CHAPTER 16

Biological Dinitrogen Fixation: Symbiotic

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An' I am blessed becos me feet 'ave trod
A land 'oo's fields reflect the smile o' God

—*The Sentimental Bloke*, C. J. Dennis

A **mutualistic symbiosis** is an association between two organisms from which each derives benefit. It is usually a longer-term relationship and, in the case of symbiotic dinitrogen (N_2) fixation, often involves the development of a special structure to house the microbial partner. Each N_2-fixing symbiotic association involves a prokaryotic organism that is N_2-fixing (e.g., *Rhizobium*, *Klebsiella*, *Nostoc*, or *Frankia*) and a eukaryotic, usually photosynthetic host (e.g., leguminous or nonleguminous plant, water fern, or liverwort). Together, microsymbiont and host convert inert dinitrogen gas from the atmosphere into ammonia, benefiting the host, while the microsymbiont has a protected environment and a supply of photosynthate and other nutrients for growth and N_2 fixation. Estimates of the contribution of these symbioses to the global nitrogen economy vary, but crop and pasture legumes alone are grown on more than 250 million hectares worldwide and fix about 90 Tg N $year^{-1}$, accounting for almost 50% of the nitrogen used in agriculture. Rates of symbiotic N_2 fixation in legumes vary with host, microsymbiont, and environment, but rates as high as 600 kg N fixed ha^{-1} $year^{-1}$ have been reported in temperate clover pastures, while grain legumes fix from 165 to 450 kg N ha^{-1} $year^{-1}$ (Unkovich and Pate, 2000).

The direct availability of the N_2 fixed to the host allows it to grow in environments that are low in nitrogen, and to minimize nitrogen losses through denitrification, volatilization, and leaching. This enhances the sustainability of agricultural and natural systems that are legume based. Greatest dependence on N_2 fixation in agriculture is usually associated with:

- small-holder farms where access to inputs may be limited,
- organic producers who use green manures rather than chemical fertilizers, and

BOX 16–1

The Importance of Symbiotic Dinitrogen Fixation

Symbiotic N_2 fixation is the single greatest contributor to the global cycle of nitrogen. Important symbiotic associations in which dinitrogen is fixed include:

- leguminous plants and their associated rhizobia,
- actinorhizal plants in symbiosis with *Frankia*,
- the water fern *Azolla* and its microsymbiont *Anabaena*, and
- lichen symbioses involving cyanobacteria.

- environments where rainfall is unreliable and extensive use of fertilizer is risky.

With the world's population projected to increase to 8.3 billion by 2025, and with much of that increase to occur in developing countries where fertilizer usage is already limited, a marked increase for such countries in dependence on N_2 fixation may be needed to maintain food security. In contrast, where fertilizer nitrogen is still relatively inexpensive, agriculture intensive, or manures land applied, N_2 fixation in agriculture will be important for reasons of soil quality and agricultural sustainability, but less emphasized and studied.

THE SYMBIOSIS BETWEEN LEGUMES AND RHIZOBIA

Legumes have been used in crop rotations since the time of the Romans, Theophrastus (370 to 285 B.C.) stated "... beans are not a burdensome crop to the ground, they even seem to manure it ... wherefore the people of Macedonia and Thessaly turn over the ground when it is in flower." However, it was not until detailed nitrogen balance studies became possible that legumes were shown to accumulate nitrogen from sources other than soil and fertilizer. In 1886 Hellriegel and Wilfarth associated the ability of legumes to convert dinitrogen from the atmosphere into compounds that could be used by the plants with the presence of swellings or **nodules** on the legume root (Fig. 16–1). They related this to the presence within the nodule of particular bacteria, now collectively known as **rhizobia**. It was a series of short, but important, steps to the isolation of rhizobia from nodules by Beijerinck in 1888 and to the completion of **Koch's Postulates** (Chapter 1) by the demonstration of their ability to reinfect the legume and to fix dinitrogen in symbiosis.

Groupings of Rhizobia and Their Separation into Species

Early studies demonstrated that each rhizobial **strain** or nodule isolate had a finite host range, nodulating certain legumes but not others. This led to the con-

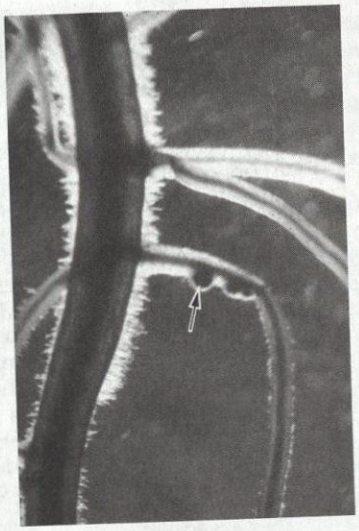


FIGURE 16–1
Early nodule development (arrow) on the roots of *Phaseolus vulgaris*. *Photo courtesy of M. H. Chaverra. Used with permission.*

cept of **cross-inoculation groups**, with legumes clustered according to the different rhizobia with which they formed nodules. Thus, rhizobia isolated from alfalfa (*Medicago sativa*) would also nodulate plants from the genera *Melilotus* and *Trigonella*, and vice versa, though none of these rhizobia would nodulate clovers (*Trifolium* spp.). More than 20 different cross-inoculation groups were identified, with the bacteria associated with the clover, medic, bean, lupin, pea, and soybean groups each named as separate species within a single genus *Rhizobium* (e.g., *R. trifolii* for clover and *R. meliloti* for medics). Host specificity is still important in the characterization of rhizobia, but more recently other traits have assumed greater significance in their classification (Table 16–1). The classification of rhizobia is still controversial and there are a number of reasons for this, including:

- Initial studies emphasized mainly legumes of agricultural importance. Study of other species has now blurred the original cross-inoculation boundaries. For example, the rhizobial strain NGR234 (originally isolated from *Lablab purpureus*, the hyacinth bean) nodulates at least 112 different species of legume and the nonlegume *Parasponia andersonii*. Even now, though, less than 15% of the roughly 19,000 species of legumes have been evaluated for nodulation.
- Many anomalous results have been reported. Thus some hosts are promiscuous, nodulating with rhizobia from other cross-inoculation groups, whereas others fail to nodulate all legumes within their particular group.
- Nodulation genes in many rhizobia are plasmid-borne (Chapter 4). Strains cured of this symbiotic plasmid lose the ability to form nodules, and for many years could not be identified as rhizobia. In soils from Mexico, rhizobia lacking this plasmid can outnumber those capable of nodule formation with beans by 40 to 1.

TABLE 16–1 Characteristics Used in the Phenotypic and Phylogenetic Characterization of Rhizobia

Phenotypic traits*
Range of substrates usable as sources of energy (e.g., sugars, sugar alcohols, complex carbohydrates)
Range of substrates usable as sources of nitrogen (e.g., amino acids, urea, and nitrate)
Resistance to specific antibiotics
Electrophoretic mobility of different cell enzymes
Tolerance to different stresses (e.g., salt, temperature, and pH)
Fatty acid methyl ester (FAME) analysis of cell membranes
Phylogenetic traits** (refer also to Chapter 4)
Pattern of banding of DNA restriction fragments (RFLPs)
Degree of hybridization with specific DNA probes
16S rRNA sequence analysis

*Phenotypic traits can be observed in culture.
**Phylogenetic traits are related to cell DNA or RNA composition.

- Taxonomic methods were developed that compare strains on the basis of many different traits. Computer-based numerical classifications or methods based on differences in cell DNA or RNA often give results at odds with those based on host range.

Changes in classification suggested over the past 40 years now divide the original genus *Rhizobium* into 6 genera and more than 35 species, as shown in Table 16–2. However, some scientists maintain that there is insufficient evidence to warrant such separation among rhizobia, and even propose adding species of *Agrobacterium* to the genus. Agrobacteria do not nodulate legumes, but sometimes occur as nodule contaminants. They also include saprophytes such as *A. radiobacter,* and the organisms responsible for crown gall and hairy root disease. The significant difference between fast-growing rhizobia and slow-growing bradyrhizobia is recognized by most rhizobiologists. The latter organisms have been shown to grow as hydrogen autotrophs, and to produce bacteriochlorophyll. Recent reports in which five genera of beta-protebacteria including species of methyl-oxidizing bacteria have also been shown to nodulate specific legumes have not helped the confused taxonomic situation.

THE INFECTION PROCESS

Nodule Initiation and Development

Rhizobia can infect their hosts and induce root- or stem-nodule formation using several different mechanisms, including:

- penetration of root hairs and formation of infection threads, as found in plants such as clovers and beans,

TABLE 16–2 Validly Described Genera and Species of Root-Nodule Bacteria of Legumes. Changes to this taxonomy suggested by Young et al. (2001) are in bold type. Genera in the square brackets refer to better-known host legumes nodulated by each species of root-nodule bacteria. Common names are included for well-known legume genera. In several examples in this list, different species of root-nodule bacteria nodulate the same legume.*

*Azorhizobium***
- *A. caulinodans [Sesbania]*

Bradyrhizobium
- *B. elkanii* [*Glycine*, soybean]
- *B. japonicum [Glycine]*
- *B. liaoningense [Glycine]*
- *B. yuanmingense [Lespedeza]*

Mesorhizobium
- *M. amorphae [Amorpha]*
- *M. chacoense* [*Prosopis*, mesquite]
- *M. ciceri* [*Cicer*, chickpea]
- *M. huakuii* [*Astragalus*, milkvetch]
- *M. loti [Lotus]*
- *M. mediterraneum [Cicer]*
- *M. plurifarium* [*Acacia, Leucaena*, Ipil-ipil]
- *M. tianshanense [Glycyrrhiza, Sophora]*

Rhizobium
- *R. etli* [*Phaseolus vulgaris*, bean]
- *R. galegae [Galega, Leucaena]*
- *R. gallicum [Phaseolus, Dalea, Onobrychis, Leucaena]*
- *R. giardinii [Phaseolus]*
- *R. hainanense [Stylosanthes, Centrosema]*
- *R. huautlense [Sesbania]*
- *R. indigoferae [Indigofera]*
- *R. leguminosarum*
  - *bv trifolii* [*Trifolium*, clover]
  - *bv viciae* [*Pisum*, peas, *Vicia*, field beans, *Lathyrus; and Lens*, lentil]
  - *bv phaseoli* [*Phaseolus*]
- *R. loessense [Astragalus]*
- *R. mongolense [Medicago, Phaseolus]*
- *R. sullae [Hedysarum]*
- *R. tropici [Phaseolus; Leucaena, Dalea, Macroptilium]*

Allorhizobium
- *A. undicola*, ***R. undicola*** *[Neptunia]*
- ***R. radiobacter*** [non-nodulating saprophyte], ***R. rhizogenes*** [causes hairy root disease],
- ***R. rubi, R. vitis***

Sinorhizobium
- *S. abri [Abrus]*
- *S. americanus [Acacia]*
- *S. arboris*
- *S. fredii [Glycine]*
- *S. indiaense [Sesbania]*
- *S. kostiense*
- *S. kummerowiae [Kummerowia]*
- *S. medicae [Medicago]*
- *S. meliloti [Melilotus*, sweetclover; *Medicago*, alfalfa; *and Trigonella*, fenugreek]
- *S. morelense [Leucaena]*
- *S. saheli, S. sahalense [Sesbania]*
- *S. terangae [Sesbania, Acacia]*
- *S. xinjiangense [Glycine]*

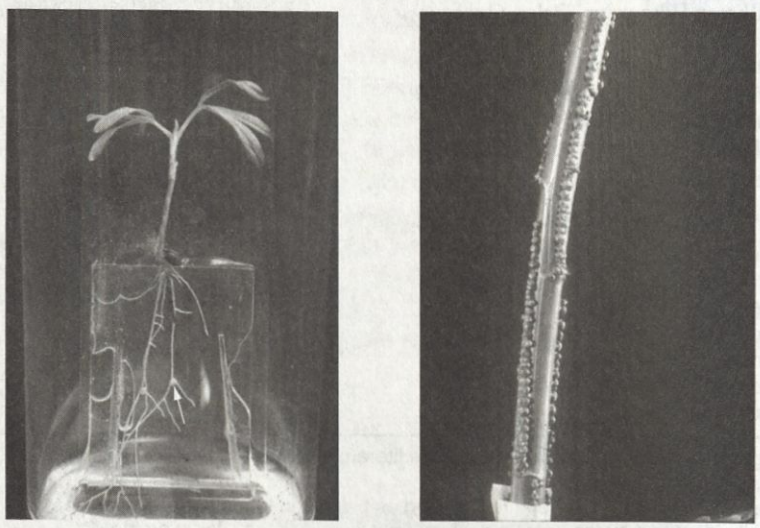
*Other genus and species names exist in the literature. Some predate the present names; others have not been accepted as valid.

**Strains which have not yet been recognized as belonging to any named species are usually identified by the host from which they were isolated—for example, *Rhizobium* spp. (*Acacia*) or *Bradyrhizobium* spp. (*Lupinus*).

- entry via wounds or sites of lateral root emergence, as found in peanuts and the pasture legume *Stylosanthes* (Fig. 16–2a), and
- penetration of root primordia found on the stem of plants such as *Sesbania* (Fig. 16–2b).

The Fåhraeus slide technique, where small seeded legumes were inoculated with rhizobia, embedded in agar, and grown between glass slides (Fåhraeus, 1957), and the root-tip marking procedure (Bhuvaneswari, Bhagwhat, and Bauer, 1981) were seminal to our understanding the process of nodule formation, outlined in the following discussion. The first method allowed repeated observation of the infection process, while the second showed differences in the susceptibility of immature and mature root hairs to infection, and focused research on those parts of the root where infection by *Rhizobium* was most common.

Visible Changes During Root-Hair Infection. Infection begins with attachment of rhizobia to immature, emerging root hairs of a compatible host. This becomes evident within minutes of inoculation, with attached rhizobia capping the root-hair tip, and often oriented end-on to their host. Deformation and curling of the root hair follows (Fig. 16–3a), with the root-hair surface at the point of infection hydrolyzed to permit penetration of the rhizobia. Rhizobia then move down the root hair toward the root cortex. Rhizobia do not gain direct intracellular access to



a. b.

FIGURE 16–2
Nodule formation in *Stylosanthes* (a) and *Sesbania* (b). In *Stylosanthes*, infection occurs through wounds left at sites of lateral root emergence, with the resultant nodules produced in the angle between main and lateral roots (arrow). In *Sesbania*, rhizobia infect through adventitious root primordia found on the stem, and give rise to nodules that contain chlorophyll. *Photos courtesy of P. H.Graham and Y.Dommergues, respectively. Used with permission.*

their host. During infection, and as they move down the root hair, they remain enclosed within a plant-derived **infection thread** (Fig. 16–3b). In some of the more primitive legumes, for example *Chamaecrista*, rhizobia may never escape this containment. Root-hair penetration and infection-thread formation is paralleled by visible changes including host-cell proliferation in the root cortex adjacent to infected root hairs. In indeterminate nodules (see following discussion) this includes

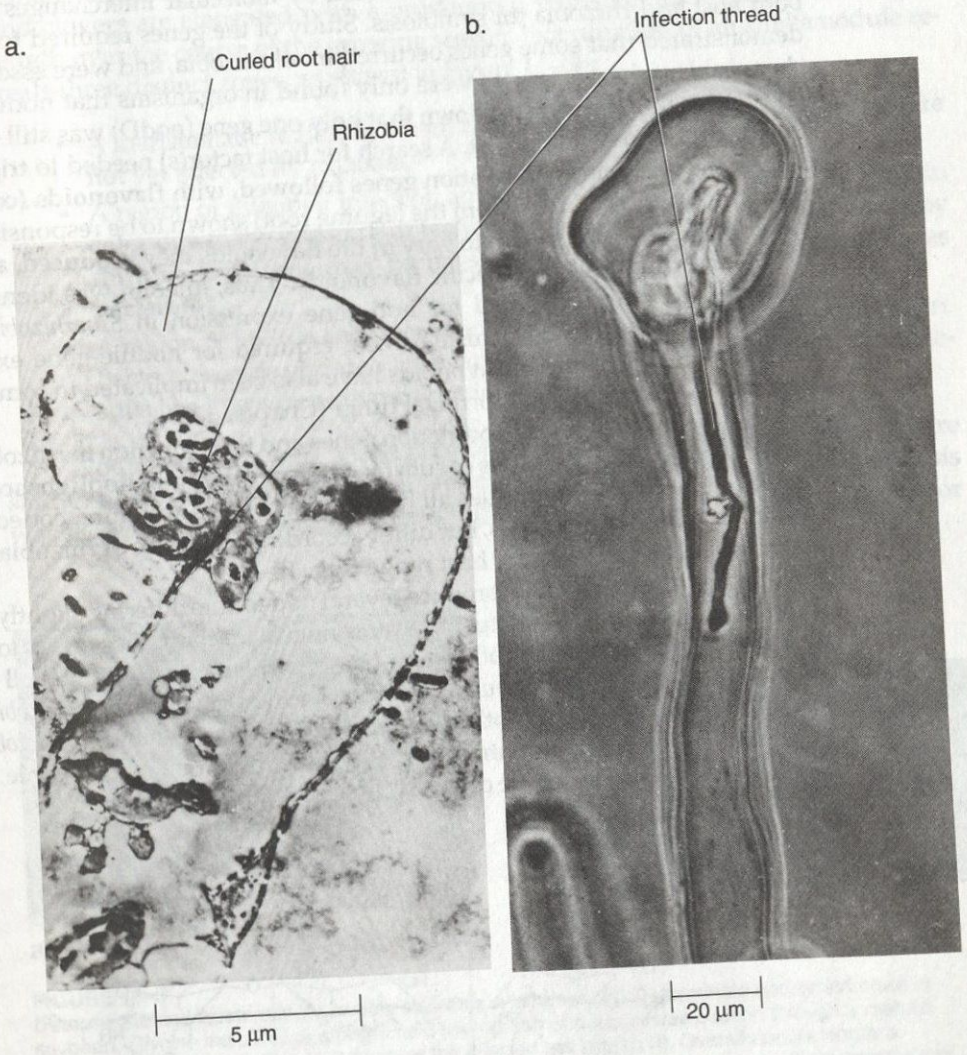


FIGURE 16–3
Root-hair deformation, curling, and infection; an early stage in the nodulation of clover by rhizobia. (a) The initiation of infection-thread formation showing initial penetration of the curled root hair by 10 to 20 cells of rhizobia, and the redeposition around them of plant-derived gelatinous material. *From Sahlman and Fåhraeus (1963). Used with permission.* (b) Rhizobia enclosed within the infection thread move down the root hair toward the root cortex. *From Fåhraeus (1957). Used with permission.*

formation of a nodule primordium, with rhizobia then released into modified cells of the root cortex, where again they are enclosed within a plant-derived **peribacteroid membrane**. These membranes protect the bacteria from the defense responses of the host.

Molecular Changes During Root-Hair Infection. The visible changes evident during root-hair infection are paralleled by molecular interchanges that prepare both host and rhizobia for symbiosis. Study of the genes required for nodulation demonstrated that some genes occurred in all rhizobia, and were essentially interchangeable, whereas others were only found in organisms that nodulated a specific legume(s). It was also shown that only one gene (nodD) was still expressed in the absence of a suitable host. A search for host factor(s) needed to trigger expression of the remaining nodulation genes followed, with **flavonoids** (complex phenolic compounds exuded from the legume root) shown to be responsible. Further, legume species were shown to vary in the flavonoids they produced, and rhizobia to respond differently to specific flavonoids. Thus, luteolin was identified as the principal flavonoid required for nod-gene expression in *Sinorhizobium meliloti*, whereas naringenin and genistein were required for nodule-gene expression in *Bradyrhizobium japonicum*. Flavonoids have also been implicated in some aspects of infection by arbuscular mycorrhizal fungi (Chapter 12).

Study of the different nodulation genes and their function in rhizobia then led to the identification of a series of substances, termed **lipo-chitooligosaccharides** or "**nod factors**." These molecules all have the same core structure, coded for by the "common" nodulation genes, but differ according to species of rhizobia in the side chains each carries, affecting host range (Fig. 16–4).

Strains of rhizobia may produce several nod factors differing slightly in composition. They act as very powerful plant morphogens, at concentrations as low as 10^{-11} molar they can induce many of the root-hair deformation and cortical cell changes in the root that are typical of nodule formation. The nod-genes in *Bradyrhizobium* are not located on plasmids, but are otherwise analogous to those found in *Rhizobium*. Even in *Rhizobium*, some genes contributing to nodule formation (for example, the genes regulating lipopolysaccharide composition) are not located on plasmids.

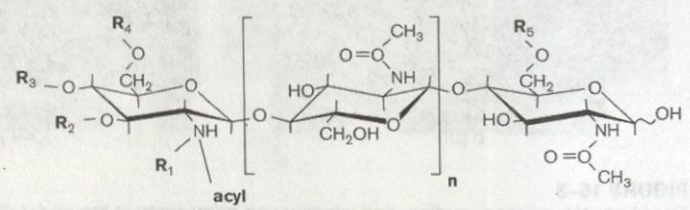


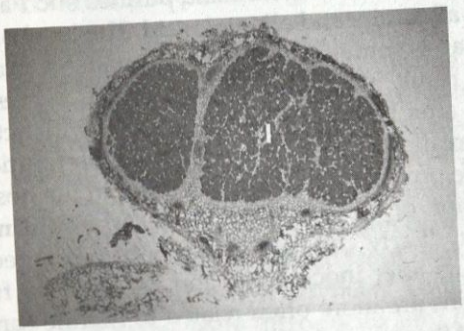
FIGURE 16–4
General structure of "nod-factors" produced by rhizobia. The basic core structure coded for by the "common" nodulation genes is the same in all rhizobia. Components R_1 to R_5 vary with species and affect host specificity. *From Schultze and Kondorosi (1996). Used with permission.*

Nodule Development and Function

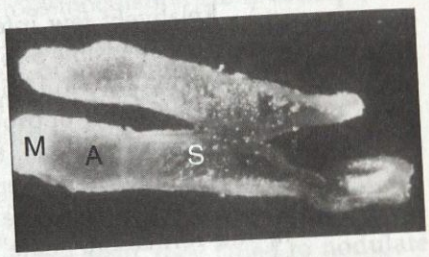
As the infection thread penetrates the root cortex, host root-cell division and enlargement result in the formation of a visible nodule. Root nodules differ in appearance and structure, traits determined by the host legume. *Determinate* nodules, such as occur in soybean and *Phaseolus*, are round and have no pronounced meristematic region (Fig. 16–5a). In contrast, the *indeterminate* nodules of peas, medics, and clovers are elongated with a pronounced meristematic region, increasing in length over the course of the growing season. Examination of an alfalfa nodule reveals three distinct zones, as shown in Figure 16–5b:

- A meristematic region in which host cells undergo active division but are not yet infected by rhizobia.
- A region of rhizobial infection and active N_2 fixation, often red or pink in color due to the presence of **leghemoglobin**. Host cells will contain many rhizobia and these may be misshapen. Such bacteria are referred to as **bacteroids**.
- A region of nodule **senescence** in which the symbiosis is breaking down. Bacteroids may undergo lysis, and the degradation of leghemoglobin results in a green or brown coloration.

Nodules that are pink or red in color are usually active in N_2 fixation, and are said to be *effective*. If the nodule is white or greenish brown, either the symbiosis was *ineffective* or the nodule is senescing. A typical nodule may remain active for



a.



b.

FIGURE 16–5
Determinate and indeterminate nodule structure in legumes. (a) Determinate nodule structure in soybean, *Glycine max*. This is a bright field micrograph of a transverse section through a mature nodule stained with toluidine blue to show the infected cell region (I). Overall nodule length is approximately 0.4 cm. *Photo courtesy of P. H. Graham and D. Espinosa Victoria.* (b) Indeterminate nodule structure in alfalfa, *Medicago sativa*. Note the progression from a white, meristematic area (M) in which few cells are infected with rhizobia, through a pink-red region containing leghemoglobin and active in N_2 fixation (A), to a region of senescence (S). Nodule attachment to the root would be to the right of the photo. Overall nodule length is approximately 0.5 cm. *Photo courtesy of C. P. Vance. Used with permission.*

BOX 16–2

Events Leading to Nodulation and Dinitrogen Fixation in Legumes

- Attachment of rhizobia to the root begins within 1 minute of inoculation.
- Number of attached rhizobia increases with time up to several hours.
- Root hair curling begins within 5 hours.
- Infection threads visible in the root hair within 3 days of inoculation.
- Nodules become visible within 5 to 12 days.
- N_2 fixation is often evident in 15-day-old plants.

only 50 to 60 days, meaning that plants in the field may have more than one flush of nodule formation during the growing season.

In indeterminate nodules such as those produced on alfalfa, bacteroids produce ammonia which is exported to the host cell and there converted via glutamine, glutamate, and aspartate to asparagine. Asparagine is then exported to the shoot. The overall equation for this series of reactions is:

$$2 \text{ ammonia} + 3\text{ATP} + \text{oxalocetate} \rightarrow \text{L-asparagine} + \text{AMP} + 2\text{ADP} + 2P_i + \text{NAD} + Pp_i.$$

Determinate nodules export a very different end product. Glutamate and aspartate are produced, but are then used to synthesize purines such as xanthine. These are converted to ureides, allantoin, and allantoic acid. The level of these substances in nodules and xylem sap is sometimes used to estimate N_2 fixation.

Relatively few infections result in root-nodule formation. Successful infections may be visible 5 to 6 days after inoculation with rhizobia, with active N_2 fixation beginning 8 to 15 days thereafter. During infection, nodule formation, and N_2 fixation a number of proteins are produced that are not found in the host or bacteria when grown alone. Expression of these substances, called **nodulins**, may be both time and tissue dependent. "Early" nodulins have been recovered from infected root hairs less than 6 hours after inoculation, and are likely to function in nodule formation. "Late" nodulins are more often related to nodule function and N_2 fixation. Functions for most of these proteins are yet to be determined, but those characterized to date include leghemoglobin and the nodule enzymes nitrogenase, uricase, and glutamine synthetase. Increasingly we can deduce functions for some of these compounds through sequence, genomic, and bioinformatic analysis.

Host-Rhizobium Specificity

Given the signaling that occurs between host and root nodule bacteria during nodule formation, it is not surprising that specificity can occur at several stages of nodule formation. The range of this specificity is described as follows.

Legume Species That Never Nodulate. Among the subfamilies of the Leguminosae, 97% of the Papilionoideae bear nodules, but less than 20% of Caesalpinioideae are nodulated. Because of such differences in the frequency of nodulation, and because some legumes can be nodulated by rhizobia from several different genera, it has been suggested that ability to nodulate with rhizobia might have arisen on more than one occasion in the evolution of legumes. In one often cited example the genus *Cassia* is not nodulated, whereas the very closely related *Chamaecrista* bears nodules ranging in structure from primitive to quite highly developed.

Nodulation Mutants Within Normally Nodulated Plant Species. A number of different nodulation mutants have been identified in legumes, particularly soybean and pea. In some cases these plants will nodulate only with specific rhizobia; in others they are never nodulated. Non-nodulating soybean, bean, and pea lines are often used as control plants in inoculation studies.

Cross-Inoculation Group Specificity. No rhizobia nodulates all legumes, though some are promiscuous and will nodulate hosts from a number of different species and genera of legumes. The example of strain NGR234 has already been mentioned. *Rhizobium tropici* strain UMR1899 is also promiscuous, nodulating species of *Leucaena, Dalea, Phaseolus, Macroptilium, Onobrychis,* and *Coronilla*. Other strains may only nodulate hosts belonging to one genus of legume (e.g., *Trifolium*), and may not even nodulate all species of that genus. As a consequence more than 100 different strains of rhizobia are needed to satisfy the inoculation requirements of currently important legume species.

Infectiveness Subgroups. According to the cross-inoculation group concept, rhizobia isolated from one host in a cross-inoculation group should nodulate all members of that group. However, particular species, or even cultivars, of legume can require different rhizobia. A well-documented example is the pea landrace "Afghanistan" that is highly specific in *Rhizobium* requirement. It nodulates freely with pea rhizobia from the center of origin of *Pisum sativum* in the Middle East, but not with rhizobia recovered from peas in other areas (Table 16–3).

Similarly, when soybean varieties such as "Bossier" developed in the United States were introduced into Africa around 1980, they failed to nodulate with indigenous soil rhizobia and had to be inoculated. In contrast, soybean varieties such as "Orba" and "Malaysian" from Asia nodulated with the indigenous strains and rarely responded to inoculation (Table 16–4). It has been suggested in South Africa that small farmers without access to good-quality inoculants should use the promiscuously nodulating cultivars, whereas larger-scale farmers would be better off using the more productive American cultivars and inoculants.

Such symbiotic specificities can be a problem in the introduction and evaluation of new plant germplasm. For this reason collectors of legume germplasm should also collect nodule or soil samples from which the appropriate rhizobia can be isolated.

Host-rhizobial interactions also influence levels of N_2 fixation. Peanut and cowpea, for example, are nodulated by and fix dinitrogen with a range of soil rhizobia, whereas *Centrosema* and *Desmodium* species will often nodulate with these strains but fix little dinitrogen (Box 16–3). A consequence is that when cowpeas or peanuts are introduced into a new area, they will often grow well without inoculation, whereas *Centrosema* and *Desmodium* species may have many nodules, but grow poorly.

TABLE 16–3 Number of Strains of Pea Rhizobia from the Middle East and Other Regions with the Ability to Nodulate the Pea Landrace, Afghanistan

Country	Number of Soils Tested	Number with Rhizobia Nodulating Afghanistan Pear
Afghanistan	1	1
Turkey	120	115
Israel	55	52
Greece	8	0
Egypt	8	0
Tunisia	1	0
Europe	55	0
South Africa	4	0

From Lie (1978). Used with permission.

TABLE 16–4 Response to Inoculation in the American Soybean Cultivar "Bossier," and the Indonesian Landrace "Orba" Under Field Conditions in Nigeria

Cultivar/Treatment	Nodule Dry Mass (mg Plant^{-1})	Yield (kg ha^{-1})
Bossier		
Noninoculated	14	1643
Inoculated	485	2970
Nitrogen addition	46	2726
Orba		
Noninoculated	287	1924
Inoculated	514	1844
Nitrogen addition	275	1848

From Ranga Rao et al. (1982).

BOX 16–3

Infectiveness and Effectiveness

- Infectiveness is the ability of a rhizobial strain to induce nodule formation with a particular legume.
- Effectiveness is the ability of those nodules to fix dinitrogen.

Even different varieties of the same legume species can vary in ability to fix dinitrogen with a particular rhizobial strain. This is commonly related to differences in maturity, with early-flowering varieties more likely to be limited in their N_2-fixing ability. However, genetic differences in earliness of nodulation, nodule mass, nodule senescence, and enzyme function have also been reported, and attempts to improve the levels of N_2 fixation through plant breeding are under way in a number of laboratories. Greatest success has been with soybeans in Brazil where the crop may derive in excess of 90% of its nitrogen needs from N_2 fixation, with no additional benefit from nitrogen fertilization. Perhaps the simplest approach to improving the levels of N_2 fixation achieved is to inoculate plant-breeding nurseries with rhizobia and to grow and select promising lines under conditions of nitrogen deficiency. The plant biomass and seed yield of the germplasm evaluated will then depend heavily on their ability to fix dinitrogen.

ENVIRONMENTAL FACTORS AFFECTING SYMBIOSIS IN LEGUMES

Environmental factors influence all aspects of nodulation and symbiotic N_2 fixation, in some cases reducing rhizobial survival and diversity in soil, in others affecting nodulation or N_2 fixation or even growth of the host. Critical factors include acidity, temperature, mineral nutrition, salinity, and alkalinity.

Acidity

In Latin America alone there are more than 800 million hectares of Oxisols and Ultisols having a pH less than 5.0. Dinitrogen fixation in these soils can be markedly reduced due to:

- hydrogen ion concentration *per se*,
- toxic levels of aluminum and manganese, and
- induced deficiencies of calcium, phosphorus, and molybdenum.

Soil acidity can directly limit rhizobial growth and persistence in soil with isolates of *Sinorhizobium meliloti* particularly acid sensitive. In one study 89,000 *S. meliloti* cells g^{-1} were reported in soils of near neutral pH, but only 37 cells g^{-1} in soils of pH < 6.0. Fast-growing rhizobia are generally more sensitive than are the slow-growing bradyrhizobia, but relatively low pH-tolerant strains exist in many species (Fig. 16–6). Surprisingly, not all of the strains recovered from acid soils are acid tolerant, suggesting that microsites of more favorable pH can occur within the soil (Chapter 2).

Failure to nodulate is common in acid soils, in part because fewer rhizobia persist, but also because acid pH affects attachment of the rhizobia to their host. For many strains, problems in nodulation can be expected once soil pH falls below 5.2. Acid soils in the United States are often limed to near neutrality. The cost and

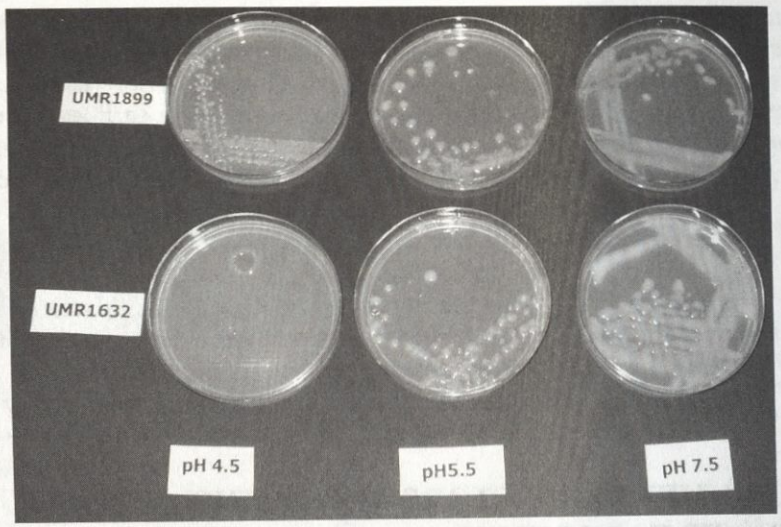


FIGURE 16–6
Differences in the pH tolerance of two strains of *Rhizobium* used for the inoculation of *Phaseolus* beans. *Rhizobium tropici* CIAT899 grows well at pH values below 4.5 producing isolated colonies each derived from an individual cell. In contrast, *R.etli* CIAT632 does not grow well below pH 5.2.

availability of limestone, however, precludes this approach in many other countries where a more normal practice is to lime to a pH of 5.5 to 5.8. In Brazil, even this practice can require 6 to 8 tons of lime per hectare. Other ways to limit the negative effects of soil pH include the use of acid-tolerant inoculant strains and host cultivars, and the pelleting (coating) of inoculated seed with ground rock phosphate or limestone. In Australia, the use of relatively acid-tolerant *S. meliloti* strains such as WSM419, together with *Medicago* species collected from acid soils in Sardinia, has permitted extension of the area sown to annual medics by some 350,000 ha (Ewing and Howieson, 1989). Similarly the acid-tolerant *R. tropici* strains, UMR1899 and PRF81, are now recommended for inoculation of *Phaseolus vulgaris* in the acid soils of Brazil.

Plant species vary in tolerance to aluminum and manganese but are generally more affected by these ions than are rhizobia. Some rhizobia tolerate 100 µM aluminum and 300 µM manganese, but reduced root growth of alfalfa (*Medicago sativa)* occurs at only 8 µM aluminum, and nodulation in cowpea can be inhibited at 25 µM aluminum.

Temperature

Rhizobia are mesophiles and most do not grow below 10°C or above 37°C. Exceptions are the rhizobia from some Arctic legumes, and bradyrhizobia collected from the hot, dry Sahel savannah of Africa. Temperature during the shipment and storage of rhizobial inoculants, and after seed inoculation and planting, is particularly critical. Exposure to high temperatures at these times can lead to the loss of

the symbiotic plasmid in *Rhizobium* or can reduce cell numbers below the levels needed for good nodulation.

Temperature also influences nodule growth, N_2 fixation, and the period of time over which nodules remain active. The optimum temperature for many legumes is around 25°C, and exposure to temperatures in excess of 40°C for even short periods can cause irreparable loss of nodule function. In contrast, minimum tillage in the American Midwest can result in cool-wet soil conditions in the early spring, and delayed nodulation.

Mineral Nutrition

Although well-nourished legumes generally nodulate and fix dinitrogen better than those that are nutrient limited, several elements have specific functions in nodulation and symbiotic N_2 fixation (Table 16–5). Adequate levels of these elements are essential for effective N_2 fixation and failure to supply them will often result in the generalized yellow chlorosis typical of nitrogen deficiency. Several of these elements warrant specific mention.

Phosphorus. Dinitrogen-fixing leguminous plants usually require more phosphorus than similar plants supplied with fertilizer nitrogen. Nodules are an important phosphorus sink, and commonly have the highest concentration of that element in the plant. The high-energy cost of N_2 fixation, with its need for ATP, along with the phosphorus required to build and maintain functioning nodules, leads to this elevated requirement. Under P-deficient conditions, phosphorus fertilization will usually result in enhanced nodule number and mass and greater N_2 fixation per plant (Table 16–6). Problems of phosphorus fertilization are particularly severe in Africa, where the abandonment of traditional slash-and-burn methods, and the limited supply and high cost of fertilizers for small farmers, have resulted in significant phosphorus loss from soils. Complicating this issue, global phosphorus reserves are only projected to

TABLE 16–5 Elements Having Specific Functions in the Nodulation or N_2 Fixation of Legumes

Molybdenum	FeMoCo-protein of nitrogenase
Phosphorus	Energy transformations in the nodule
Iron	Fe- and FeMoCo-proteins of nitrogenase
	Leghaemoglobin
	FeS centers of nitrogenase
	Unspecified function in nodule development
	Attachment of rhizobia to root hairs
Calcium	Cell wall integrity in *Rhizobium*
	FeS centers of nitrogenase
Sulfur	Nodule coenzyme function
Cobalt	Hydrogenase function
Nickel	

TABLE 16–6 Effect of Three Levels of Phosphorus Fertilization on Selected Parameters of Nodulation and N_2 Fixation in *Phaseolus* Beans*

	Phosphorus in Solution (μg L^{-1})		
	1	4	16
Nodule dry mass (mg $plant^{-1}$)	76.5	265.2	581.1
$N_2(C_2H_2)$ reduction (μmol $plant^{-1}$ h^{-1})	3.01	11.43	57.70
Nodule phosphorus (%)	0.22	0.25	0.29
Total nodule phosphorus (mg $plant^{-1}$)	0.20	0.65	1.70
Specific nodule activity (μmol C_2H_2 reduced g $nodule^{-1}$ h^{-1})	0.039	0.043	0.099
μmol C_2H_2 reduced mg nodule P^{-1} h^{-1}	15.05	17.58	33.94

*Results are the average for four cultivars (four replicates per cultivar) and were from plants sampled 40 days after planting. Plants were grown in sand culture under glasshouse conditions and were supplied with a plant nutrient solution containing 1, 4, or 16 μg P L^{-1} twice daily.

last 60 to 90 years. Strain and cultivar differences in phosphorus-utilization efficiency have been demonstrated, and will need to be emphasized in the future.

Field-grown legumes form tripartite symbioses with both *Rhizobium* or *Bradyrhizobium* and arbuscular-mycorrhizal fungi (Chapter 12). This has an added energy cost for the host, but can result in striking growth improvement due to enhanced phosphorus uptake.

Molybdenum. Molybdenum is as a component of the **nitrogenase** enzyme complex, with only 100 to 500 g ha^{-1} of sodium or ammonium molybdate needed to meet this requirement. How to supply this small amount can be a problem, especially under acid soil conditions where adsorption reduces the availability of soil molybdenum to the plant. Molybdenum salts have sometimes been included in the rhizobial inoculants, but this commonly kills the rhizobia and is not recommended. Liming the soil can also enhance molybdenum availability. An alternate approach adopted in Brazil is to fertilize plants being grown for seed with molybdenum applied to the foliage. This provides enough molybdenum for the next growing cycle.

Iron. Iron is a component of leghemoglobin, which functions in the regulation of oxygen supply to bacteroids. It also occurs in both the Fe- and FeMo- proteins of the nitrogenase complex, and is essential for early nodule development. Plants that are iron-deficient can develop hundreds of nodule initials but few mature nodules. Both host and microsymbiont can differ in efficiency of iron utilization. In the case of the bacteria this is because some strains produce iron-sequestering **siderophores**, and so compete more effectively for iron in the rhizosphere. For example, in peanuts, bradyrhizobial strains with the ability to produce catechol can significantly enhance the growth and iron nutrition of iron-inefficient cultivars.

Salinity and Alkalinity

The effects of saline or alkaline conditions are likely to be greater on the host than on the microsymbiont. Alkaline soil conditions limit the availability of iron, zinc, manganese, and boron in the soil, thereby reducing plant growth and N_2 fixation. Foliar fertilization with micronutrients is often an effective remedy. Legumes, as a group, are also markedly sensitive to salt, with some species affected by concentrations as low as 80 m*M* salt. In contrast, strains of rhizobia from *Medicago* and *Acacia* often tolerate 500 m*M*. Cells of rhizobia exposed to high salt concentrations may accumulate compatible solutes such as glutamic acid, trehalose, glycine betaine, and proline that help to maintain turgor in the cell, and limit the damage caused by salts.

LEGUME INOCULATION

When a new legume species is introduced into a region the soil is unlikely to contain appropriate rhizobia, and inoculation is usually needed for adequate nodulation and N_2 fixation. Yield increases following this initial inoculation can exceed 50%, with clear differences evident between inoculated and noninoculated plants, as seen in Figure 16–7 and in Table 16–4.

Inoculation in subsequent years is often not needed. In fact, where a legume has a long history of cultivation in an area, most soils will contain appropriate rhizobia, and even noninoculated plants will be heavily nodulated. Strains used in any subsequent inoculation usually give rise to only a small fraction of the nodules formed, and a yield response is unlikely. This is the situation with soybeans in the American Midwest (Box 16–4). Unfortunately, many indigenous soil rhizobia are less than fully effective in N_2 fixation. Because response to inoculation in these areas may be limited, adoption of new inoculant technologies is usually much less than for areas where legumes are of relatively recent introduction. For example, in Thailand, Hall and Clark (1995) report that inoculants were used on 80% of farms

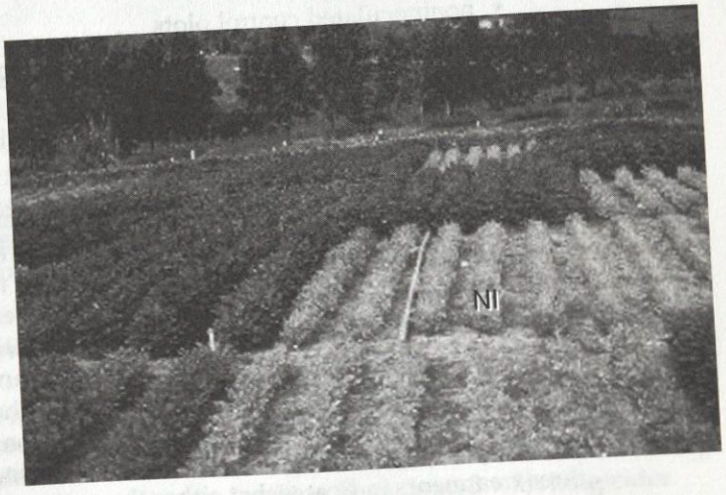


FIGURE 16–7
Response to inoculation of soybean in Puerto Rico. Plants in noninoculated (NI) or ineffectively nodulated treatments are smaller and chlorotic, and have clear symptoms of nitrogen deficiency; those inoculated with effective strains of *Bradyrhizobium* are green and vigorous. *Photo courtesy of R. S. Smith, Liphatec. Used with permission.*

BOX 16–4

Inoculation in the American Midwest

Inoculation of soybeans in the American Midwest was widely practiced in the early 1900s. Continued cultivation of soybeans in the region led to a buildup in soil rhizobia such that most soils, including some that had never been inoculated, now contain 10^3 to 10^4 soybean rhizobia g^{-1} soil. These indigenous rhizobia limit nodulation by superior inoculant rhizobia to less than 20% of the nodules formed, and yield response to inoculation is uncommon. Because the indigenous strains are often less than fully effective in symbiosis and soil nitrogen levels can be high, soybeans in the Midwest region may derive only 30% to 40% of their nitrogen needs from symbiosis, and inoculation is rarely practiced. However, in recent years soybeans have replaced cereals over significant areas of the northern Midwest. Few of these soils have soybean rhizobia, and some striking responses to inoculation have been obtained. Extension programs in these areas are beginning to stress inoculation rather than nitrogen fertilization, and practices that ensure good nodulation and N_2 fixation.

in new soybean production areas, but that this figure dropped to only 30% where soybeans had been cultivated for some time. One area where reinoculation has been beneficial is Brazil, where the acid soils and high temperatures limit rhizobial survival between crops. Presterilized higher count peat and liquid inoculants are now available in the USA, Australia and Canada, and might justify the reinoculation of soils that contain indigenous, less than fully effective rhizobia.

Need for Inoculation

A simple, three-treatment experiment will establish the need for inoculation:

- noninoculated control plots,
- plots inoculated with a strain of rhizobia effective on the host legume being evaluated, and
- plots inoculated with the same strain, but also supplied with fertilizer nitrogen.

Extensive nodulation of the noninoculated plants means that the soil already contains indigenous rhizobia able to nodulate the host. The contrast between the noninoculated plants and those supplied with nitrogen will then be a measure of the effectiveness in N_2 fixation of the indigenous rhizobia. If the noninoculated plants are green and vigorous, inoculation is probably not necessary. Absence of nodulation in the noninoculated plants, coupled with heavy nodulation of plants receiving inoculation, indicates the need for inoculation. The difference in plant growth in the three treatments is then an indicator of the efficiency in N_2 fixation of the inoculant strain. Excellent plant growth in all three treatments indicates that either the native rhizobia are highly effective, and

thus inoculation is not necessary, or the site is high in available nitrogen. Poor growth in all treatments would imply that a factor other than nitrogen is limiting plant growth.

Methods of Inoculation

When inoculation is necessary there are a number of approaches that can be used. These have as their goals to:

- supply the number of rhizobia needed for good nodulation and effective N_2 fixation,
- ensure that the highly effective rhizobial strains used in inoculants form most of the nodules produced, and
- favor inoculant strain persistence in soil and domination in nodulation over subsequent years.

Prior to the twentieth century, inoculation was often achieved by taking soil from a productive site and applying it to a new crop or revegetation area. However, this often resulted in the transfer of undesirable soil organisms as well as those that were beneficial. Inoculant rhizobia are now commercially produced and packaged, with different formulations available to meet different seeding requirements. The following four inoculation procedures are common.

Seed Inoculation. The inoculant is mixed with milk or some other slightly adhesive material, and the seed is uniformly covered with this suspension. The seed is dried in the shade and sown the same day. This procedure requires that the inoculant strain be packaged in a relatively fine carrier material (commonly finely ground peat) or liquid that will adhere to the seed. A disadvantage is the time it takes to inoculate seed during the busy planting season.

Seed Pelleting. A stronger sticker, such as gum arabic or methyl cellulose, is used with the inoculated seed and then rolled in ground limestone or rock phosphate. Pelleting combats unfavorable soil conditions such as low pH or high temperature, and is used for aerial sowing. The aim is to provide a microenvironment around the seed favorable to rhizobial survival. Preinoculation of legume seed for subsequent sale is sometimes advocated by commercial vendors on the grounds of time constraints at seeding. This is not recommended, however, as rhizobial numbers on the seed can decline dramatically during storage.

Soil Inoculation with a Granular Peat or Liquid. The inoculant is banded into the seed furrow so that it can make contact with the emerging root. The inoculant is packaged in a coarse peat or a liquid that is dribbled into the row alongside or below the seed. Granular soil inoculation is less time consuming, allows higher rates of inoculation when soil conditions are unfavorable, and permits separation of seed and inoculant when the seed has been treated with fungicides.

Inoculation in the Planter Seed Box. The inoculant is mixed directly with seeds in the planter box. This simple method is sometimes used as insurance when soils already contain rhizobia. However, inoculant and seed tend to separate, providing uneven coverage, and the practice is not recommended.

Strain Selection and Testing

Where inoculation is required, it must be carried out using an inoculant preparation having a rhizobial strain with the following characteristics:

- able to form highly effective nodules with all commonly used varieties of the legume species for which it is recommended,
- competitive in nodule formation and persistent in the soil,
- able to tolerate soil environmental stresses such as pH and temperature,
- have good growth in simple, inexpensive culture media,
- be genetically stable and not subject to mutation,
- able to survive well on the seed prior to seed germination, and
- have the ability to persist in soil between crops.

Less than 1 strain in 100 is likely to meet these criteria, so rhizobia for inclusion in inoculants must undergo extensive testing. The initial step is usually a growth chamber or greenhouse evaluation of numerous strains obtained from other research laboratories or from nodules collected in the field. Marked variation in nodulation and N_2 fixation will usually be evident.

Strains that perform well in the initial testing must be further evaluated using other cultivars or species of legume for which the inoculant strain may be recommended. Host variety-strain interactions are common, and the goal is to identify strains that are broadly effective. As the number of strains being evaluated is reduced, testing under field conditions is recommended. Field trials should be conducted at sites varying in soil type and numbers of indigenous rhizobia. The latter helps to provide a measure of strain competitiveness over time and, in succeeding seasons, can be used to determine inoculant rhizobia persistence in soil. Finally, environmental and cultural factors that could influence strain performance, for example, acid or temperature tolerance, need to be considered.

Designation of a strain as being of inoculant quality should not be done lightly. Once added to the soil they can be difficult to displace, as is evident with soybean strains of serogroup 123 that are relatively ineffective but persist in the soils of the American Midwest.

Inoculants and Inoculation

The industry that now manufactures rhizobia for seed and soil inoculation generates approximately $20 million in sales annually. Inoculants range from simple

tube cultures sufficient for small quantities of exotic seed, to large-scale, fermenter-grown cultures mixed with peat or other carrier material. The latter are used in the commercial inoculation of the large areas planted to soybean, bean, peanut, and clover. The large-scale production of inoculants is a relatively simple process that should result in at least 10^9 highly effective rhizobia per gram of product.

Once the inoculant culture has been produced, different carrier materials can be used to keep these bacteria viable until used by the farmer. Most allow rhizobial survival for periods of at least 6 months. Characteristics of a good inoculant carrier are:

- high water-holding capacity,
- nontoxic to rhizobia,
- available, inexpensive, and easily processed,
- sterilizable by autoclaving (pressurized steam) or preferably radiation,
- adheres well to seed, and
- good buffering capacity.

The most commonly used carrier is peat, but because peat is not universally available, compost, bagasse (derived from the milling of sugarcane), coal, polyacrylamide, vegetable oils, clays and clay granules, liquid preparations, and soil have all been used successfully. No listing of physical or chemical properties can fully explain why some materials make suitable inoculant carriers and others do not. Previously sterilized carriers generally support a higher population of rhizobia, and have longer shelf lives than nonsterilized materials.

Inoculant Quality and Inoculation Failure

The number of rhizobia desired per seed varies with environmental conditions, and is affected by seed size. In countries where inoculant quality is regulated by law, such as Canada and Australia, the usual standard is for 10^4 rhizobia per seed for small-seeded legumes including clover, but for 10^6 rhizobia per seed for larger-seeded species such as bean and soybean. Inoculants from many countries do not meet this standard. Gomez et al. (1997) found counts of rhizobia in commercial soybean inoculants in Argentina to range from 0 to more than 10^9 rhizobia per gram, and in several cases to have more contaminants than rhizobia. Only one of the 18 inoculants evaluated would have satisfied the standards for a Canadian inoculant. Factors contributing to the poor quality of many inoculants include:

- inadequate testing in the selection of the inoculant strain,
- mutation in the inoculant strain after repeated subculture or storage at high temperature,
- use of inappropriate culture media,
- contamination of the rhizobial culture,

- use of carrier materials which will not support suitable populations of rhizobia, and
- poor storage and transport conditions.

Inoculants can fail for other reasons including high levels of soil nitrogen, contact with acidic fertilizers or with pesticides, use of inappropriate gums or stickers, and use of the wrong inoculant culture. Catroux, Hartmann, and Revellin (2001) in a review of trends in inoculant production and use concluded: "We enter the era of biotechnology knowing more and more about the mechanisms of N_2 fixation at the gene level, but except for some manufacturers in developed countries . . . still lacking good quality and reliable inoculants (p. 27)." Only by continued research in strain selection and evaluation, and the imposition of quality standards, can inoculation success be improved.

FRANKIA AND THE ACTINORHIZAL SYMBIOSIS

Frankia is an actinomycete (Chapter 5) forming N_2-fixing (actinorhizal) nodules with a range of angiosperms. A number of the hosts involved are important in agroforestry, in the ecology and nitrogen economy of marginal soils, and in mine-spoil reclamation or sand dune stabilization. Rates of N_2 fixation are highly variable but in *Coriaria arborea* have averaged 90 kg^{-1} N ha^{-1} annually over a 20-year period (Silvester, 1975).

Frankia

Isolation of *Frankia* from nodules was not achieved until 1978 and from some hosts, such as *Datisca*, is still difficult. Increasing numbers of isolates are now available, and information on their morphological, genetic, and specificity differences is beginning to accumulate.

Frankia is a Gram-positive, filamentous organism characterized by multilocular **sporangia** and N_2-fixing **vesicles** *in vitro*. Few isolates produce spores within the nodule, and those doing so are generally less effective in N_2 fixation. Vesicle production occurs under conditions of nitrogen limitation with mature vesicles in the nodule having a pronounced lipid envelope that protects the nitrogenase from oxygen. In the symbioses in which vesicles are not formed, such as with *Casuarina*, lignification of infected and adjacent cells provides an oxygen diffusion barrier.

Sequence analysis of 16S-rRNA (Chapter 4) from cultured and uncultured nodule endophytes has permitted identification of four main subgroups within the genus *Frankia*:

- a large group including isolates from *Alnus* and *Casuarina*,
- uncultured isolates from *Dryas*, *Coriaria*, and *Datisca*,
- strains from *Eleagnus*, *Hippophae*, and *Gymnostoma*, and
- atypical and non-N_2-fixing strains.

Specificity and Symbiosis

Knowledge of the *Frankia*-actinorhizal symbiosis lags behind that for the nodulated legume. However, as legumes and actinorhizal species share common ancestors, contrasts between the two symbioses have allowed significant recent progress (Pawlowski and Bisseling, 1996). Commonalities with nodulation by rhizobia include:

- Flavonoids from seed washes of *Alnus rubra* affect nodulation.
- Root-hair deformation assays suggest involvement of "nod-factors" in nodulation (however, in at least some strains, these appear to be heat stable and chitinase resistant, suggesting a structure quite different from that found in the rhizobia).
- Regulation of nodulation has been shown in *Discaria* and *Alnus*.
- Nodule-specific gene products have been identified including expression of a hemoglobin conserved between actinorhizal *Casuarina* and legumes.

Strains of *Frankia* form root nodules on eight families of dicotylenous plants, including *Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae*, and *Rosaceae*. Infection is via root hairs in some genera (*Casuarina* and *Myrica*), but proceeds intracellularly in others (*Discaria, Dryas*, and *Ceanothus*). Nodules are perennial modified lateral roots with lobes up to 5 cm in length (Fig. 16–8).

Cross inoculation studies with *Frankia* have been complicated by difficulties in isolating the microsymbionts from some actinorhizal hosts. Though many more

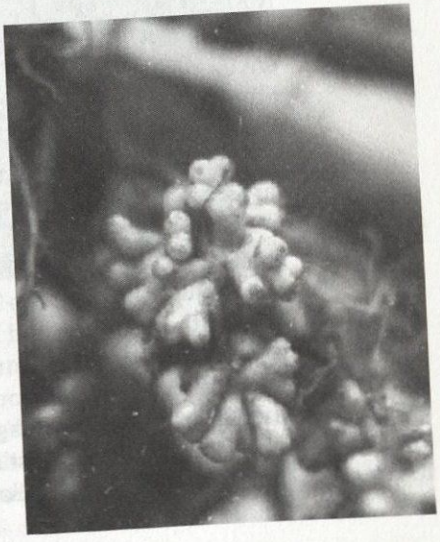


FIGURE 16–8
Nodules of *Alnus* showing a typical multilobed structure that is derived from modified lateral roots. *Photo courtesy of P. O. Lundquist. Used with permission.*

tests are needed, it appears that microsymbionts differ quite markedly in promiscuity. *Alnus* and *Casuarina* are specific in *Frankia* requirement, whereas isolates from *Myrica, Gymnostoma, Eleagnus,* and *Atriplex* are much more promiscuous. Soil factors seem to play a large role in cross-inoculation. Simonet et al. (1999) found seven phylogenetically distinct *Frankia* groups in the nodules of Australian *Casuarina* and *Allocasuarina* species, yet recovered only one group from *Casuarina* plants grown outside Australia. These isolates were more easily cultured and survived better in soil apart from their host.

ANABAENA (*NOSTOC*) AND THE *AZOLLA* SYMBIOSIS

Cyanobacteria, with the ability to both photosynthesize and fix dinitrogen, are ideal pioneering species (Chapter 7). However, some also form symbioses and their hosts include lichenous fungi, liverworts such as *Blasia*, the aquatic fern *Azolla,* and the angiosperm *Gunnera*. Of these, *Azolla* is the most important commercially and will be emphasized here. In many rice-growing areas, *Azolla* is maintained and propagated in slow-flowing creeks or overwintered in protected beds, then introduced into paddies between plantings of rice. After some period for growth, the fern can be incorporated into soil before the transplanting of rice seedlings, or left to be shaded out as the rice canopy develops. The low C/N ratio of the fern ensures rapid mineralization after incorporation, with yields in the subsequent rice crop enhanced by up to 1000 kg ha^{-1}. Under favorable conditions rates of N_2 fixation can reach 2 kg N ha^{-1} day^{-1}, but in the field rates of 100 kg N ha^{-1} $year^{-1}$ are more likely.

Dinitrogen fixation in *Azolla* has traditionally been attributed to the heterocystous cyanobacterium *Anabaena azollae* growing within cavities in the dorsal leaf lobe. Some recent studies suggest that the microsymbiont is really a species of *Nostoc*. The dorsal lobe of the *Azolla* frond contains mucilaginous ellipsoidal cavities within which are located 2,000 to 5,000 cyanobacterial cells, surrounded by an envelope of plant origin (Rai, Soderbuck, and Bergman, 2000) (Fig. 16–9). Unlike other cyanobacterial symbioses, *Anabaena* cells are persistent in *Azolla,* and pass to the progeny during fragmentation of the host thallus.

Dinitrogen fixation in *Anabaena* species occurs predominantly in specialized larger cells termed **heterocysts**. When these organisms are grown in culture, only 6% to 10% of the cells in the filament are heterocysts, though this frequency increases under nitrogen deprivation. In the mature *Azolla* frond, heterocysts occur at a frequency of 20% to 30% irrespective of nitrogen level (Fig. 16–10).

Although the yield benefits from the use of *Azolla* can be appreciable, the intensification of agriculture, increased labor costs, and the availability in some areas of relatively cheap sources of fertilizer nitrogen have resulted in reduced usage of green manures such as *Azolla*. This has dramatically reduced the land area devoted to *Azolla*, some estimates placing current usage in China at only 1.5 million hectares annually (Rai, Soderback, and Bergman, 2000).

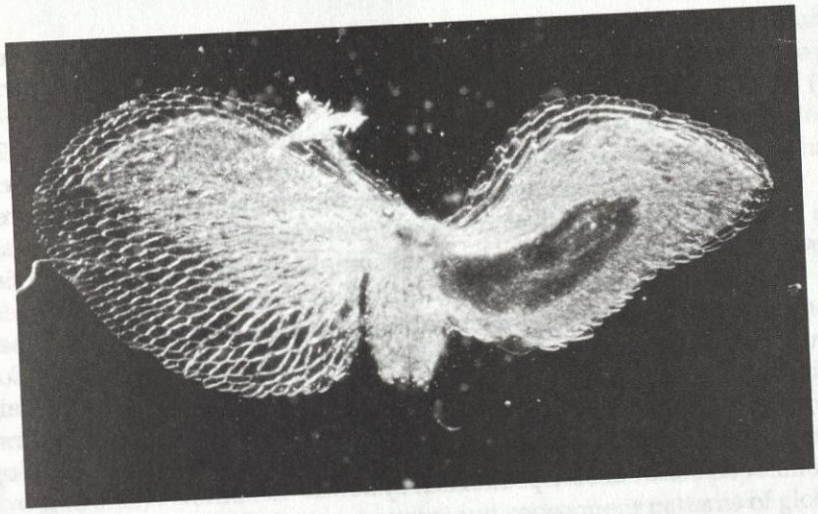


FIGURE 16–9
Location of the symbiotic cyanobacterium *Anabaena azollae* within the leaf frond of the water fern *Azolla*. The leaf frond has been cleared, with the filaments of the microsymbiont evident as a darker region toward the center of the frond. Frond diameter varies with species from 1.5 to 15 cm (Lumpkin and Plunkett, 1980). *Photo courtesy of T. A. Lumpkin. Used with permission.*

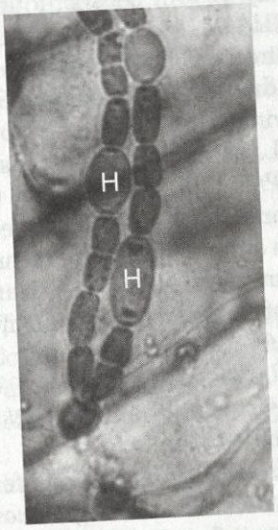


FIGURE 16–10
In the *Azolla* frond, much of the N_2 fixation occurs within larger, modified cells known as heterocysts (H). Vegetative cells are 4 to 8 μm wide and 5 to 12 μm long; heterocysts measure 6 to 10 μm by 7.5 to 11.5 μm (Lumpkin and Plunkett, 1980). Heterocyst frequency within the frond may be 3 to 4 times greater than is found in free-living filaments of *Anabaena*. *Photo courtesy of T. A. Lumpkin. Used with permission.*

SUMMARY

This chapter emphasizes the potential for N_2 fixation in legumes as well as some of the problems associated with using this symbiosis. It also introduces the *Frankia*-actinorhizal and *Anabaena-Azolla* symbioses. The greater exploitation of

all three symbioses will require a multidisciplinary approach and a better understanding of microbial ecology.

Symbiotic N_2 fixation currently accounts for almost 50% of the nitrogen used in agriculture. With world population increases in the period to 2025 concentrated in developing countries, that contribution will have to increase. Many of the research skills needed to resolve specific problems in N_2 fixation are already in place. They include methods to breed for enhanced N_2 fixation, fertilization practices appropriate to the inoculated legume, limiting the effects of environmental stress, and better integration of pesticide and inoculation methodologies. Although inoculant production in many regions of the world leaves much to be desired, quality inoculant preparations are within the reach of most countries. Consistent support for applied research in N_2 fixation, for technology transfer and training, and for the better understanding of the nitrogen needs of both modern and traditional systems should improve the benefits coming from this important technology in both intensive and extensive production systems.

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STUDY QUESTIONS

1. As a Peace Corps volunteer in Nepal you are assigned the task of helping to develop an inoculant industry. What steps might be necessary in completing such an undertaking?