with two other HUP+ strains did not affect plant growth compared with that of the HUP− control.[236] The absence of a significant difference between HUP+ and HUP− strains in *Cajanus* was later confirmed with 37 strains of *Rhizobium*.[237] Similar results were also reported for mung beans.[238]

In another group of results, a specific effect of the hydrogenase activity on the nitrogen-fixation process was observed. Under field conditions, no increase in plant dry weight, but a marked increase in the percentage of nitrogen (an average of 9% increase in four experiments) and in seed protein yield, was observed with soybean inoculated with HUP+ strains.[239] Under field conditions, plants are often not limited by nitrogen but are by temperature, phosphorus, and pH, which adversely restrict plant growth.[240] Consequently, the energy saving resulting from a low hydrogen evolution will be reflected only in increased percentages of nitrogen in the plants.

Some results, however, have shown increases not only in the nitrogen content but also in seed yield or in plant dry weight. Most of these experiments were performed under greenhouse conditions, and sometimes the increase in plant growth was much greater than the maximal theoretical energy saving possibly promoted by an active hydrogenase system (Section III.A).

In greenhouse-grown soybeans, the inoculation with low hydrogen-evolving strains increased plant dry weight by 24%, percentage of nitrogen in the tissues by 7%, and the total nitrogen by 31% compared with high hydrogen-evolving strains;[241] various other reports corroborated the beneficial effect of low hydrogen-evolving strains to plant growth[242,243] and total shoot nitrogen.[242] On the other hand, the inoculation with a HUP− mutant strain of

R. japonicum decreased soybean dry weight and total nitrogen compared with the isogenic HUP + original strain.[244] Similar differences in dry matter and nitrogen accumulation were reported for mung beans[245] inoculated with mutant strains possessing hydrogenase activity compared with the original strains.

The inoculation of soybeans with strains showing a RE value between 0.94 and 0.98 increased the seed yield by an average of 37% and the nitrogen harvest index by 23% in relation to inoculation with strains with RE between 0.30 and 0.74.[246] This experiment was performed under field conditions in a sandy soil of extremely low nitrogen content where plants were severely dependent on nitrogen fixation for growth and seed production.

In *Phaseolus* beans, a very high correlation was found between the RE and the nitrogen content of pods of 30 symbiotic systems,[225] as can be seen in Figure 4. The poorest symbiotic combination, with a RE of 0.38 recorded at flowering, accumulated 0.20 mg nitrogen per plant as young fruits at 50 days after emergence, whereas the best symbiotic combination, with a RE of 0.96, accumulated 64.32 mg nitrogen per plant as young fruits.[225]

All these results have in common the fact that plants were raised under nitrogen-limiting conditions. Consequently, plant growth and seed yield respond to any increase in nitrogen availability. But because we know biological systems do not respond "arithmetically" when the limiting factor is overcome, plant response can be greater than expected; that is, the increased supply of nitrogen to the plant shoot may in return allow for a greater supply of carbon substrates to nodule growth and activity. Therefore, the elucidation of the importance of uptake hydrogenase in bacteroids to the symbiotic performance has become an intriguing problem.

The importance of hydrogenase is greater under carbon-limited conditions,[230,247] but under these conditions nitrogenase-specific activities of HUP − isolates are increased, not differing from that of HUP + isolates of *R. leguminosarum*.[230] Another important factor to be considered is the effect of nitrate-nitrogen on the proportion of electrons allocated to hydrogen.[177,248] It was speculated that the presence of nitrate in the nodules as an additional sink for electrons would result in decreased turnover rates of the MoFe:Fe protein, which then causes increased allocation of electrons to protons.[177] In practical terms, since nitrate is always available in soils, it is important to select symbiotic systems less sensitive to nitrate.

E. Hydrogen Metabolism and Other Metabolic Functions of Nodules

The possibility that other metabolic pathways are linked to the complex metabolism of hydrogen in the nodules cannot be disregarded. A possible relationship between the activity of hydrogenase and nodulation capacity is not yet established, but in one strain of *R. leguminosarum* the genetic information for hydrogenase activity and nodulation ability were found to be linked, and both seemed to be carried by one plasmid.[249] Similarly, between 108 isolates of *R. leguminosarum*, none of the ineffective strains had hydrogenase activity, whereas the HUP + strains were always good nodulators.[232]

Among strains capable of producing nodules, differences in nodule-specific activity between high and low hydrogen-evolving strains may also be seen. In soybeans, strains with a RE above 0.97 had nodule efficiencies higher than those of the high hydrogen-evolving strains.[246] In *Phaseolus* beans, a strain with a RE of 0.88 at flowering had a nodule efficiency of 382.2 mg nitrogen per gram of nodule, in comparison with a nodule efficiency of only 45.56 mg nitrogen per gram of nodule of a strain with a RE of 0.43 (Table 6).

The relationship between the activity of hydrogenase and the presence of leghemoglobin is still confused. In soybeans, the leghemoglobin content of nodules formed by HUP + strains was significantly greater than the comparable mean for the HUP − strains.[243] However, in peas[232] and within the cowpea "miscellany" hosts,[220] low levels of leghemoglobin were related with greater activity of the hydrogenase, which led the authors to suggest a role for the uptake hydrogenase in maintaining an appropriate redox potential within the

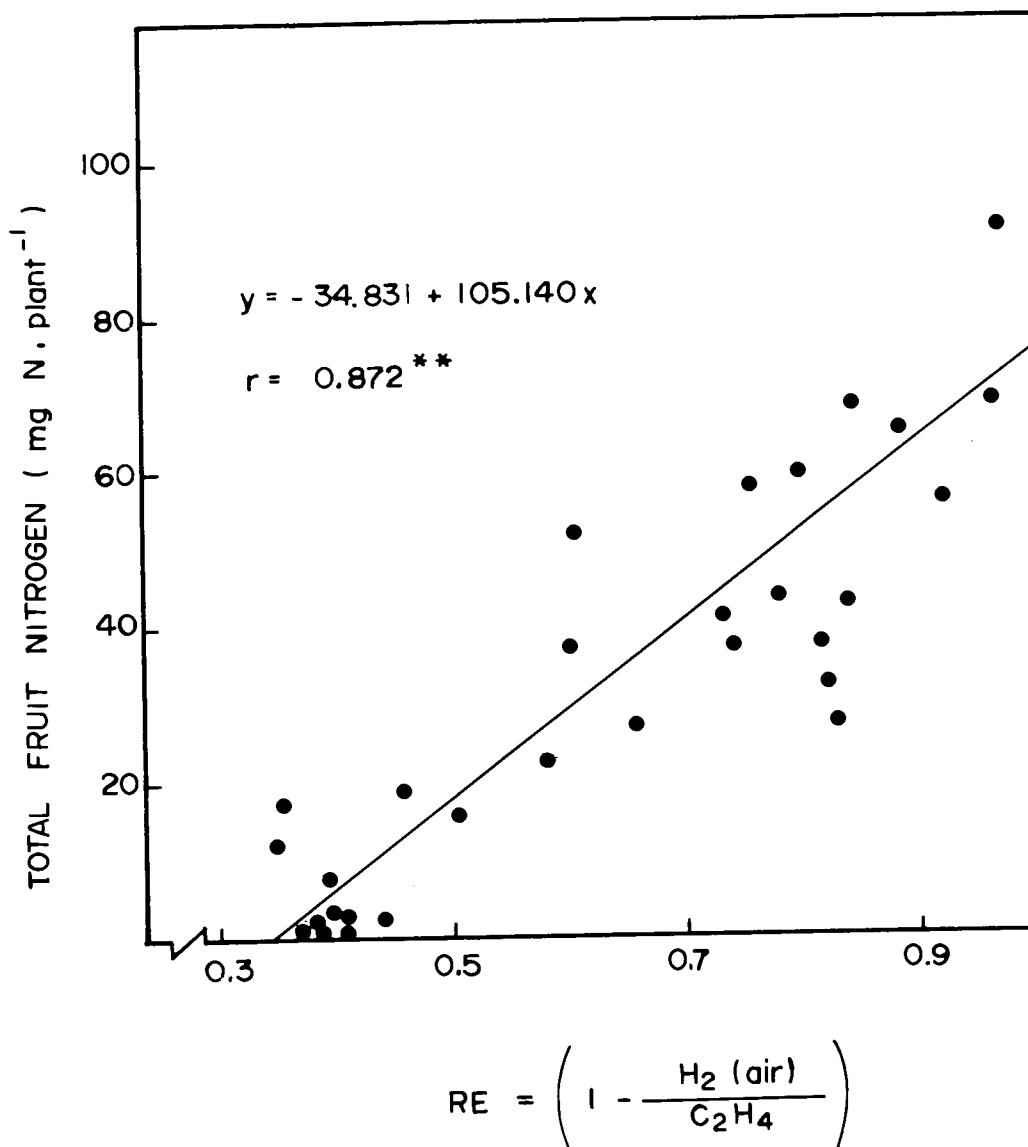$$RE = \left(1 - \frac{H_2 \, (air)}{C_2 H_4} \right)$$

FIGURE 4. Relationship between nodule relative efficiency (RE) at 35 days after emergence and total nitrogen in fruits of *Phaseolus* beans at 50 days after emergence. A total of 30 symbiotic combinations were used, including 5 plant cultivars and 6 *Rhizobium* strains. Points are means of four replicates. Acetylene reduction was estimated by incubation of detached roots in a continuous flow of 12% acetylene in air. (From Hungria, M. and Neves, M. C. P., *Pesqui. Agropecu. Bras.*, 21, 127, 1986. With permission.)

nodules. A possible relationship between CO_2 fixation in nodules and hydrogen metabolism has also been suggested.[250,251] The inoculation of soybean plants with HUP+ strains increased by 95% the incorporation of ^{14}C into aspartic acid measured in the sap (which is derived from oxaloacetic acid produced by the carboxylase system [Section II.D]) when compared with plants inoculated with HUP− strains.[251] Therefore, CO_2 fixation by nodules formed by HUP+ strains played an important role in furnishing the carbon skeletons for ammonia assimilation once the nitrogen fixation was increased.

The rate of transport of fixed nitrogen was also found to be different between soybean plants nodulated with HUP+ and HUP− strains,[252] and these differences were mainly

related to a higher exudation rate of plants inoculated with the HUP+ strains. Similar results were obtained with *Phaseolus* beans inoculated with high and low hydrogen-evolving strains, as can be seen in Table 6.

Finally, a relationship between nitrogen assimilation and hydrogen metabolism has been also suggested. Analysis of the amino acid fractions of xylem sap of soybeans inoculated with HUP+ and HUP− strains showed that in plants inoculated with the HUP+ strains the nitrogen assimilation via aspartic acid was operating much more actively.[251] In another experiment, using the same strains, the amide-nitrogen fraction, especially asparagine, was increased with the inoculation of the HUP+ strains, while the relative amount of ureide-nitrogen to total nitrogen in the sap was almost unchanged. However, the percentage of nitrogen as asparagine accounted for a maximum of 8.6% of the total nitrogen in the xylem sap in comparison with a maximum of 5.5% with the HUP− strains.[252] Minamisawa and co-workers[253] also observed that the HUP− strains accumulated a large quantity of an unknown compound in the nodules, probably a basic amino acid derivative.

Although it was initially observed that an efficient (high RE) strain of *R. japonicum* produced less ureides than an inefficient strain,[254] inoculation of soybeans with two groups of strains, one with a high RE and another one with a low RE, showed that the efficient strains transported a greater proportion of the xylem sap nitrogen as ureides.[246] Similar observation was reported for *Phaseolus* beans (Table 6). The highly significant positive correlation between the RE and the percentage of ureide in the xylem sap of *Phaseolus* beans (Figure 5) confirms the relationship between RE and ureide production. However, it may possibly represent an indirect relationship because the ATP produced during hydrogen oxidation would increase the rate of nitrogen fixation and, as a consequence, the assimilation of fixed nitrogen would require either more carbon skeletons or incorporation in nitrogenous compounds which have low carbon-to-nitrogen ratios such as the ureides (see Section V.B). Otherwise, evolved hydrogen may interfere with the enzymes responsible for nitrogen assimilation; but there are many other alternatives to explain this surprising correlation, including all kinds of genetic linkage.

V. NITROGEN ASSIMILATION, TRANSPORT, AND DISTRIBUTION

A. Assimilation of Fixed Nitrogen

The starting material for the incorporation of fixed nitrogen into organic compounds is ammonia, which was found to be the first stable product of nitrogenase[255,256] and, although the studies were performed with nodules of only two plant species (soybean and *Ornithopus sativus*), this is generally considered to be true for most legume/*Rhizobium* symbioses (see reviews of Dilworth[257] and Bergersen[104]).

Active isolated bacteroid suspensions[258] and diazotrophic cultures of free-living *Rhizobium*[259,260] liberate most of the fixed ammonia into the surrounding medium, which in functional nodules is rapidly assimilated into organic forms. Glutamate was the first labeled organic compound found following exposure of nodules to $^{15}N_2$.[261,255] However, Kennedy[256] and, more recently, Meeks and co-workers[262] and Ohyama and Kumazawa[263] demonstrated that amide groups become labeled sooner and more intensively than amine groups. After a period of controversy about the role of the ammonia-assimilating enzymes glutamate dehydrogenase, glutamine synthetase, and glutamate synthase (see review of Rawsthorne et al.[7]), it has become accepted that ammonia is excreted from bacteroids and assimilated directly into glutamine in the host-cell cytosol via the glutamine synthetase/glutamate synthease pathway,[262,264,265] although a possible role for bacteroids in the assimilation of ammonia cannot be entirely excluded[265] (see also the reviews of Dilworth[257] and Bergersen[76]).

A computer-simulation model for ammonia assimilation in nodules[266] demonstrated a role for glutamate dehydrogenase as a way to circumvent an irreversible buildup of keto
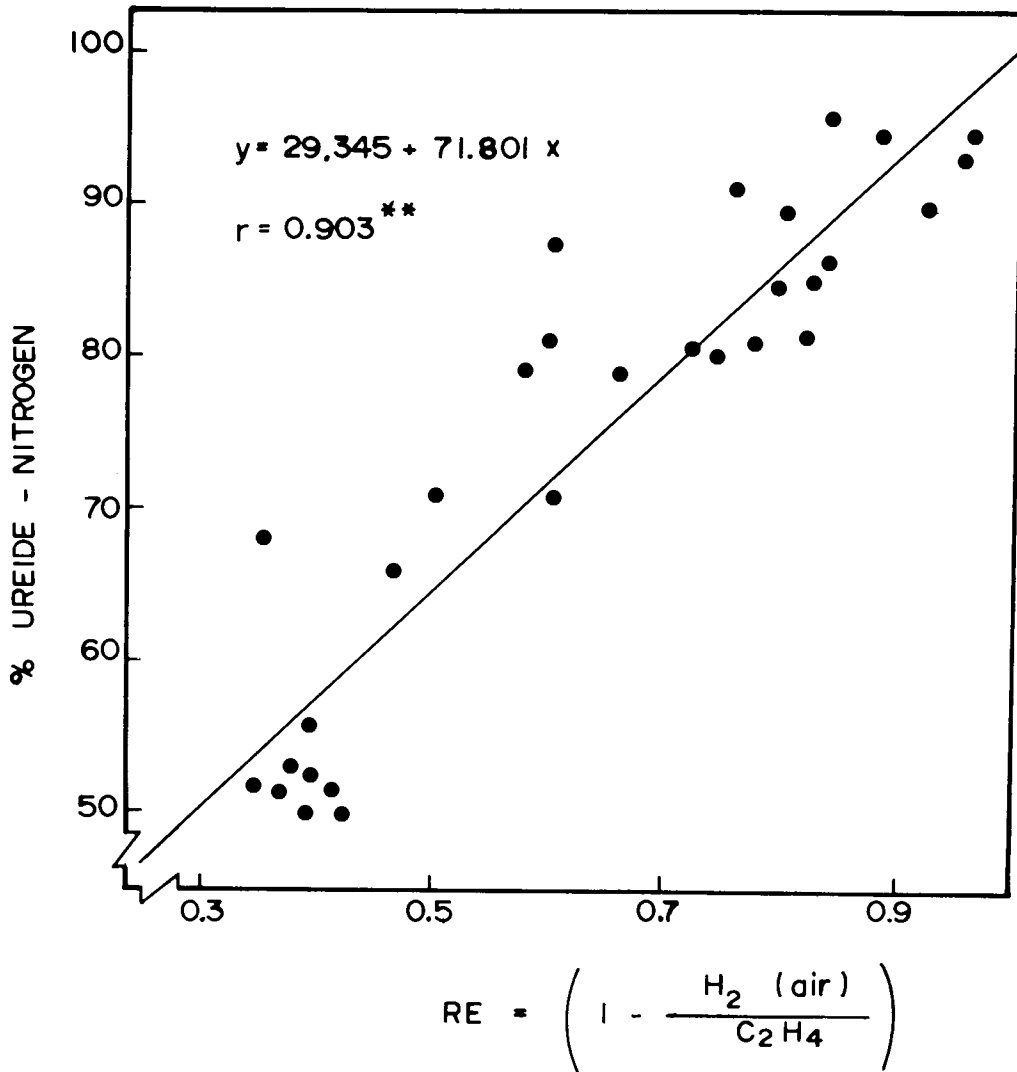
FIGURE 5. Relationship between nodule relative efficiency (RE) and percentage of ureide-nitrogen in the xylem sap of *Phaseolus* beans at 35 days after emergence. A total of 30 symbiotic combinations were used, including 5 plant cultivars and 6 *Rhizobium* strains. Points are means of four replicates. (After Hungria, M. and Neves, M. C. P., *Pesqui. Agropecu. Bras.*, 21, 127, 1986. With permission)

acids occurring whenever the supplies of ammonia and/or ATP where limited. Under these conditions, no restarting of ammonia assimilation was possible unless glutamate dehydrogenase was added to the model. Furthermore, the labeling pattern after incubation of nodulated roots of *Vigna unguiculata* with ^{13}N-labeled ammonia was consistent with assimilation via glutamate dehydrogenase in parallel with glutamine synthetase, which seems to indicate that species may differ in the way they assimilate the fixed nitrogen.[267]

Although glutamate and glutamine are the primary amino products of nitrogen fixation, legume species differ in the composition of the nitrogenous compounds which they export from the nodules (Table 8). The major exported compounds include the amides asparagine and glutamine, the ureides allantoin and allantoic acid, and, to a minor extent, amide derivatives such as 4-methylene glutamine and various amino acids.

Although this may prove to be an oversimplification, two categories of legumes, in terms

Table 8
MAJOR NITROGENOUS COMPOUNDS REPORTED IN XYLEM SAP OF NODULATED GRAIN LEGUMES

Species	Compounds[a]	Type of xylem analyzed	Ref.
Arachis hypogea	ALAN + ALAC, amides	Root exudate	330, 411
	γ-me-GLN	Root exudate of non-nodulated plant	412
Cajanus cajan	ALAN + ALAC	Root exudate	91, 413
Glycine max	ALAN + ALAC	Root exudate,	274
	ASN, ASP, HIS, GLN	nodule exudate	414
Lupinus albus	ASN, GLN, ASP, SER, GLU	Root exudate	352
Phaseolus	ALAC	Root exudate	275
vulgaris	ALAN + ALAC	Root exudate	101
Pisum sativum	ASN, GLN, HOM, ASP	Root exudate	309, 340
Psophocarpus tetragonolobus	ALAN + ALAC	Root exudate	297, 330
Vicia faba	ASN, GLN, ASP	Nodule exudate	311
Vigna unguiculata	ALAN, ALAC, GLN, ASP, ALAN + ALAC	Root exudate	283
	ALAN + ALAC	Root exudate	297
V. mungo, V. angularis, V. radiata	ALAN + ALAC	Root exudate	297

[a]   Abbreviations: ALAN, allantoin; ALAC, allantoic acid; ASN, asparagine; ASP, aspartic acid; GLN, glutamine; γ-me-GLN, methylene glutamine; HIS, histidine; HOM, homoserine; SER, serine.

of the major nitrogenous compound exported, have been proposed: the ureide- and the amide-producing species.[104,268] In general, legumes in which the major exported nitrogenous compounds are ureides are species of tropical origin belonging to the tribe Phaseoleae[269,270] which, with the exception of soybeans, have not received much research attention. Amide-producing symbioses, on the other hand, are temperate species (e.g., peas, lupins, *Ornithopus*) which have been intensively studied for a long period of time. The proposed pathway for ammonia assimilation into amides involves the initial glutamine synthetase/glutamate synthase pathway, followed by asparagine production via aspartate amino transferase and asparagine synthetase.[264,271]

Whereas amide synthesis has been the subject of intense investigation, very little attention has been given to how the metabolism of nitrogen in nodules proceeds (see Rawsthorne et al.[7]). This is probably one of the reasons why ureide metabolism in nodules was largely ignored, although ureides were detected by Fosse in leguminous plants as early as 1926.[272] Ureides were shown to constitute a major proportion of the nitrogenous solutes exuded from excised nodulated roots of soybeans[273,274] and *Phaseolus* beans,[275] but it was only after a number of papers by Japanese workers were published relating ureide accumulation with the presence of nodules[276-281] that the importance of ureides in nodule nitrogen metabolism of some leguminous species was realized. A decade of intense investigation on ureide production in nodulated legumes has shown that nodules are the sites of ureide synthesis[263,278,280,282,283] and that ureide production and export from nodules are reduced when plants are supplied with combined nitrogen.[101,172,274,278,281,284,285]

It has been also shown that in soybean nodules ureides are not directly synthesized via condensation of glyoxylic acid and urea[280] but via synthesis and degradation of purines.[286-288] This pathway also operates in nodules of cowpea[289] and *Phaseolus* beans.[271] The metabolic pathways involved in ureide synthesis in tropical legumes have been the subject of a recent review by Reynolds and co-workers.[290] Although there are some conflicting
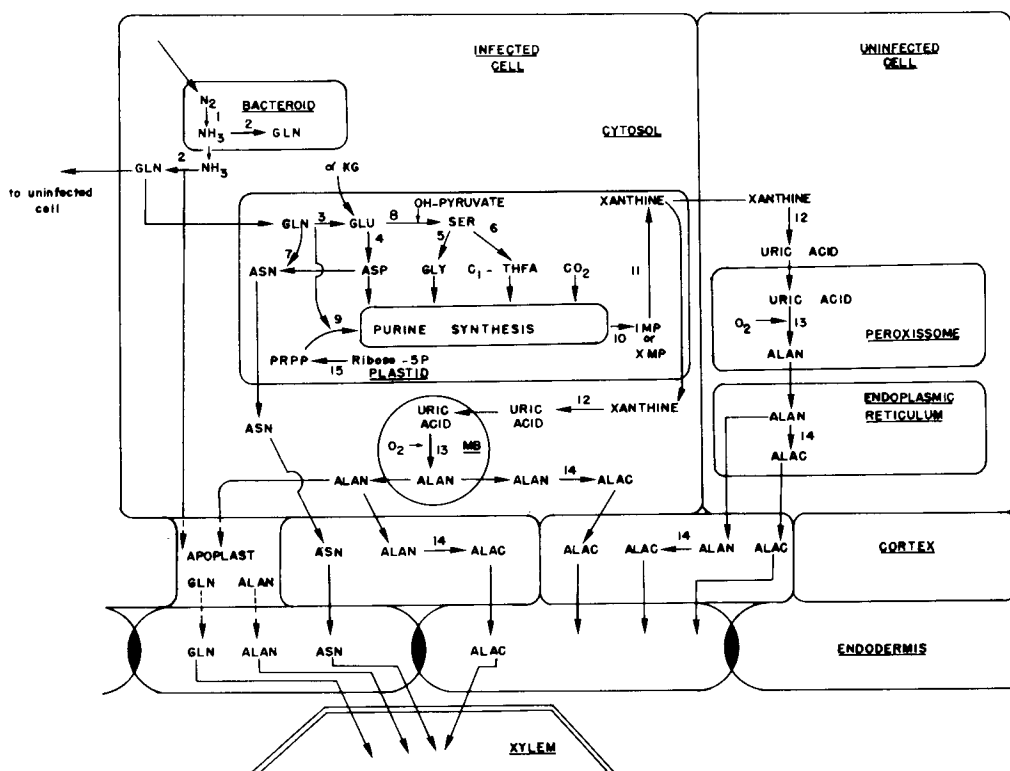
FIGURE 6. Model for the nitrogen assimilation and export in ureide-producing legume nodules. (1) Nitrogenase (E.C. 1.18.6.1); (2) glutamine synthetase (E.C. 6.3.1.2); (3) glutamate synthase (E.C. 2.6.1.53); (4) aspartate aminotransferase (E.C. 2.6.1.1); (5) serine hydroxymethylase (E.C. 2.1.2.1); (6) methylene tetrahydrofolate oxidoreductase (E.C. 1.5.1.5); (7) asparagine synthetase (E.C. 6.3.54); (8) phosphoglycerate oxidoreductase (E.C. 1.1.1.95); (9) phosphoribosyl amido-transferase (E.C. 2.4.2.14); (10) inosine monophosphate oxidoreductase (E.C. 12.1.14); (11) various steps; (12) xanthine oxidoreductase (E.C. 1.2.3.2); (13) urate oxidase (E.C. 1.7.3.3); (14) allantoinase (E.C. 3.5.2.5); (15) phosphoribosylpyrophosphate synthase (E.C. 2.7.6.1). Abbreviations used: ALAN, allantoin; ALAC, allantoic acid; ASN, asparagine; ASP, aspartic acid; GLY, glycine; OH-pyruvate, hydroxypyruvate; IMP, inosine monophosphate; MB, microbody; PRPP, phosphoribosylpyrophosphate; SER, serine; THFA, tetrahydropholic acid; XMP, xanthosine monophosphate. (Modified from Shelp, B. J., Atkins, C. A., Storer, P. J., and Canvin, D. T., *Arch. Biochem. Biophys.*, 224, 429, 1983. With permission.)

reports on the localization of the enzymes related to ureide synthesis (in bacteroid or in the host plant cytosol)[282,283,289] which arise from the complexity of the nodule tissue, a model for the compartmentalization of the purine pathway[291] has been proposed (Figure 6) based on studies of the cellular and subcellular location of some key enzymes[292-294] and also on electronmicrographs of soybean nodules.[295] Newcomb and co-workers observed the presence of peroxisomes mainly in the uninfected cells as well as greater smooth endoplasmic reticulum, suggesting a key role of these cells in the final steps of ureide production.[295] It has also been shown that the enzymes for the conversion of uric acid to ureides are located in both the infected and uninfected cells.[291,296] Since this is an oxygen-dependent step of purine degradation, the location of the enzyme in the uninfected cells is more probable because of the greater availability of oxygen in those cells compared with the infected ones.

Many reports on the metabolism of ureide-producing nodules have shown higher levels of glutamine relative to asparagine in the transpiration stream leaving the nodules.[283,297] Ureide production based on purine biosynthesis requires two amide nitrogen atoms originating from glutamine or asparagine and one nitrogen each from aspartic acid and glycine.[298] Since the amide group of glutamine is also used in the synthesis of asparagine by transamination,[264]

the pathway of ureide synthesis may in fact be competing with asparagine synthesis, as suggested by Pate and co-workers.[297] This would in turn explain the low levels of asparagine among the exported products. However, many other reports have failed to confirm the prevalence of glutamine over asparagine,[254,285,299-301] although in all these studies, the plants were grown entirely dependent on nitrogen fixation.

B. Transport of Fixed Nitrogen

The products of nitrogen fixation are not retained in the nodules.[302] In fact, fixed nitrogen is quickly exported to the shoot,[120,283,303,304] and the retention of some nitrogenous compounds, such as γ-amino butyrate in nodules of clover produced by some strains of *Rhizobium*, is related to the low effectiveness of these strains, which tend to form a greater mass of nodules than the nonaccumulating strains.[305] However, no data are available for tropical grain legumes, although, in soybeans, *Rhizobium* strains were shown to affect the nodule efficiency (nitrogen fixed per nodule weight),[306] which was later found to be reflected in increased seed yields of plants inoculated with those strains forming a smaller but more efficient nodule mass.[246]

Both in legumes and nitrogen-fixing nonlegumes, fixed nitrogen is transported from nodules to the host shoot via the xylem[307-310] through the transpirational stream. Indeed, this upward transport of fixed nitrogen is so efficient that portions of the root below the nodulated zone depend almost entirely on nitrogen recycled through the shoot and back, via phloem, to these distal regions, as was very elegantly shown by Oghoghorie and Pate.[310] The export process involves the pericycle cells of the vascular tissues which, in some species, are modified as transfer cells through the formation of elaborate finger-like wall ingrowths.[311] Transfer cells have been reported to occur surrounding the vascular bundles throughout the length of the nodules of *Vicia, Pisum, Trifolium, Ononis, Medicago,* and *Lupinus,*[311] which are species forming the indeterminate type of nodules (nodules with an apical meristem and vascular strands free at the apical end),[269] but are not found in legumes possessing nodules of determinate type (nodules of determinate growth, with vascular strands fused at the apex, forming a closed loop),[269] except at the junction of the nodule bundles with the root vascular system.[312]

Water required for the transport of nitrogenous solutes is acquired from mass flow of sugars into nodules via the phloem.[194] In addition, nodules may also absorb water from the surrounding medium and bleed easily from the cut vascular tissue when detached from the root[283,311,313] in a manner similar to the osmotically induced bleeding of decapitated roots. Nodules of the determinate type, which prevail among the tropical grain legumes, have also been reported to effectively retrieve water from adjacent root tissue.[314,315] In fact, the nature of the vascular system of the determinate nodules, forming a closed loop, may facilitate water flow through these nodules.[269] But whether this confers greater efficiency in terms of water uptake, relative to the indeterminate type of nodules with their open vascular strands, remains to be determined.

Great diurnal changes in the transport of fixed nitrogen are likely to occur in intact plants due to the variation in the transpiration rates, with more nitrogen being delivered to the shoot during the day.[112,316,317] The importance of the transpirational flow in the nitrogen-export process is illustrated by the diurnal patterns of nitrogen concentration in nodules and in the xylem sap. During the daytime, when transport is proceeding rapidly, the amides and amino acids of pea nodules are maintained at a basal level; but during the night, the concentration of these compounds greatly increases, indicating that the transport process is unable to keep pace with the rate of fixation in this legume species.[90] The same effect was observed when phytotron-grown plants were transferred to a more humid environment during the photoperiod.[90] In cowpeas and *Phaseolus* beans, the concentration of nitrogen in the xylem sap remains low during the day, but at night, sap concentration greatly increases until

the next morning when the onset of transpiration dilutes the nitrogen.[112,317] However, no apparent accumulation of nitrogen within the nodules has been observed, indicating that the osmotically attracted water can cope with the transport of fixed nitrogen during the night.[112]

The export of nitrogenous compounds from nodule to shoot is a selective and active process. In fact, the exported compounds are in much greater concentration in the nodule bleeding sap than in the bacteroidal tissues,[311,313,318] while others, such as glycine, valine, and γ-amino butyric acid,[311] appear to be actively excluded from export and accumulate in large amounts within the nodule of *V. faba* but not in the host xylem sap. The transfer cells have been implicated as the sites from which nitrogenous compounds are selectively secreted to the extracellular space of the nodule bundle.[311] In determinate nodules, an apoplastic transport of nitrogenous solutes which could simply leak out of the central tissue cells and selectively enter the xylem bundles through the endodermis has been proposed.[291] This was based on the evidence that only the existence of apoplastic transport can explain why allantoin and glutamine are found in the xylem sap; even so, the widespread occurrence and the high activities of allantoinase and of enzymes utilizing glutamine in nodule cells could easily prevent most of the allantoin and glutamine formed from reaching the xylem via a symplastic route.[291]

Analysis of xylem sap using the root pressure exudation technique has been used for a long time for studying the transport of nitrogenous compounds in legume plants.[309] Although the technique is not without criticism,[319] it is accepted as providing a reasonably accurate picture of the xylem transport of nitrogen. The vacuum-extracted "tracheal sap" from shoot segments is an alternative technique if spontaneous bleeding from roots does not occur.[320] However, it must be carefully checked if it resembles the true spectrum of nitrogenous compounds exported from nodules because lateral abstraction and/or addition of compounds are likely to alter the ratios.[321-323]

A series of experiments have been performed to study the transport of nitrogen from nodules to the shoot as well as the effect of physiological, nutritional, and environmental factors on the forms of nitrogen exported in the xylem. As mentioned before (Table 8), a variety of products are employed to transport the fixed nitrogen to the shoot. The carbon to nitrogen (C:N) ratio of these compounds ranges from 5:1 (glutamic acid) to 1:1 (allantoin or allantoic acid). The small C:N ratio of the ureides implies an economy in the carbon supplied by the host to transport fixed nitrogen. In fact, as discussed previously (Section III.B), the amount of carbon used per unit of nitrogen fixed is greater for lupin, an amide-producing plant, than for cowpea, a ureide-producing plant[100] (see Table 4); but since many other physiological traits also differ, it is not clear whether only ureide production contributes to the observed difference. Although the transport of ureides may represent a minor savings in terms of carbon recycling through the roots for plants of low nitrogen requirements[324] or fixing nitrogen at low rates,[100] it could be of major importance for a plant with a high nitrogen requirement and short crop duration, such as cowpeas, which may pump nitrogen upward at a rate of 20 to 23 mg per plant per day.[172,188]

Variations in sap composition have been observed during plant development of soybeans,[99,246,313,325] cowpeas,[172,283,326] and *Phaseolus* beans,[101,154,327] with the highest concentrations and/or percentages of total nitrogen as ureides being recorded at or after flowering. Whenever separation of allantoin and allantoic acid was performed, the latter has always been shown to be the predominant ureide in the xylem throughout plant growth.[154,252,283,285,328,329]

The effects of host plant genotype and *Rhizobium* strain on the transport of fixed nitrogen has produced some conflicting reports. In soybeans, cultivars were observed to affect not only the nitrogen flux to the shoot, but also the proportion of ureides in the sap of plants grown in the greenhouse or in the field.[254,330] Others have, however, failed to observe significant differences in the sap composition of soybean cultivars grown in the greenhouse

with supplemental lighting.[299] In field- or greenhouse-grown *Phaseolus* beans, cultivar effects on nitrogen-exudation rates and on the percent of ureides in the xylem sap were observed and related to increased plant growth and seed yield.[154,225,327]

As to the effects of *Rhizobium* strain on nitrogen transport, the reports are also diverse. The nitrogen-exudation rate has been shown to be affected by the inoculated strain in soybeans[252,254,328] and *Phaseolus* beans.[154] Significant differences in the percentage of nitrogen transported as ureides were observed in field-grown soybeans inoculated with different *Rhizobium* strains[246,330] and also in greenhouse-grown *Phaseolus* beans[154,225,248] and soybeans.[252,331] However, other authors have reported no significant effect of *Rhizobium* strain on the sap composition of plants grown in the greenhouse with supplemental lighting or in phytotrons.[186,299,328] *Rhizobium* strain effects on sap composition and exudation rates were also observed in peas, a temperate legume.[309,332] The lack of a significant strain effect on sap composition in some greenhouse and phytotron studies may be related to the fact that many plant processes are affected by light quality.[333,334] In the case of ureides, there are indications that light is either directly or indirectly involved in ureide metabolism, with the highest amounts accumulating in plants exposed to green and far-red light[335] (see also the review of Thomas and Schrader[336]).

Whereas cultivar and strain effects on exudation rates may only reflect changes in the root system, the observed effects on sap composition may indicate changes in either the nitrogen-assimilatory enzymes of nodule cytosol or in the availability of carbon skeletons to — or even a role of bacteroids in — the nitrogen-assimilatory processes of nodules (and see Kahn et al.[79]).

The nitrogen transport to the shoot is also affected by source-sink manipulations. The effect of light and consequently of carbon availability on the nitrogen metabolism of roots has long been demonstrated.[337] Under carbon-limited conditions, plants supplied with ammonia assimilate nitrogen into organic compounds having a low C:N ratio.[338] In such extreme situations, the ureides represent a mechanism for ammonia detoxification.[278,339] Shading to 75% of full light has been shown to increase the rate of nitrogen transport and to affect the composition of the xylem sap of *Phaseolus* beans, increasing the percentage of nitrogen transported as ureides.[44] While the effects on nitrogen fixation and transport were due to the lower temperatures associated with the partial shading, the effect on sap composition may be reflecting an adaptation to a carbon-limited condition. On the other hand, partial defoliation and flower removal did not alter the composition of the xylem sap, although the nitrogen flow rate was initially increased by flower removal and decreased by leaf removal.[49]

Several studies have provided data which have shown that nitrogen-fixing and nitrate-utilizing plants employ the same set of solutes for xylem transport[340,341] and therefore are physiologically similar.[342] While this seems to be the case with amide-producing species, a remarkable difference in xylem composition is observed in those producing ureides, depending on the source of nitrogen supplying the plants,[101,172,274,285,300,327,343] making the nitrogen-fixing plants physiologically distinct from those utilizing mineral nitrogen. Furthermore, mineral nitrogen affects the rate of exudation in both peas and *Phaseolus* beans,[248,341] probably as a result of the increased root dry matter production relative to nodule-dependent plants.[188] Although nonnodulated plants of *Phaseolus* beans, cowpea, or soybean produce ureides when grown on mineral nitrogen,[172,254,285,344] these compounds represent just a minor fraction of the total nitrogen transported in the xylem. Therefore, when nitrate is applied to nodulated plants, it not only inhibits nitrogenase activity but also significantly reduces ureide production, and nitrate becomes the predominant nitrogen form transported.[285,343,344] When ammonia is applied to nodulated plants, nitrogen fixation is also reduced, but although most of the ammonia is assimilated into amino acids, ureides are also produced from ammonia by plant roots.[343,344]

The environmental effects on nitrogen transport in plants have so far received little at-

tention. Temperature is an important factor when considering the solubility of compounds. Among the products most frequently exported by nodules, the ureides are the least soluble in pure solution,[345] and this could be a disadvantage to ureide-producing species when grown at low temperatures. However, the absence of ureide accumulation in nodules or other plant organs of *Phaseolus* plants grown at 15/10°C compared with those grown at 25/15°C[346] indicated that low temperatures have no specific inhibitory effect in either the assimilation of fixed nitrogen into ureides and subsequent transport from nodules to the shoot or their assimilation in the shoot organs.

Nitrogen transport from nodules to shoot largely depends on the transpirational stream, as previously discussed. Therefore, variations in water flux through the roots are likely to affect the rate of nitrogen export from nodules and/or the composition of xylem sap. In cowpeas and *Phaseolus* beans, a diurnal variation was observed in the concentration of ureides in the xylem sap, with a faster rate of nitrogen transport recorded during the day.[112,299,317] However, unlike peas, which are an amide-producing species where, as discussed before, nodules accumulate the products of nitrogen fixation whenever the transpiration rate is reduced,[90] cowpea nodules do not show any significant accumulation of ureides.[112] Furthermore, in cowpeas, more ureides relative to amides are transported during the night period.[112] Water-stressed cowpeas, however, showed significant retention of ureides within the nodules.[330] Ureide concentration of stressed nodules was almost three times that of control plants, while nitrogenase activity was reduced by 92%. Accumulation of ureides was also observed in roots and stems of water-stressed plants compared with controls. One day after the water supply was restored, the ureide concentration in the nodules was still high, while the levels of ureides in stems and roots did not differ from those of control plants.[330]

C. Nitrogen Distribution within the Shoot

Accumulation of nitrogen in non-nitrogen-fixing plants is greatly dependent on the mobilization of nitrogen from senescing vegetative organs, especially if little or no nitrogen is available for direct uptake and incorporation during pod development[347] or if the activity of the assimilatory enzyme (e.g., nitrate reductase) is reduced due to leaf senescence.[348,349]

In nodulated grain legumes, the process of nitrogen accumulation in the protein-rich seeds is far more complex. In addition to the above-mentioned sources, biological nitrogen fixation during the critical period of pod development may provide nitrogen for increased seed yields,[170,188,202,350,351] as long nodules receive a supply of carbon substrates. Therefore, nitrogen accumulation in developing legume fruits involves not only uptake of compounds directly supplied by the transpiration stream, but also intake from the phloem stream of compounds released from protein turn-over or mobilization of nitrogen reserves in the leaves.[352-355]

When considering the nitrogen transport through the xylem stream, emphasis must be given to the dynamic nature of the xylem composition. As discussed before, lateral abstraction of solutes from the ascending xylem sap has been observed in a number of plant species,[319] including some legumes. The changes in composition of the xylem sap collected at different heights of *Pisum arvense* demonstrate not only selectivity in the lateral abstraction, but also the capacity of the surrounding tissues to release nitrogenous compounds into, or remove them from, the stream.[321]

More detailed studies of nitrogen transport using labeled amino acids transpirationally supplied to detached shoots of lupins have revealed the diverse fate of different amino acids.[323] Some compounds (e.g., arginine) were effectively absorbed by the stems, which retained up to 78% of the labeled material accumulated in the shoot. Asparagine, glutamine, and valine are absorbed by stems with moderate effectiveness (43 to 58% of the total, respectively). Such compounds are involved in xylem-to-phloem transfer and are important

to the nutrition of the growing apex. Aspartic and glutamic acids are not loaded as such into phloem and are retrieved ineffectively by stems with as much as 80 to 90% reaching the leaf blades, therefore sustaining the more mature organs of the shoot.[323] Similar findings were reported for peas.[322] In soybean, the partitioning of labeled nitrogen supplied as nitrate revealed little retention in stem and petioles, and most was distributed to young and mature leaves.[356] Glutamine and asparagine were distributed relatively more actively to the young leaves with strong retention in the stem and petioles. Allantoin showed a pattern of distribution intermediate between that of nitrate and amides. Additional experiments at the grain-filling stage provided further evidence of asparagine retention in stems and petioles, with only little transformation and retranslocation to the grain, while allantoin was found to be transferred to and metabolized in both leaves and pods.[357] The fate of the anionic form of allantoic acid is, however, likely to be diverse. The selection process determining the contrasting patterns of distribution of nitrogenous compounds seems to involve ionic interactions of the cell walls with the solutes, promoting adsorption of cationic forms (e.g., arginine), and also membrane-based uptake sites in cells lining the xylem.[319] Great differences among plant species are likely to occur. It is interesting to note that when solutions of equal molarity for nitrogen in the form of nitrate, asparagine, or allantoin were transpirationally supplied to detached vegetative soybean shoots during a 2-hr period up to four times more nitrogen was taken up from allantoin in solution than from asparagine or nitrate,[356,357] indicating a somehow facilitated transport of allantoin in the xylem stream.

The nitrogenous compounds arising from the root system by the xylem stream are, therefore, not uniformly distributed between shoot organs. It follows that any cultivar, strain, or environmental effect on sap composition will affect the relative availability of the various nitrogenous forms to different plant tissues, which will ultimately affect the proportion of plant nitrogen allocated to the grain and hence is likely to affect seed yield. For nodulated peas, an amide-producing species, greater seed yield was associated with strains promoting higher levels of asparagine and aspartic acid in xylem sap,[332] whereas in soybeans and *Phaseolus* beans, greater seed yields and a more favorable nitrogen distribution to seed was recorded for those symbioses transporting nitrogen predominantly as ureides.[154,225,246] In *Phaseolus* beans, the most efficient strains not only promoted ureide production but also provided higher rates of nitrogen transport to the shoot,[154,225] whereas in soybeans, strain effects were mainly observed in terms of ureide production without any significant effect on the rate of nitrogen transported to the shoot.[246]

It has been claimed that because fruits have a relatively slow transpiration rate[62] direct nitrogen uptake from xylem represents just a minor contribution to seed nitrogen. The protein-rich seeds of grain legumes have, however, a higher requirement for nitrogen than the phloem alone can provide. A process leading to nitrogen enrichment of the ascending streams has been demonstrated as consisting of removal of nitrogenous solutes from the xylem stream and their subsequent reloading into the phloem (i.e., a xylem-to-phloem transfer),[52,323,353,358] and thus provides a continuous supply of nitrogen from nodules to the growing apices. The process plays an important role in lowering the C:N ratio of the sap delivered to the fruits and was found to be selective. While asparagine[358] was shown to actively participate in the xylem-to-phloem transfer, some amino acids (e.g., aspartic acid) participate very little in both xylem/phloem or direct phloem transport to the shoot apex.[358]

Similar studies with cowpeas demonstrated the effective transfer of ureides from xylem to phloem followed by translocation in the phloem to developing fruits and growing apices.[354] In soybeans, xylem-to-phloem transfer was estimated to provide 52% of the total seed nitrogen,[300] whereas in cowpeas, direct xylem transport supplied 28%; the remaining nitrogen is thought to be acquired from transport involving the phloem, which would also include the compounds transferred from the xylem.[355] More detailed data on the nitrogen flow are available for lupins. This legume was shown to acquire 40% of the fruit nitrogen directly

from the xylem and 58% from the xylem-to-phloem transfer, whereas only 2% is estimated to be supplied directly by the phloem.[353]

The direct xylem transport also provides a source of nitrogen which enters the fruit early in development[352,355] and may play an important role during a critical stage in pod growth[359] when abscission of buds, flowers, and immature pods is a major factor limiting grain legume yields.[360-362]

Among the factors implicated to explain the extensive loss of reproductive structures during the early stages of reproductive development of grain legumes is a lack of available assimilates and essential nucleic acids.[363,364] It is possible that the higher seed yields and nitrogen harvest indexes which have been observed associated with higher ureide production and transport in soybeans[246] are due to increased retention of reproductive structures promoted by a greater availability of ureide nitrogen to pods, although a hormonal effect of the *Rhizobium* strains cannot be discounted.[365] Many studies have provided data showing that fixed nitrogen is preferentially utilized for pod growth,[202,366] especially during the initial stage of pod development.[367] Fixed nitrogen is transported more quickly to pods and seeds than nitrate nitrogen, as was shown by more intense labeling of those organs soon after ^{15}N feeding.[368]

Balance sheets for nitrogen during plant growth and ^{15}N studies have been used to estimate the contribution to the seeds of the nitrogen recycled through or mobilized from vegetative organs, as well as to determine the efficiency with which nitrogen assimilated at different stages of growth is subsequently mobilized to the seeds. However, information is available for only a few cultivars of a limited number of species (Table 9). While the data stress the importance of direct nitrogen assimilation (mainly from fixation) during the pod-filling stage, which may provide from 40 to 78% of the seed nitrogen requirement, it is interesting to observe that a more favorable partitioning of nitrogen to seeds, and also higher seed yields, generally occurred in plants where direct transport (or a rapid flux through the vegetative structures[352]) of the nitrogen derived from fixation supplied more than one half of the total nitrogen incorporated into the seeds.

In many legume species, leaves appear to represent a more important source of mobilized nitrogen than the supporting organs,[3] either because they accumulate more nitrogen than any other vegetative tissue[369] or because the accumulated nitrogen can be more easily retrieved from leaves than from supporting structures.[351] However, in soybeans the remobilization of nitrogen from leaves and stems was found to be affected by the inoculated strain.[246,370,371] Furthermore, plants inoculated with the most efficient strains promoted a slow but more efficient remobilization of the nitrogen previously accumulated in the leaves, and more nitrogen was made available for seed production.[246] The observed effects of *Rhizobium* strain on nitrogen remobilization from soybean leaves to pods are probably due to differences in the nitrogen composition of the sap, which can promote either a leaf bypass or a rapid cycling through the leaves of the nitrogen coming through the transpirational stream. Morris and Weaver[371] observed substantial differences between the strains tested in terms of cycling of nitrogen through the leaves, and a slightly higher seed yield was related to little or no nitrogen being exchanged in the leaves, that is, more nitrogen being directly transported to the fruits.

In *Phaseolus* beans, the nitrogen harvest index was low (0.55 to 0.66), even in the best plant/*Rhizobium* symbiosis,[203] although 59 to 66% of the fruit nitrogen is thought to have been obtained directly from nitrogen fixation during pod development (Table 9). The superior performance of cultivar Negro Argel inoculated with strain CO5 (Table 9) resulted from the increased availability of fixed nitrogen to seed production, which was 20% higher than that of cultivar Rio Tibagi inoculated with the same strain and, as a result, nitrogen partitioning to seeds was increased. No significant difference was observed in the fraction of seed nitrogen originating from direct transport of fixed nitrogen, but plants inoculated with strain CO5

Table 9
SOURCES OF NITROGEN TO SEEDS, NITROGEN HARVEST
INDEXES, AND SEED YIELD OF GRAIN LEGUMES

Species/strain	Direct uptake	Remobilization (%)	Nitrogen harvest index	Seed yield (g/plant)	Ref.
Arachis hypogea	63	37	0.80	—	After 3
Cicer arietinum	57	43	0.73	—	After 3
Glycine max					
cv. Ranson/USDA 110	88	12	0.70	11.7	370
/USDA 31	85	15	0.68	4.4	370
cv. Davis/USDA 110	61	40	0.78	10.6	370
/USDA 31	81	19	0.74	4.4	370
Phaseolus vulgaris					
cv. Negro Argel/CO5	66 (8)[a]	10 (16)	0.66	7.8[b]	202
/127 K 17	66 (12)	7 (15)	0.61	6.6[b]	91
cv. Rio Tibagi/CO5	63 (19)	0 (18)	0.57	5.4[b]	91
/127 K 17	59 (18)	1 (23)	0.55	4.7[b]	202
Vicia faba	50	50	0.76	—	After 3
Vigna unguiculata					
cv. Vita 3	40	60	0.67	6.4[c]	117
cv. K2809	44	55	0.63	71.8[c]	369
	47	53	0.63	71.8[c]	415
/CB756	53	47	0.75	28.2	172
/+ 200 ppm N	31	69	0.65	38.4	172
cv. TVu 1503/CB1024	69	31	0.68	22.4	188
/R5028	78	22	0.72	31.5	188
cv. TVu 1469/CB1024	—	—	0.46	54.3	373
/R5008	—	—	0.59	133.3	373
/R5008 + N	—	—	0.56	116.4	373
cv. TVu 2321/CB1024	—	—	0.60	94.4	416
/R5008	—	—	0.65	147.2	416
/R5008 + N	—	—	0.56	121.7	416
cv. TVu 4552	47	53	0.64	22.2[c]	415

[a]   Numbers within parentheses are percentage of N derived from applied nitrogen.
[b]   Calculated assuming 3% N in seeds of *Phaseolus beans*.
[c]   Calculated assuming 3.8% N in seeds of cowpea.

remobilized the nitrogen previously accumulated in vegetative tissues more effectively (17% more than plants inoculated with strain 127K17).[203] Cultivar Rio Tibagi did not obtain enough nitrogen to satisfy its potential seed yield from the symbiosis with *Rhizobium*, even when supplemented with 2.5 mg nitrogen per plant per day.[203] The greater dependence of this cultivar on mineral nitrogen is characterized by the 18% lower contribution to seed production of the nitrogen derived from fixation.[203]

In soybeans, the picture is very complex (Table 9). Whereas higher seed yields of cultivar Ramson were related to strains promoting greater availability of recently fixed nitrogen to seeds, cultivar Davis attained higher yields when associated with strains promoting remobilization from vegetative structures.[370] On the other hand, two Brazilian cultivars, Santa Rosa and Savana, attained higher seed yields with strains promoting more efficient nitrogen partitioning to seeds.[246,372]

In cowpeas, the available data are for plants raised entirely dependent on biological nitrogen fixation (Table 9). The improved seed yield of the American cultivar TVu 1503 inoculated with strain R 5028 resulted from the maintenance of nodule activity throughout the pod-filling stage compared with plants inoculated with CB 1024.[188] The longevity of nodule

activity increased by 13% the fraction of seed nitrogen originated directly from fixation and improved nitrogen partitioning to seeds.[188] Nodulating plants, however, did not yield as much as plants supplied with mineral nitrogen, which probably reflects the breeding history of this cultivar. Higher seed yields of improved cowpea cultivars which originated from IITA's breeding program were obtained by those symbioses which maintained higher rates of nitrogen fixation well into the period of later pod-fill stage (Table 9). For cultivar TVu 1429 inoculated with strain R5008, higher seed yields corresponded to improved nitrogen partitioning to seeds, and the application of supplemental nitrogen equivalent to 60 kg nitrogen per hectare stimulated excessive vegetative growth in detriment of seed production.[373] Nitrate nitrogen applied to inoculated plants reduced seed yields by decreasing the rate of nitrogen assimilation during the later reproductive growth stage even though nitrogen assimilation was, on average, increased during the earlier vegetative stages of growth.[373]

The maintenance of an active transport of fixed nitrogen to the developing pods either directly or after a rapid flux through the vegetative structures seems, therefore, to play an important role in the improvement of the seed yields of grain legumes based on biological nitrogen fixation. The curtailment of the flow of fixed nitrogen to the developing pods as a result of an intrinsicaly premature nodule senescence soon after flowering in cowpea cultivar Caloona,[187] or by imposing complete leaf shading at mid-pod-fill stage to nodulated cowpea plants[351] or even by growth in an adversely hot environment,[169-171] dramatically restricted seed yields because the previously accumulated nitrogen could not be efficiently retrieved to benefit seed growth.

Then again, the remobilization of previously acquired nitrogen to supply the seeds seems also to be affected by the origin of the nitrogen accumulated in the shoot.[366,374] Symbiotically fixed nitrogen has been shown to be more mobile than nitrogen originating from soil or fertilizer,[375,376] thus suggesting the existence of different pools for fixed nitrogen in soybean, probably accumulated as ureides for later transfer to developing fruits. The products of nitrate reduction may have to satisfy the demands of the reducing organs before they are distributed to the growing pods,[304,322] therefore benefiting the vegetative rather than the reproductive growth in soybeans,[366] cowpeas,[350,373] *Phaseolus* beans,[202,377] and chickpeas.[170]

The observed failure of foliar-applied nitrogen to increase soybean yield although the concentration of nitrogen in leaves was increased[378] has been recently attributed to a limited capacity of the plants to load the nitrogen previously accumulated in the vegetative structures and transport it to the seeds,[379] and further illustrates the necessity of selecting cultivars which maintain active nitrogen fixation for as long as possible during the reproductive period, as well as selecting strains which produce active secondary nodule populations.

The fate of ureides in the shoot of tropical grain legumes is, therefore, of utmost importance. Accumulation of ureides in the shoot of nodulated plants actively fixing nitrogen has been observed in many tropical grain legumes.[273,276,278,279,281,283] The stems usually contain higher amounts of ureides on an organ basis than the leaves, amounting to as much as 60 to 75% of the total soluble nitrogen. Ureide contents of stems have been shown to decrease rapidly during seed development,[276,278,279,313] probably as a result of either ureide remobilization from stem to pods or degradation and reassimilation before transporting to fruits. This can be achieved by the ureide-degrading enzymes allantoinase and allantoicase present in stem.[380,381] The breaking down of allantoic acid may not even rely on enzymic activity occurring spontaneously due to the unstable character of this compound.

Ureides also accumulate in pods in amounts which may even exceed those of leaves and stems[278,279,382,383] and may represent from 50 to 70% of their soluble nitrogen[279] or 15% of their total nitrogen.[313]

The ureide level in the phloem of nodulated cowpeas was found to be very low (11% of total nitrogen) compared with the xylem sap (77% of total nitrogen) and did not differ from the phloem of plants supplied with nitrate nitrogen.[384] This seems to indicate that the ac-

cumulation of ureides in the pods of cowpea plants greatly depends on xylem transport. However, the fruit phloem in well nodulated soybeans was found to contain up to 55% of its nitrogen in the form of ureides,[300] thus indicating extensive transfer of ureides from xylem to phloem before entering the fruits. This transfer was also shown by the use of tracers.[354]

Recent studies about the fate of ureides in the fruits indicate that they are partly metabolized in the pods and partly transported to the seeds, where they are assimilated into amino acids and proteins.[354,381,383] The high activity of allantoinase in developing seeds suggests a rapid metabolism of ureides in this organ.[278,381,383] However, ureides are a poor source of nitrogen for the growth of isolated soybean embryos compared with glutamine and asparagine.[301,385,386] The data suggest that pods and seed coats play important roles in the metabolism of the incoming ureides in soybeans. In fact, allantoate-degrading activity, most probably mediated by an allantoate amidohydrolase, was more than five times greater in seed coats than in soybean embryos, with the activity being high enough to metabolize all available ureides before they entered the embryos.[387] A similar role for the seed coats was also reported by Coker and Schaeffer,[386] who observed that all nitrogen from allantoin was incorporated into seed protein in a manner similar to that of glutamine.

In *Phaseolus* beans, however, the allantoin-degrading activity is higher in the cotyledons and embryos than in the seed coats, in contrast with asparaginase activity, which was found to be higher in the cotyledons and seed coats. This suggests that at least for this species ureides may directly support embryonic growth.[388]

The products of ureide metabolism in the shoot are ammonia, glyoxylate and its derivatives, urea, and CO_2.[387] Loss of labeled carbon as CO_2, as was shown to occur by Atkins and co-workers[354] and by Coker and Schaeffer,[386] is probably the reason why tracer studies using ^{14}C-labeled allantoin resulted in low activities associated with the various seed tissues[301] and were unable to estimate the real importance of ureides to protein production of developing soybean seeds.

VI. STRATEGIES FOR IMPROVING SEED YIELD BASED ON BIOLOGICAL NITROGEN FIXATION

To obtain maximum seed yields based on biological nitrogen fixation, nodules must provide adequate nitrogen to the plant during early vegetative growth and then continue to fix at sufficiently high rates for a prolonged period throughout pod development. Unless the available soil nitrogen is very high, early nodulation is important to provide good plant establishment. Large variations among *Rhizobium* strains have been reported for many legume species in the various steps of nodulation and nitrogen fixation, but increased nitrogen assimilation into vegetative plant parts may not always be translated into increased seed yields.

The role of vegetative organs as storage tissue for providing nitrogen to developing fruits is important. Far more important, though, is the maintenance of nitrogen fixation during pod development and the form of nitrogenous products exported from nodules to shoots, which affects the partitioning of nitrogen in the shoot and consequently affects seed yields, as it was observed with some tropical grain legumes, where the incorporation of fixed nitrogen into ureides seemed to increase the nitrogen harvest index and seed yields.

Maintaining nitrogen fixation during the period of grain filling depends on a continous supply of photosynthates to the nodules. Strategies envisaging a more efficient use of energy by the nodules seem to be more promising than the increase in plant photosynthesis. There are possibilities of improving plant photosynthesis, but other functions may interact with carbon supply and be implicated as possible limitations of nodule functioning. Also, plants seem to increase the photosynthetic efficiency to meet nodule needs in a source/sink relationship. Nodules are richer in protein than other plant organs, and the costly synthesis of

proteins[389] consumes carbon that otherwise would be used for plant growth. However, strains have been reported which form nodules with greater specific activity. Energy utilization in the process of nitrogen fixation depends also on an active uptake hydrogenase system, but this process is still controversial even though a majority of the results show a positive effect of low hydrogen evolution on various growth parameters. To date, however, no data are available yet which directly relate differences in carbon utilization with differences in seed yield of grain legumes.

Possible relationships among uptake hydrogenase activity and the nodulation capability, nodule efficiency, nodule senescence, CO_2 fixation activity, metabolism, and transport of the nitrogen fixed seem to show that the hydrogen metabolism of bacteroids can affect a much broader spectrum of nodule metabolic processes than was initialy imagined. High activities of phosphoenol pyruvate carboxylase were found in nodules of many legumes and may play an important role in the processes of assimilation of the fixed nitrogen, in the synthesis of carbon compounds to be used as energy sources, and in the synthesis of compounds used for pH control.

The importance of all these physiological factors leads to the consideration that efforts should be directed to quantify the various processes involved in nodule functioning and their mutual relationships in order to result in the best plant performance.

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