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Some Aspects of the Biology of Nitrogen-Fixing Organisms [and Discussion]

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Some aspects of the biology of nitrogen-fixing organisms

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[Plate 1]

Eukaryotic organisms do not fix nitrogen. Animals generally have no need to do so because of their complex food-acquisition and waste-disposal systems. Plants, by using carbon polymers for structural purposes, minimize their need for nitrogen. When very nitrogen-limited, to enter into symbiosis with nitrogen-fixing microorganisms may be the most controllable method for eukaryotes to obtain fixed nitrogen.

Filamentous, heterocystous nitrogen-fixing cyanobacteria may be better adapted to a free-living than to a symbiotic existence, because of their complexity. In symbioses, their photosynthetic machinery becomes redundant and the need to differentiate heterocysts as well as derepress *nif* genes may be a disadvantage. This could in part account for the greater success of symbioses involving the structurally simpler genera *Frankia*, *Rhizobium* and *Bradyrhizobium*.

Nitrogen fixation by legume nodules can be controlled by varying the oxygen supply. This control may be effected by a variable diffusion resistance, enabling oxygen required for ATP synthesis to be matched to available photosynthate. Such a resistance, which is probably located in the nodule cortex, may also be used to reduce nitrogen fixation in the presence of combined nitrogen and could also facilitate rapid responses to other forms of stress. Alternative resistances to gaseous diffusion may operate when water supplies are restricted.

Rhizobium and *Bradyrhizobium* follow different patterns of differentiation into nitrogen-fixing bacteroids. These patterns are coupled with retention or loss of viability and with significant or no natural enrichment of the bacteroids with ^{15}N respectively. The basic patterns of each type are subject to host-modification.

Recent studies on structures of primitive legume nodules show some parallels both with actinorhizas and with nodules on *Parasponia* induced by *Bradyrhizobium*. In particular, distribution of rhizobia in nodule tissues is intercellular and infection threads are formed only when bacteria 'enter' host cells; there is no intracellular 'bacteroid' stage. These threads are retained in the active nitrogen-fixing cells. Many legumes and some actinorhizas are not infected via root hairs. Therefore two of the stages often considered typical of the development of effective legume nodules, i.e. 'release' of bacteria into vesicles bounded by peribacteroid membrane and infection through root hairs, can be omitted; these omissions may be of use in attempts to transfer nodulating ability to new genera.

1. Introduction

All but one (the archaebacteria) of the major categories of nitrogen-fixing organism were described by the turn of the century. However, many new examples within these categories are still being recorded and much remains to be discovered about their biology and their role in global nitrogen cycling. This paper begins by addressing the problem of why nitrogen fixation does not extend to eukaryotes, then considers selected aspects of nitrogen-fixing symbioses, and finally discusses briefly the problems of estimating the extent of nitrogen fixation.

2. Why do only some types of organism fix nitrogen?

There are no confirmed reports of nitrogen fixation by eukaryotic organisms. Prokaryotes (eubacteria) may have evolved the ability to fix nitrogen shortly after they began to fix carbon dioxide (by non-oxygen-evolving pathways) and when non-biological supplies of combined N, (NH_3 or NO_x) were insufficient to sustain simple organisms having a C:N ratio with apparently fixed limits between 3 and 6 (Sprent & Raven 1985; for alternative points of view see Postgate (1982, 1985)). Recent evidence that some methanogenic archaebacteria can fix nitrogen (Murray & Zinder 1984) is consistent with early evolution of nitrogen fixation. None of these nitrogen-fixing organisms is obligately so; all can use combined nitrogen in preference to N_2 when both are available.

Why do no eukaryotes fix nitrogen? In animals, their mode of nutrition involves a food-acquisition system (mouth, pseudopodia, etc.) and a waste-disposal system (anus, contractile vacuoles, etc.). This enables them to live on diets with a very wide range of C:N ratios and, more especially, available-C:available-N ratios. Excess of one or the other is voided. Under certain excess-C diets (which usually involve cellulolytic endosymbionts), N_2-fixing organisms may be found in the gut (termites, pigs, humans) (see discussion in Postgate (1982)). When these animals have a very low-N diet (for example, humans whose staple diet is sweet potatoes) nitrogen fixation may be of significance to the host, but the benefit is usually biased towards the microsymbiont. Only two situations are currently known where the host animal naturally derives a *significant* fraction of its nitrogen from a symbiont. In both these cases – arboreal termites (Prestwich & Bentley 1981) and shipworms (Waterbury *et al.* 1983) – the host has a carbon-rich diet and a potential waste-disposal problem (see Sprent & Raven (1985) for further discussion).

Plants are nutritionally quite different. Their ability to fix carbon dioxide has enabled them to use carbon-based polymers as structural material so that their need for N (compared with that of animals) is reduced. Unlike animals, most plants can assimilate nitrate, by a process very similar to that of many aerobic microorganisms. Did plants acquire this ability from prokaryotic ancestors? If so, why did they not also acquire the ability to fix nitrogen? Biologically, the assimilation of nitrate is a very versatile process, being variable in both space and time within a plant (Sprent 1987). Associated problems, such as pH regulation, can be dealt with in a variety of ways (Raven 1985). The problems of nitrogen fixation, particularly the sensitivity of nitrogenase to oxygen, are far less easily overcome, although suggestions have been made as to how this could be achieved by genetic engineering if *nif* could be introduced into plants (Merrick & Dixon 1984). In natural systems, this does not yet appear to have occurred, plants always relying on some form of symbiosis when they have a major N-deficiency problem (note that when this is accompanied by deficits of other nutrients, including water, nitrogen fixation is unlikely to be the best solution (Sprent 1985)). Plant symbioses are arguably the most advanced extant terrestrial nitrogen-fixing systems. However, even free-living nitrogen-fixing organisms seldom occur in pure culture (blooms of heterocystous cyanobacteria approach this, but may be a special case). Some facets of the relationships between nitrogen-fixing organisms and their cohabitants will now be discussed. For reasons of space, and also because there have been numerous recent reviews of the subject (see, for example, Skinner & Uomala 1986), loose associations between plant roots and azospirilla or other nitrogen-fixing bacteria will be omitted.

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Metabolic complementation: syntrophy

Microbes which do and do not fix nitrogen may exist in such close physical proximity as to make it difficult to separate the individual species in pure culture. One common type of association consists of an oxygen-consuming component (providing a low-oxygen environment) and a nitrogen-fixing component, for example *Pseudomonas* and *Clostridium* respectively (Line & Loutit 1973). Because the only known nitrogen-fixing organism that can hydrolyse cellulose is the highly specialized shipworm endophyte (Waterbury *et al.* 1983) a natural association between cellulolytic microorganisms and nitrogen-fixing bacteria may be of ecological significance and also suitable for exploitation in, for example, the conversion of waste straw into fertilizer. Two such systems presently being studied are the fungus–bacterium pair, *Trichoderma harzianum* and *Clostridium butyricum* (Veal & Lynch 1984) and the bacterium–bacterium pair, *Cellumonas* sp. and *Azospirillum brasilense* (Halsall & Goodchild 1986).

Heterocystous cyanobacteria are the most highly differentiated nitrogen-fixing organisms. Heterocysts are well constructed to control oxygen entry and yet admit sufficient nitrogen (Walsby 1985), thus enabling nitrogen fixation to proceed there and leaving the vegetative cells to fix carbon dioxide. Thus cyanobacteria have little need for associated microorganisms and indeed a major problem could be competition from other cyanobacterial species. The possible ecological role suggested by Flores & Wolk (1986) for bacteriocins produced by some heterocystous cyanobacteria (e.g. *Nostoc* sp.) thus seems entirely appropriate.

Advantages of symbiosis with a plant

The major advantages are associated with life in a terrestrial habitat. Inside a plant sporophyte (the only known gametophytic host plants are genera of liverworts (e.g. *Blasia*) and hornworts (*Anthoceros* spp.)) a nitrogen-fixing microorganism is protected from desiccation, and gains potential access to all the nutrients available to an extensive root system. There are three broad categories of such symbioses, those involving cyanobacteria, *Frankia* and *Rhizobium* (this term will, in general discussion, be used to include *Bradyrhizobium*); these will be considered in turn.

Cyanobacterial symbioses

These are formed with fungi (in some two- and all three-membered lichens) and all major green plant groups (for a recent review see Peters *et al.* 1986*a*). With the exception of cycads, where symbiosis seems to be universal, occurrence is very uncommon. This, together with recent evidence on evolutionary relationships between green plant groups (Hori *et al.* 1985) (figure 1) makes it difficult to envisage a common ancestor for cyanobacterial symbioses. Separate events are certainly consistent with a rather simple mode of entry of cyanobacteria into leaf pockets, as in the liverworts, hornworts and *Azolla*. They may share these pockets with, and may even be outnumbered by, certain eubacteria (Wallace & Gates (1986) and references therein). Other apparently casual associations occur, for example when *Nostoc* inhabits crevices and curled 'leaf' margins of the leafy liverwort *Porella navicularis* (Dalton & Chatfield 1985) or the water-filled hyaline cells of the bog moss *Sphagnum* (Granhall & Hofsten 1976). In cycads, the cyanobacteria are localized between cortical cells of specially modified coralloid roots (occasional entry into host cells has been found, for example in *Cycas revoluta* (Grilli 1963)). The only angiosperm genus (*Gunnera*) known to harbour cyanobacteria does so intracellularly, in

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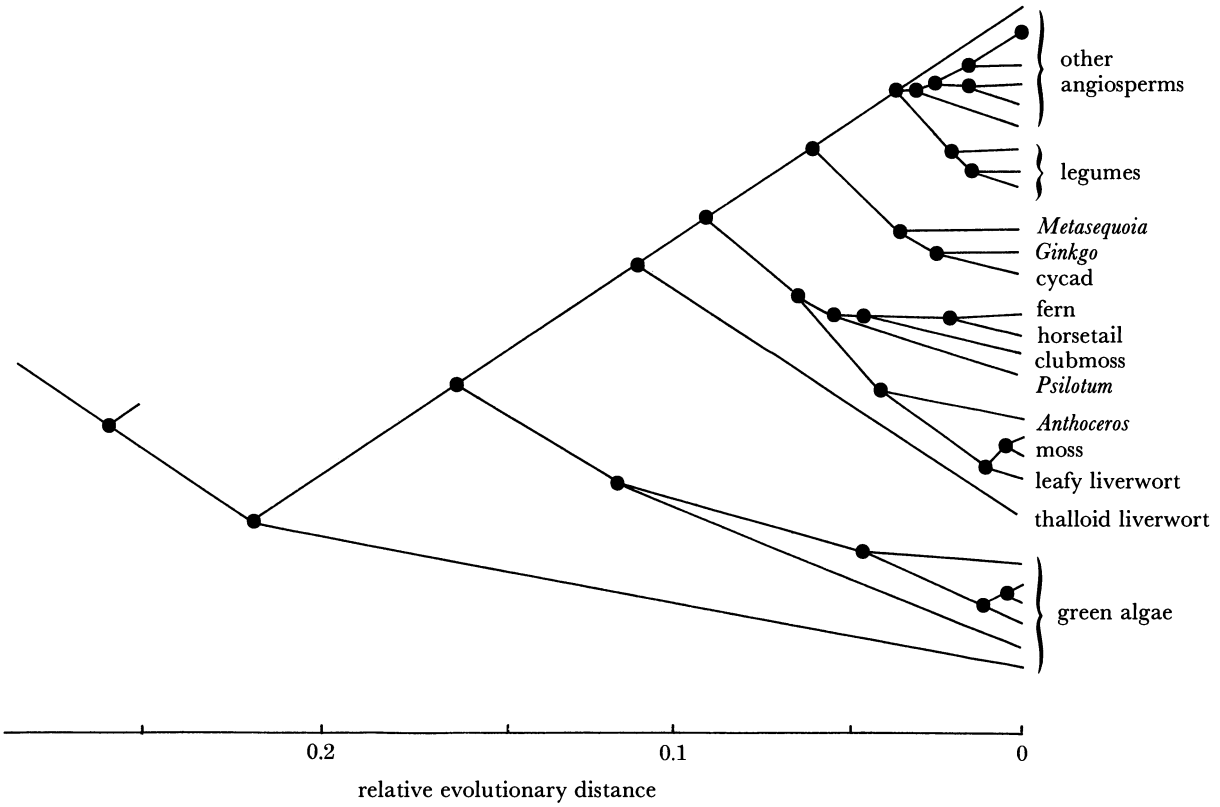

FIGURE 1. Possible evolutionary relations between green plants, based on 5S rRNA sequences. (Modified from Hori *et al.* (1985).)

cells deep inside the bases of the leaf petioles. In the more advanced plants the endosymbiont is located (i) in more specialized structures and (ii) further away from the host outer surface. These features give increased protection and decreased access to light. For example, in cycads, especially those such as *Zamia riedlii* in which the coralloid roots are subterranean (Halliday & Pate 1976) and *Gunnera*, the cyanobacterial component *cannot* photosynthesize and yet the vegetative cells possess all the necessary machinery (Lindblad *et al.* 1985). Even when light is available, as in the liverwort, hornwort and *Azolla* systems, the cyanobacteria photosynthesize very little, if at all. Instead, they obtain carbon from their host, as shown clearly for *Anthoceros* by Stewart & Rodgers (1977). In return, about 90% of fixed N may pass to the host for assimilation (Meeks *et al.* 1985). This suggests that selection pressures favoured nitrogen fixation at the time these plants began to colonize land. It is not yet known whether the host plant suppresses endophyte photosynthesis. Because, when actively fixing nitrogen, the endophyte has ceased growth, demand for photosynthate is low and mainly confined to the needs of the nitrogenase-enzyme complex.

In liverworts, hornworts and *Azolla*, structural modifications are found in the host cavities housing the cyanobacteria. For example, in *Azolla* (Calvert *et al.* 1985), there is a marked developmental sequence in the formation of hairs by the host. In the apical portions of the fronds, where the endophytic *Anabaena* is still growing, host hairs supply it with fixed nitrogen; later, after nitrogen fixation has begun, hairs transport ammonium fixed and secreted by the endophyte to other host cells for assimilation. A different population of hairs is developed to

supply *Anabaena* with fixed carbon. Modifications of cells are also found in other hosts (Stewart *et al.* 1983). In *Zamia skinneri*, for example, transfer cells traverse the cyanobacterial zone (Lindblad *et al.* 1985): this type of cell is normally found where there is intense local exchange of nutrients.

The cyanobacteria also show modifications, most notably an increase in frequency of heterocysts, to which nitrogen fixation is probably restricted (Bergman *et al.* 1986). An increase in the proportion of cells that are heterocysts is not, however, always correlated with increased nitrogen fixation (Lindblad *et al.* 1985). Further, in cycad coralloid roots and other cyanobacterial symbioses, akinetes may be found. These resting cells are normally associated with adverse conditions (Sutherland *et al.* 1979). In *Gunnera* many of the *Nostoc* cells appear to be degenerate at all stages of the development of the symbiosis (Towata 1985). All endophytic cyanobacteria are heterocystous, filamentous species, forming motile hormogonia which may assist in invasion of the host (Stewart *et al.* 1983). This degree of differentiation, important for free-living forms in aerobic environments, may, however, be disadvantageous in symbioses because of the presence of essentially redundant photosynthetic cells and the need to switch on genes for heterocyst differentiation as well as those for nitrogen fixation (Haselkorn 1986). Collectively, these facts suggest that cyanobacterial endophytes may not be particularly well adapted to symbiosis. Perhaps it is not surprising that only one angiosperm genus has retained (or ever had ?) an endosymbiotic cyanobacterium. Why unicellular nitrogen-fixing cyanobacteria (e.g. *Synechococcus*) (Huang & Chow 1986) did not enter into symbiosis is a mystery; apart from their lack of motility, they seem to be better suited than heterocystous forms.

Actinorhizas and legume nodules

There is little evidence that either *Frankia* or *Rhizobium* fixes significant amounts of nitrogen *ex planta* in the field. Nitrogen fixation requires the plant and is to a large extent under the control of the plant. This control can be exerted at many levels; one mechanism found in some functional legume nodules is by control of oxygen supply. In the early stages of nodule differentiation, control of oxygen level may also be necessary for nitrogenase induction (see, for example, Regensburger *et al.* 1986).

Once a nodule has started to fix nitrogen, the endophyte needs matched supplies of energy-yielding substrates (ultimately derived from host photosynthesis) and oxygen to generate ATP from these substrates. Fine control of oxygen supply to bacteroids is mediated in legumes by haemoglobin, which ensures a high flux at a low concentration (Appleby 1984). In non-legumes, low levels of haemoglobin may be supplemented or replaced by other substances with an apparently similar role (e.g. in *Parasponia* (Sandeman & Gresshoff 1985)). In addition, there are structural controls on oxygen diffusion, such as vesicle walls in most *Frankia* symbioses (Murry *et al.* 1985) and host-cell barriers in legume nodules. The latter may exert an overriding control on nitrogen-fixing activity and stem from the fact that, because photosynthesis varies in a fluctuating environment, the oxygen supply must also be varied. The most efficient way to do this is to have a variable oxygen-diffusion resistance (analogous to stomata in leaves (Sheehy *et al.* 1983, 1985)). This avoids oxygen inactivation of nitrogenase when photosynthate supplies are low and ensures that, when plentiful, photosynthate supplies are fully utilized. Such a variable resistance could also act as a control point to suppress nitrogen fixation when supplies of combined nitrogen are available, thereby saving energy. This has been shown for white clover supplied with nitrate, when the oxygen-diffusion resistance in nodules rose by a

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factor of five (Minchin *et al.* 1986). Because there is evidence that the diffusion resistance includes a water-filled pathway of variable length, it is not surprising that water deprivation has a direct effect on nodule activity (Durand *et al.* 1987 and references therein). To protect a water-filled pathway mechanism, it may be necessary for the cellular reaction to water deficit to be located at a different site. Collapse of surface lenticels and/or shrinkage of intercellular spaces have been implicated (Pankhurst & Sprent 1975*a*, *b*; Ralston & Imsande 1982); recent simulations of the effects of water deficits, based on the diffusion resistance model, have reproduced previous experimental results (Sheehy *et al.* 1985).

The variable oxygen-diffusion barrier, which is located in the cortex of nodules, has only been shown for advanced legume species. Whether it also occurs in the primitive legume nodules described below, or in actinorhizas, remains to be determined. Many perennial nodules have cortical phellogen, which may produce cork or aerating tissue according to the environment (Sprent 1985). Thus nodules may control oxygen diffusion in a variety of ways which both optimize nitrogen fixation and minimize stress damage. This type of on-site control is very effective, allowing, for example, nitrogen fixation after water stress to be resumed more quickly than photosynthesis, presumably by using photosynthate reserves (see, for example, Albrecht *et al.* 1984).

Comparative physiology of legume nodules

Legume nodules vary greatly in size, shape and structure and in the rhizobia which they host. Although only a few have been studied in sufficient detail, it is becoming possible to evaluate some the different ways in which the two symbionts interact to form the effective nodules which may be vital to the ultimate survival of both. This section will discuss some recent findings and attempt to integrate some diverse data into a general framework.

(*a*) *Comparison of Rhizobium- and Bradyrhizobium-induced nodules on the same host*

These two genera, roughly corresponding to the old categories of 'fast'- and 'slow'-growing rhizobia (see Trinick 1982; Pankhurst 1986 for general reviews) probably diverged early in bacterial evolution (Stackebrand & Woese 1984; Hennecke *et al.* 1986). Several cases are known where both slow- and fast-growing rhizobia nodulate the same host, the most widely studied being those nodulating *Lotus* spp., *Glycine max* (soybean) and *Lupinus* spp. Because the data for *Lotus* are the most comprehensive, they will be considered in detail (table 1). Clearly, the genus of endophyte affects almost all characters measured, both structural and metabolic. Note, however, that bacteroid viability and ^{15}N enrichment are similar.

(*b*) *Comparison of nodules formed by the same strain of endophyte on different hosts*

The host genotype generally controls nodule morphology. Because rhizobia show host specificity, nodule morphology is often confounded with bacteroid morphology. However, there are some cases where the same rhizobial strain will nodulate several hosts, and may inhabit nodules of different morphologies. Bradyrhizobia tend to be more promiscuous than rhizobia and thus have been more widely studied in this context, especially the various cowpea strains which nodulate either (i) *Vigna* species and *Arachis hypogaea* or (ii) various tropical legumes, such as *Macroptilium* and the non-legume *Parasponia*. Discussion of the latter will be deferred to the next section.

Table 2 lists the differences in bacteroid properties between *Vigna* and *Arachis* containing the

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TABLE 1. FEATURES OF NODULES INDUCED BY *BRADYRHIZOBIUM* SP. (*LOTUS*) OR *RHIZOBIUM LOTI* ON *LOTUS* SPP.

(Data from Wood *et al.* (1985) for strains 814 and 2037 respectively and *L. pedunculatus* except where specified.)

character	*Bradyrhizobium* sp.	*R. loti*
nodule size	smaller	larger
nodules per plant	fewer	many
proportion of nodule occupied by infected tissue	larger	smaller
N_2ase per unit nodule weight	high	low
peribacteroid space	small	large
bacteroid size/μm	0.54–1.0 × 2.0–3.1	0.23–0.46 × 1.5–2.0
bacteroid nucleoids	prominent	less distinct
bacteroid polyhydroxybutyrate	increase with nodule age	rare
bacteroid polar bodies	prominent	not observed
bacteroid viability (Sutton & Paterson (1980)	low (various strains)[a]	low (various strains)[a]
^{15}N enrichment (Steele *et al.* 1983)	moderate	moderate

[a] Various strains, growth type not always known to present authors.

TABLE 2. EFFECT OF HOST GENOTYPE ON NODULES INDUCED BY THE SAME RHIZOBIAL STRAIN

(Data for *Arachis hypogaea* and *Vigna unguiculata* inoculated with *Bradyrhizobium* sp. (cowpea) 32H1 except where specified. Characters 2, 3, 4 and 5 from Sen & Weaver (1984); characters 6 and 7 from Sutton & Paterson (1980), where *V. radiata* was classified as *Phaseolus aureus*.)

character	*Arachis*	*Vigna*
1. Nodule shape (Corby 1981)	aeschynomenoid	desmodioid
2. Nodule diameter/mm	1–3	1–3
3. Nature of infected tissue	uniform, with no interstitial cells	interstitial cells present
4. Bacteroid shape	spherical	rod-shaped
5. Volume of one bacteroid/mm^3	15.7	3.5
6. Bacteroid viability	low (CB756)	low (based on *V. radiata*)
7. ^{15}N enrichment of nodules	zero or slightly negative	moderate (176A22)

same rhizobial strain. Note that, in addition to bacteroid morphology and nitrogen fixing activity, ^{15}N is enriched in *Vigna* (typical of results obtained for ureide-exporting legumes (Shearer *et al.* 1982), but not in *Arachis*. Further, the viability of bacteroids (growth on agar plates) is affected by the host. The latter is rather a general phenomenon (Sutton & Paterson 1980) which is also correlated with other nodule features (Sprent 1980).

(*c*) *General interactions between hosts and rhizobia*

How unique are the properties associated with nodulation? Clearly there are sets of unique factors produced by the host in response to the rhizobia (nodulins) or by the rhizobia in response to the host (bacteroidins). For an excellent review of these, see Verma *et al.* (1986). Some other features, unique to particular systems, are listed in table 3. However, several authors have recently suggested that legumes and rhizobia also mutually enhance many of their inherent individual characters. Thus it has been argued (Pueppke 1984) that perhaps too much emphasis has been given to chemical specificity (e.g. lectins) in relation to adhesion of bacteria to the host surface and not enough to natural electrostatic forces (see also Miller *et al.* (1986) for a possible role for endogenous ion currents in attraction of rhizobia to plant surfaces). This line of thought is most elegantly supported by the demonstration of Peters *et al.* (1986*b*) that

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TABLE 3. SOME NOVEL COMPOUNDS (EXCLUDING NODULINS AND BACTEROIDINS) FORMED BY LEGUME (L) OR RHIZOBIUM (R) AND WHICH MAY HAVE A ROLE IN INTERACTION WITH THE OTHER SYMBIONT

compound	origin	comment	reference
luteolin	L	derepresses some *nod* genes	Smith *et al.* (1986)
glyceollin (isoflavonoid)	L	in some fix$^-$ soybean nodules, leads to a breakdown of peribacteroid membrane. May stop full host reaction to endophyte	Werner *et al.* (1985)
rhizolotine (opine-like compound)	L	formed by *Lotus tenuis* and specifically catabolized by *R. loti* NZP 2037. Similar substances in other spp. nodulated with fast-growing rhizobia	Shaw *et al.* (1986*b*)
graft-transmissible shoot factor from *Pisum sativum* cultivars	L	promotes uptake hydrogenase activity in some *R. leguminosarum* strains	Bedmar & Phillips (1984)
novel peptides (rhizobins)	R	released by *B. japonicum* into soil and from soybean nodules into soil. Found in free amino acid pool of soybean and lupin, but not alder nodules	Garay *et al.* (1986)
novel amino acids	R	formed by some strains of *R. loti*, depending on host	Shaw *et al.* (1986*a*)
rhizobitoxine (a substituted glycine)	R	produced by some strains of *B. japonicum* and induces foliar chlorosis in host. Similar substances produced by 56% of bradyrhizobia tested	Devine & Breithaupt (1980) LaFavre & Eaglesham (1986)

the common plant metabolite luteolin can activate some *nod* genes (i.e. it is not a unique property of legumes to produce such derepressing agents). The argument has been developed at a metabolic level by Kahn *et al.* (1985) in a very interesting hypothesis which is summarized and extended in figure 2. Essentially, it is proposed that rhizobia need a source of energy and that this is supplied by the plant in the form of amino compounds, which the bacteroids deaminate, returning the ammonium to the host. An integral part of such a system is a series of membrane-bound transporters, located both at the bacterial surface and at the peribacteroid membrane (see also Quispel *et al.* 1985). The latter is formed principally from host membrane allied to, but modified from, the plasmalemma. Both membranes may vary according to the particular host–rhizobial combination. For example, one of the reasons why some bacteroids become non-viable is that their surface membranes are modified in a way that makes them (amongst other things) sensitive to osmotic shock (Sutton & Paterson 1983). Modification of the bacterial outer membrane may involve changes in lipopolysaccharide (van Brussel *et al.* 1977): this would affect permeability. Additionally, Brewin *et al.* (1985) showed that components of the outer membrane of pea bacteroids may become incorporated into the peribacteroid membrane. Similarly some nodulins (host products) may be incorporated (Verma *et al.* 1986). It is therefore reasonable to expect variations in the nature of metabolites exchanged between symbionts and in the rate of such exchange, according to particular host–endophyte combinations. Furthermore, there are likely to be changes within a system as nodules differentiate and, for example, the spectrum of nodulins changes.

These suggestions can be used to extend the model of Kahn *et al.* (1985) to cover additional aspects of the legume–rhizobium interface in a functional nodule. As suggested by Kahn *et al.*

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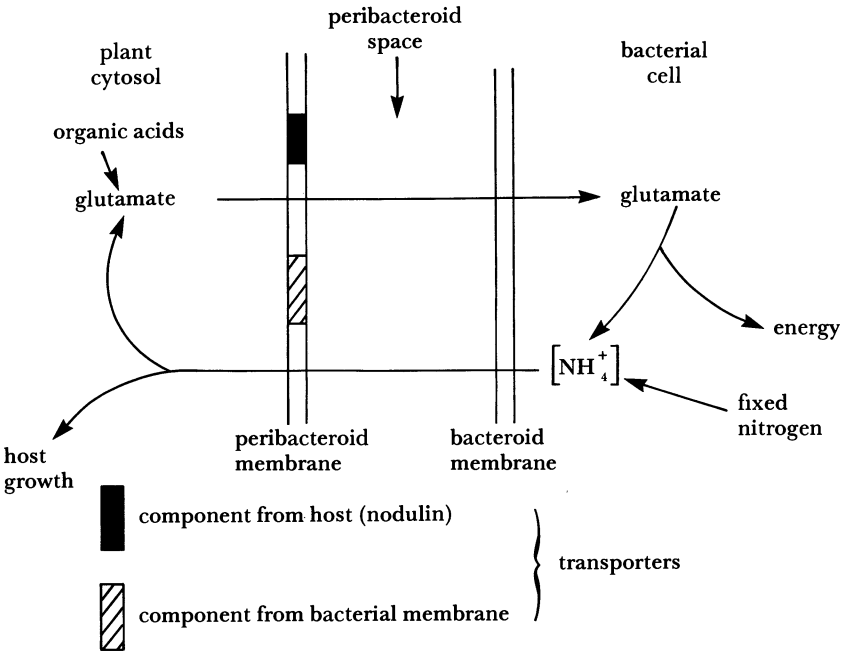

FIGURE 2. Possible relations between host legume and rhizobial cells (extended from Kahn *et al.* (1985)). Note that glutamate is only one of many possible organic N sources.

(1985) for a young nodule, bacteria will use both C and N supplied by the host, because it is necessary to make bacterial protein. Depending on the C:N ratio of the substrate supplied by the host relative to bacterial protein (remembering that some C will be lost in respiration) the plant is likely to recover a variable proportion of the N supplied. The plant then becomes N-limited; if the bacteria are to continue to obtain amino compounds as a source of energy, they must start to fix nitrogen. As the nodule develops, there are at least two possible variants, depending on whether or not the bacteria retain viability. Those that do also retain much (but not all) of their membrane integrity, their DNA and almost their entire metabolic machinery (except for some parts of the ammonium-assimilating system). To do this, they need from their host a continuous supply of organic N as well as C. If we assume that the N is enriched in the mass-15 isotope (which still begs the question of the metabolic basis of ^{15}N enrichment), it leads to a progressive enrichment of ^{15}N, i.e. enrichment of ^{15}N is associated with retention of viability. It also accompanies an increase in mass in determinate soybean nodules (Shearer *et al.* 1984), whose bacteroids show increasing viability with increased nodule age (Zhou *et al.* 1985). This argument is supported by a number of lines of evidence. First, assuming all bradyrhizobia have the potential to retain viability, then the natural ^{15}N enrichment of all determinate nodules (ureide or amide exporters) (table 4) follows. Second, if we assume that rhizobial bacteroids potentially lose viability, they do not need to maintain all their metabolic machinery and do not become greatly enriched with ^{15}N. This is generally true of indeterminate nodules on papilionoid legumes (table 5). Figure 3 summarizes these ideas.

There are several exceptions to the above two cases. First, *Lotus*, when nodulated by *Rhizobium*, becomes enriched with ^{15}N and retains viability (table 1). This means that the host can override the final differentiation stage and this could relate to the need to retain viability in determinate nodules (see below). Second, *Bradyrhizobium* loses viability in *Arachis* (table 2) and also in

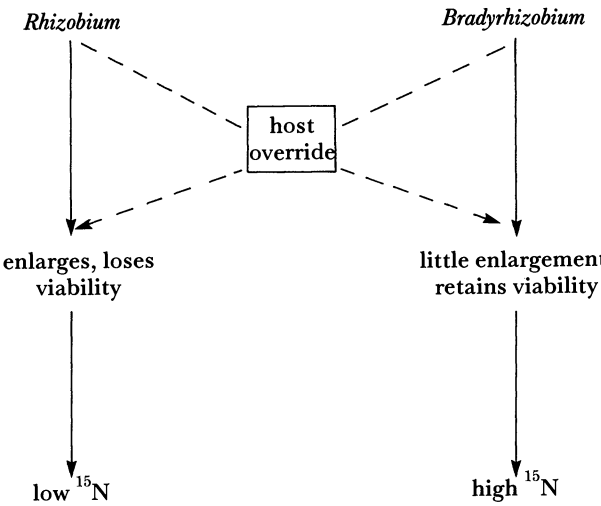

FIGURE 3. Parallel changes occurring during the transformation of free-living *Rhizobium* or *Bradyrhizobium* cells into nitrogen-fixing bacteroids. These changes can sometimes be overridden by the host.

TABLE 4. NATURAL (δ) ^{15}N ENRICHMENT IN DETERMINATE NODULES FROM THE PAPILIONODEAE

(Data only from plants grown on N-free media. Where several similar sets of data are available from one laboratory, only one is given. References are numbered as follows; 1, Steele *et al.* (1983); 2, Yoneyama *et al.* (1986); 3, Turner & Bergersen (1983); 4, Shearer *et al.* (1982); 5, Domenach (1985). *Lotus* exports amides; all others, ureides. Where a range of values is given, they cover a variety of strains.)

tribe	species	δ ^{15}N (‰)	ref.
Phaseoleae	*Centrosema pubescens*	8.43	1
	Glycine max	8.53	2
		6.7–12.5	1
		12.48	3
		9.74	4
		8.5	5
	Macroptilium atropurpureum	8.0–11.8	1
		8.82	2
	Phaseolus vulgaris	10.4	4
	Vigna umguicularis	4.7	1
Desmodieae	*Desmodium intortum*	8.82	1
Loteae	*Lotus peduncularis*	5.5–6.5	2

TABLE 5. NATURAL (δ) ^{15}N ENRICHMENT IN INDETERMINATE NODULES FROM THE PAPILIONOID TRIBES TRIFOLIEAE AND VICEAE

(Note that some values are negative; references numbers as in table 4.)

tribe	species	δ^{15}N (‰)	ref.
Trifolieae	*Medicago sativa*	3.17	3
		−1.11–−0.97	2
	Trifolium pratense	1.98–2.01	2
	T. repens	1.50–2.80	1
		−2.30–0.70	2
	T. hybridum	−1.01–0.01	2
	T. subterraneum	1.15	3
Vicieae	*Vicia faba*	−0.40	4
	V. sativa	−0.56–1.36	
	V. villosa	−0.51	2

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Stylosanthes (by implication from Chandler *et al.* (1982)). Neither shows significant enrichment of nodules with ^{15}N (table 2) (Yoneyama *et al.* 1986). Thus again loss of viability and natural ^{15}N enrichment are coupled, but this time by an overriding effect of the host on *Bradyrhizobium*. Both these genera have aeschynomenoid nodules (Corby 1981), a type with several unique features (see below) (Sprent *et al.* 1987). It has recently been shown (Bergersen *et al.* 1986) that the variation in ^{15}N found in lupin nodules is broadly related to bacterial strain, slow-growing types generally being enriched and fast-growing ones not enriched. Sutton & Paterson (1980, 1983) concluded that the host was the major determinant of viability in lupin bacteroids. However, the data showed considerable variation and the most viable strain was slow-growing. It would be interesting to make a direct comparison of natural ^{15}N and the changes in viability and amino acid incorporation known to occur during the development of bacteroids from *Lupinus* spp. nodules (Sutton *et al.* 1977). With one possible exception, appreciable ^{15}N enrichment is always associated with bacteroids from slow growing rhizobia. The exception is *Cassia nomana*, which shows only slight enrichment (Yoneyama *et al.* 1986). This caesalpinioid species is likely to have a completely different type of nodule and probably an equally different set of constraints (see next section).

Where nodules varying in effectiveness have been studied, they show structural modifications including both incomplete differentiation and enhanced senescence of nitrogen-fixing cells (see, for example, Sutton 1983). Further, the proportion of cells in different stages of differentiation in indeterminate nodules varies with rhizobial strain and environment (see, for example, Roughley 1970; Fyson & Sprent 1982). Therefore the variations in natural ^{15}N enrichment found in, for example, *Trifolium* spp., and between results from different groups working on the same species, are only to be expected (table 5) (see, for example, Yoneyama *et al.* 1984; Turner & Bergersen 1984; Steele *et al.* 1983).

Sprent & Raven (1985) argued that retention of bacteroid viability is necessary in determinate (desmodioid) nodules (where all bacteria are in approximately the same stage of development) if nitrogen fixation is to the long-term advantage of the endophyte, since soil populations must be replenished from senescent (but previously effective) nodules. Indeterminate nodules have a range of bacteria in different stages of differentiation from essentially free-living forms, to enlarged bacteroids which may be markedly pleomorphic and which have lost their viability. Loss of viability is not important for long-term survival of nitrogen-fixing strains of these rhizobia, because soil populations can be replenished from undifferentiated rhizobia within nodules. *Arachis* may be unusual in that the nodule has limited growth and virtually uniform infected tissue, but bacteroids retain very little viability. However, the rhizobia are promiscuous and thus are likely to persist in other legumes where viability can be retained.

Some unique features of nodules on primitive legumes and parallels in nodulated non-legumes

The general classification of Polhill & Raven (1981), in which the Leguminosae are divided into three subfamilies, Caesalpinioideae, Mimosoideae and Papilionoideae, will be adopted. The Caesalpiniodeae are usually considered to be the most primitive; they also have the smallest proportion of nodulated genera (Allen & Allen 1981) (see Sprent *et al.* (1987) for a summary of the current situation). Studies on the anatomy of these nodules have only recently been

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TABLE 6. SPECIES OF LEGUME HAVING PERSISTENT INFECTION THREADS IN ACTIVE, NITROGEN-FIXING CELLS

(None has been found in the subfamily Mimosoideae. For details, see Faria *et al.* (1987); Sprent *et al.* (1987).)

subfamily	tribe	genus	species	variety
Caesalpinioideae	Caesalpinieae	*Campsiandra*	sp.	
		Dimorphandra	*exaltata*	
		Melanoxylon	*brauna*	
		Moldenhaurea	*floribunda*	
		Sclerolobium	*rugosum*	
		Tachigali	*multijuga*	
			paniculata	*alba*
	Cassieae	*Chamaecrista*	*ensiformis*	*ensiformis*
			desvauxii	*glauca*
			glandulosa	*brasiliensis*
Papilionoideae	Dalbergieae	*Andira*	*fraxinifolia*	
			frondosa	
			inermis	
			legalis	
			nitida	
			racemosa	
		Hymenolobium	*algoanum*	*parvifolium*
	Tephrosieae (Millettieae)	*Cyclolobium*	*vecchii*	
		Dahlstedtia	sp.	
		Poecilanthe	*grandiflora*	
			parviflora	

started (Faria *et al.* 1986, 1987). All seven genera with confirmed nodulation have a rather uniform, but unique, nodule structure which is shared by five genera from primitive woody members of the Papilionoideae (Faria *et al.* 1986, 1987; Sprent *et al.* 1987) (table 6). Three major features of such nodules are: (i) intercellular spread of rhizobia; (ii) formation of threads bounded by host cell-wall material only when bacteria 'enter' host cells; (iii) proliferation of these threads and retention of wall material into the active nitrogen-fixing stage. This last property has considerable implications for the host–rhizobial interactions described above. Although the persistent infection threads are, like those in root hairs, bounded by host membrane, it is difficult to envisage the movement of bacterial membrane material across the wall to fuse with this membrane in the manner of that shown for pea bacteroids (Brewin *et al.* 1985). The rhizobia that induce these primitive nodules grow slowly and some have a dry type of colony, suggesting lack of exopolysaccharide. The latter is correlated with the differentiation of infected cells in some nodules, such as those on lucerne (alfalfa) (Lang-Unnasch *et al.* 1985). It is likely that further studies on these primitive nodules will reveal a different spectrum of biochemical as well as structural features.

Some of the features of primitive legume nodules are also found in the nodules formed by rhizobia with the non-legume genus *Parasponia* (Ulmaceae). In this case, initial invasion of the root is intercellular (i.e. not *via* root hairs) then at a later stage infection threads are formed (see, for example, Lancelle & Torrey 1984, 1985) to carry infections into young cells formed from the nodule meristem. Nitrogen-fixing cells contain a proliferation of threads (fixation threads) where walls become impregnated, not only with lignin (as in infection threads of this genus), but also with suberin-like substances (Smith *et al.* 1986). So far we have not found structures exactly corresponding to *Parasponia* infection threads, i.e. taking infections into young cells.

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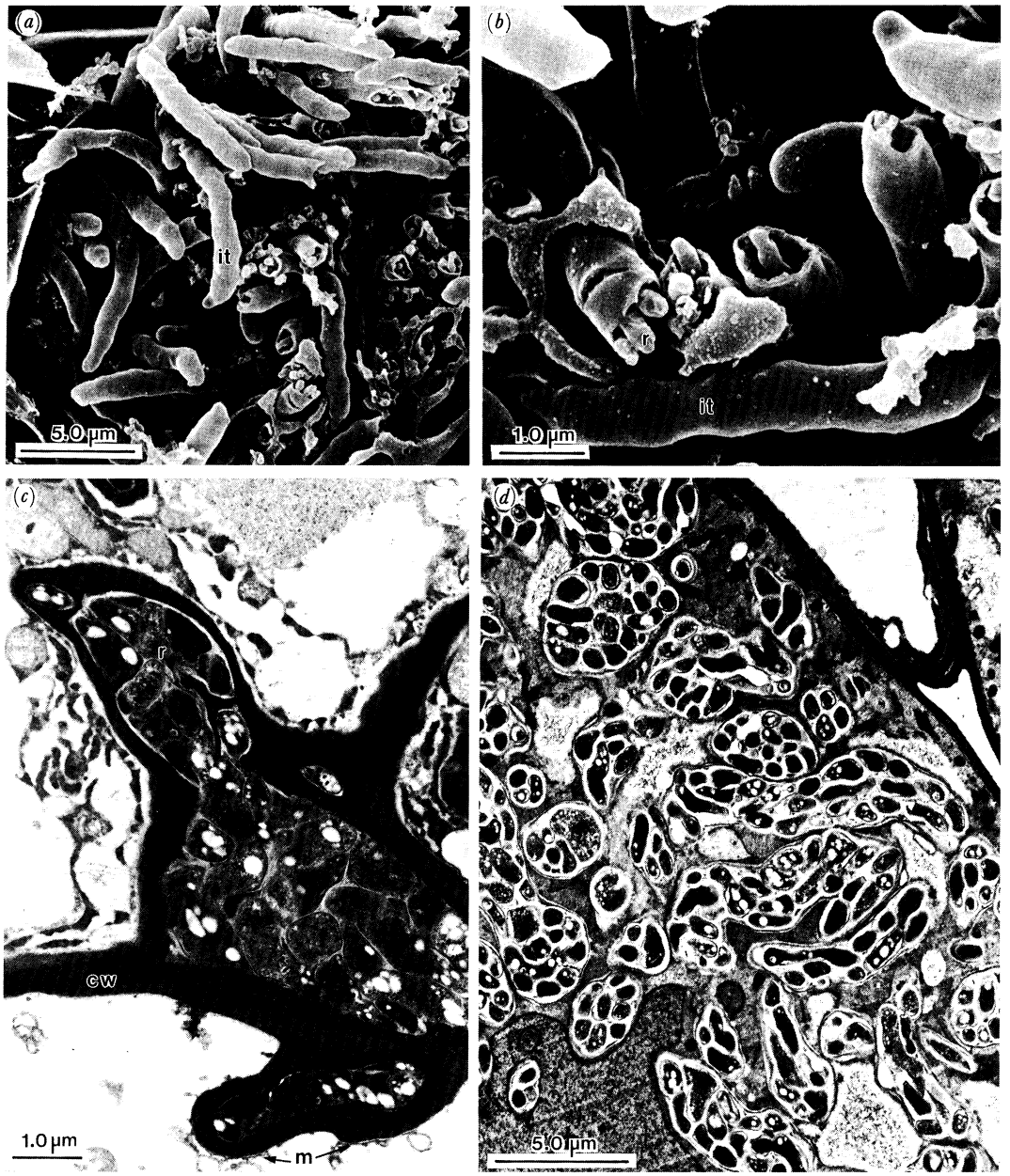

FIGURE 4. (*a*) Scanning electron micrograph of an infected cell of *Campsiandra* sp. filled with persistent infection threads (it). Magn. ×4200. (*b*) Higher magnification of part of (*a*) showing rhizobia (r) inside infection threads (it). Magn. ×15400. (*c*) Transmission electron micrograph of a nodule on *Poecilanthe parviflora* showing intercellular rhizobia (r) and penetration of rhizobia into host cells surrounded by host cell wall material (cw) and plasmamembrane (m). Magn. ×12000. (*d*) Transmission electron micrograph of a nitrogen-fixing cell of *Poecilanthe parviflora* nodule showing persistent infection threads containing rhizobia. Magn. ×5250.

(*Facing p.* 123)

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However, because we do not know how soon after cell invasion nitrogen fixation begins, we are using the term 'persistent infection threads' to cover the structures shown in figure 4 (plate 1). Rather than being the result of rhizobium in an unusual host (as suggested for *Parasponia*), these threads may be a typical part of primitive nodules and possibly represent a type of symbiosis which would be easier to extend to other genera. Even in advanced legumes, such as lucerne, *R. meliloti* produces mRNA transcripts of nitrogenase structural genes while rhizobia are still inside infection threads (Paau & Brill 1982). Some, but not all, of the active, nitrogen-fixing nodules in which we have found persistent infection threads have a pink coloration suggestive of haemoglobin (only low levels of this pigment are found in *Parasponia*) (Appleby 1984) and in all the vascular system is peripheral, whereas in *Parasponia* it is central.

In actinorhizal plants from the family Eleagnaceae (*Eleagnus* and *Hippophae*), it has recently been shown that infection by *Frankia* and passage of the endophyte through the root cortex and the young primary nodule cortex is intercellular. When the endophyte invades cortical cells before the onset of nitrogen fixation, it is confined, at least in the early stages, by host cell-wall material (Miller & Baker 1986). Thus intercellular passage of rhizobia and *Frankia* may be normal in certain host genera. In all cases studied in sufficient detail (*Parasponia* (Lancelle & Torrey 1984); *Eleagnus* and *Hippophae* (Miller & Baker 1985, 1986)), and we believe also for genera listed in table 6, it follows an infection process that does not directly involve root hairs. Whereas root-hair infection requires the presence of infection threads, the converse is not true. Further, hair infection is partly controlled by the host; the same endophyte may enter different hosts by different routes.

How common is root-hair infection?

The most widely known cases of legume infection other than that via root hairs are *Arachis* (see, for example, Chandler 1978) and *Stylosanthes* (Chandler *et al.* 1982). Other exceptions are noted above. Root hair infection can be bypassed by using genetically engineered rhizobia and agrobacteria (Hirsch *et al.* 1985; Hrabak *et al.* 1985). Where hairs are absent, as on roots of many mimosoid legumes (Sprent *et al.* 1987) and on stems of *Aeschynomene* (Vaughn & Elmore 1985) and *Sesbania* (Tsien *et al.* 1983) infection can occur directly through the epidermis or through surface breaks, such as those where lateral or adventitious roots emerge. Initial passage of rhizobia is intercellular; infection threads may never form (*Arachis*, *Stylosanthes* and other genera with nodules of a similar type (Sprent *et al.* 1987)) or may form (as in *Parasponia*) at a later stage in development (possibly true for many mimosoid legumes, including *Neptunia plena*, *Mimosa scabrella* and *Enterolobium timbouva*) (unpublished data from our laboratory). Root-hair infection appears to be an advanced character, confined to certain legume tribes which happen to be those with the most widely studied crop and forage species. The taxonomic and evolutionary implications of these findings are discussed elsewhere (Sprent *et al.* 1987). Even after a century of nitrogen fixation research, there is much to learn about the biology of nitrogen-fixing organisms. In particular, study of primitive legumes may be of use, not only for their intrinsic interest, but also in the search for ways of extending nodulation within the angiosperms.

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How much nitrogen is fixed biologically?

Numerous estimates have been made of global nitrogen fixation; these vary by orders of magnitude. Rosswall (1983) surveyed the literature and found values between 44 and 200 Tg N per year for land and 1 and 130 Tg N per year for ocean. The uncertainty arises for various reasons, including the necessity for a great deal of extrapolation. It is probably safe to say that the free-living bacteria, apart from cyanobacteria, are of only local significance, as are most cyanobacterial symbioses (even though *Azolla* may be very important to a wetland rice farmer). Because the oceans occupy 70% of the earth's surface, fixation in the open water could be very important. Does it occur? Fogg (1982) discusses this point and concludes that 15 Tg N per year is likely to be an upper limit.

Most people agree that legumes and actinorhizas contribute most to annual N fixation. However, detailed measurements of fixation have only been attempted for a few species; because there is no fully quantified method available for measuring fixation in the field, even these are subject to error. Furthermore, the potential for nodulation of the majority of legume species has not been checked (Allen & Allen (1981) summarized the position up to that time and only a few hundred other species have been examined since). Because there is no evidence of major recent changes in the N_2 content of the atmosphere, N_2 fixation (industrial or biological) must be similar to N_2 loss (industrial or biological). However, there is no good measure of denitrification either! We can perhaps console ourselves as we enter the second century of nitrogen fixation research that there is still plenty to be done.

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Discussion

M. J. Dilworth (*Murdoch University, Western Australia*). I refer to the hypothesis of Kahn *et al.*, relating to a role for glutamate in carbon supply for nitrogen-fixing bacteroids. Mutants defective in glutamate utilization remain symbiotically effective. Therefore it does not seem likely that the proposed mechanism operates significantly.

J. I. Sprent. From what Dr Dilworth says I agree, with respect to glutamate. However, Kahn *et al.* proposed a role for amino acids *such as* glutamate. In the system which you studied, another amino acid may have been involved.

H. D. L. Corby (16 *Croft Close, Bishop's Tachbrook, Leamington Spa, U.K.*). I think we must accept Dr Sprent's suggestion that infection of leguminous roots by *Rhizobium* in ways other than through root hairs may be common, if only for the reason that root hairs are often wanting, as the following instance shows.

In raising seedlings of a worldwide selection of 61 species of nodule-forming legumes, raising them both on blotting paper and in clayey soil well diluted with quartz sand, I found that most species produced a textbook display of root hairs within a day or so of germination, but that nearly a quarter of them did not.

With five species I could find no hairs at all, either on seedlings grown on blotting paper or on those grown in the diluted soil (*Acacia podalyriaefolia*, *Albizia gummifera*, *Enterolobium contortisiliquum*, *Onobrychis viciaefolia*, *Swartzia madagascariensis*).

With three species root hairs were obvious on most seedlings, but I could find none on the remainder (*Dalea leporina*, *Indigofera spicata*, *Ornithopus sativus*).

With two species root hairs were obvious on most, but not all, seedlings grown on blotting paper, but I could find none on those grown in the diluted soil (*Arachis hypogaea*, *Desmanthus virgatus*).

With two species I could find no root hairs the first time I raised seedlings, but when I tried again with seed from different sources I found that the majority had them (*Acacia sieberana*, *Neptunia oleracea*).

In *Entada arenaria*, I could find no root hairs on some 10% of the seedlings, short sparse hairs on the subordinate roots, but not on the primary root, of some 60%, and short sparse hairs on roots of all ranks of the remaining 30%.

Finally, in *Erythrophleum africanum*, I found no typical root hairs at all, but 60% of the seedlings grown on blotting paper had a puberulent rhizodermis on the primary root and, in all cases, lateral roots were stumpy, as if an ectotrophic mycorrhiza were present.

J. H. Becking (*I.T.A.L., Wageningen, The Netherlands*). In Dr Sprent's talk she mentioned that all dinitrogen-fixing cyanobacteria possess heterocyst structures. Reports are available on

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unicellular and non-heterocystous cyanobacteria such as *Gloeocapsa*, *Trichodesmium*, *Plectonema*, *Lyngbya* and some other species. Or did Dr Sprent refer in her lecture only to the symbiotic or associative cyanobacterial systems?

J. I. Sprent. The genera Dr Becking mentions seem to be notable exceptions, about which I know little, so I did not consider them in my paper.

L. A. Materon (*I.C.A.R.D.A.*, *Aleppo*, *Syria*). What are the major differences between rhizobium-nodulated *Parasponia* and legumes? Does Dr Sprent foresee more non-nodulated systems being nodulated by *Rhizobium* in nature?

J. I. Sprent. There are many similarities between infection processes and structure of infected cells in *Parasponia* and primitive woody legume nodules. However, there remains a fundamental difference between legume and non-legume nodules (*Frankia*- and *Rhizobium*-induced), namely the vascular system being central (root-like) in non-legumes and peripheral (shoot-like) in legumes. Prospects for extending nodulation to non-nodulated legumes and to non-legumes are enhanced by recent studies on primitive legumes.

J. W. Drozd (*Shell Research Ltd*, *Sittingbourne*, *Kent*, *U.K.*). What is the difference in yield between legumes fixing nitrogen and those assimilating fixed nitrogen?

J. I. Sprent. This topic will probably be addressed by later speakers. Results vary greatly. Many crops plants (e.g. soybean) have been bred from plants grown on fertilized fields and thus nitrogen fixation may have been unconsciously selected *against*. Where selection has been made from plants reliant on nitrogen fixation, lines can be obtained which grow as well (sometimes better) on N_2 as on combined nitrogen. There are, however, many complicating factors, such as length of growing season, climate, soil etc. There are many examples of crops that benefit from a dose of 'starter' nitrogen, because that often stimulates nodule production.

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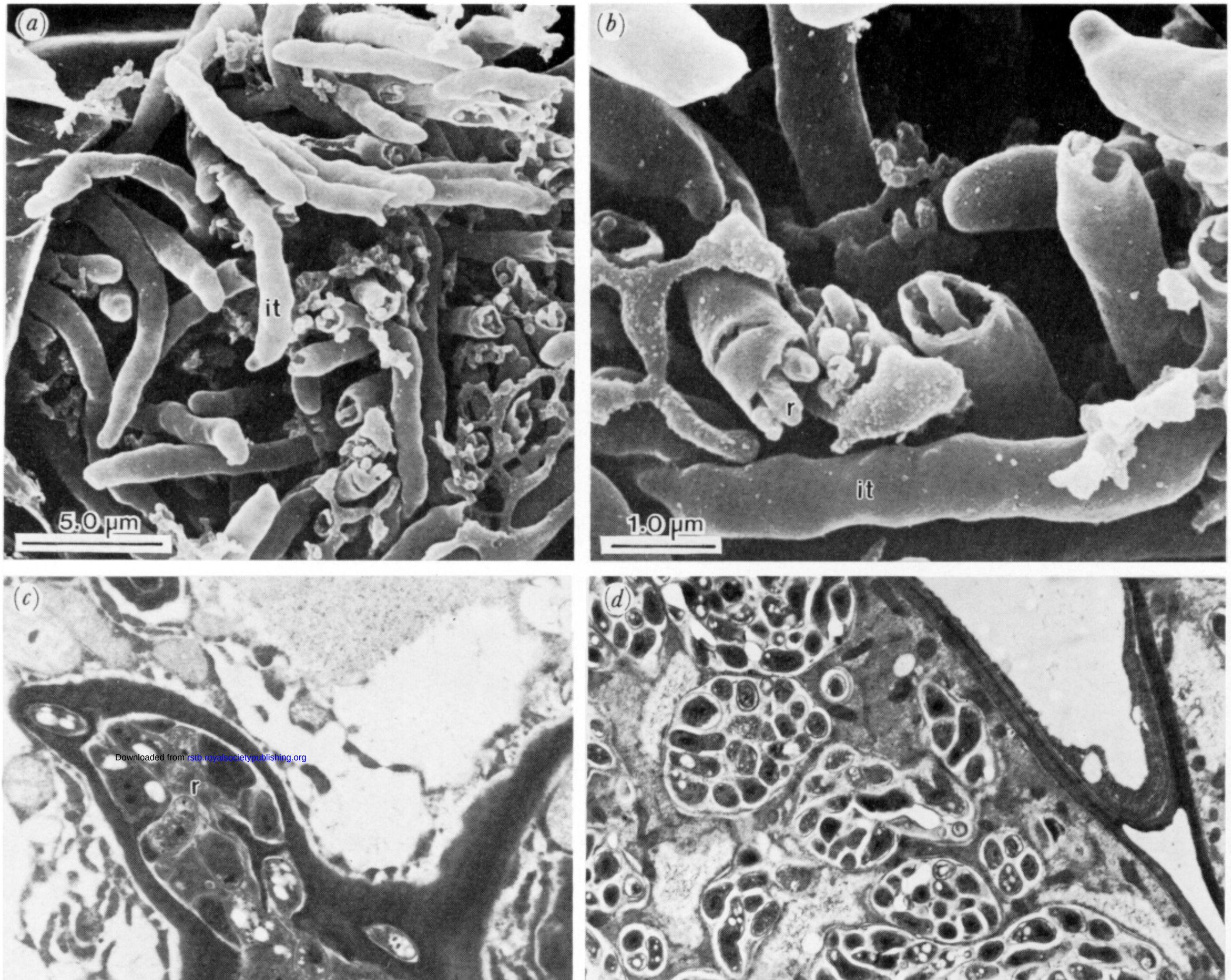

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GURE 4. (*a*) Scanning electron micrograph of an infected cell of *Campsiandra* sp. filled with persistent infection threads (it). Magn. ×4200. (*b*) Higher magnification of part of (*a*) showing rhizobia (r) inside infection threads (it). Magn. ×15400. (*c*) Transmission electron micrograph of a nodule on *Poecilanthe parviflora* showing intercellular rhizobia (r) and penetration of rhizobia into host cells surrounded by host cell wall material (cw) and plasmamembrane (m). Magn. ×12000. (*d*) Transmission electron micrograph of a nitrogen-fixing cell of *Poecilanthe parviflora* nodule showing persistent infection threads containing rhizobia. Magn. ×5250.