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Nitrogen fixation in tropical agriculture and forestry

B. L. Dreyfus, H. G. Diem, J. Freire, S. O. Keya,
and Y. R. Dommergues

Nitrogen compounds comprise from 40 to 50 per cent of the dry matter of protoplasm, the living substance of plant cells. For this reason, nitrogen is required in large quantities by growing plants and is indeed the key to soil fertility. Non-nitrogen-fixing plants, for example cereals, obtain all the nitrogen they need from the soil. In Senegalese conditions this uptake was estimated to be as follows: 79–132 kg N ha/crop for pearl millet; 74–84 kg N ha/crop for rice; 134 kg N ha/crop for sorghum; and 121–139 kg N ha/crop for maize (Blondel 1971). Nitrogen-fixing plants, essentially legumes, take a part of the nitrogen they require from the atmosphere, the other part being provided by the soil.

When nitrogen fertilizers are available, soil nitrogen levels are maintained or improved by applying these industrially-fixed nitrogen sources. Such a technology, which allows continuous crop yields, is successfully used in intensive agricultural systems, but certain limitations have been progressively observed, i.e. increasing cost especially in developing countries, low yields from leaching and denitrification, especially in tropical conditions, and pollution of underground water by nitrates. The other alternative for maintaining or improving the nitrogen content in soil is to exploit nitrogen fixation. In this chapter, the microbial and the plant components of symbiotic nitrogen-fixing systems that are encountered in the tropics are dealt with. On the other hand, an attempt is made to explain how to improve the effectivity of these systems by assuring that the infective and effective strain of *Rhizobium* or *Frankia* is present in the soil or on the seed at the proper time (section 3), by reducing the impact of the limiting factors that can be controlled (section 4), and by using plant cultivars that have been bred for improved symbiotic performance. This last approach has not yet been really exploited, but it will probably lead to major applications in the short term, at least in the case of nitrogen-fixing trees.

1. The symbionts

1.1 Tropical rhizobia

1.1.1. Taxonomy

It is now recognized that bacteria which form nodules on legumes, and which are known under the general name of rhizobia, belong to two genera: *Rhizobium* and *Bradyrhizobium* (Jordan 1982, 1984; Elkan 1984). All strains of the *Rhizobium* genus are fast-growing. The genus *Rhizobium* comprises four species: *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Rhizobium loti*, and *Rhizobium fredii*. The species *R. leguminosarum* results from the fusion of three species *Rhizobium trifolii*, *Rhizobium phaseoli*, and *R. leguminosarum* (in Jarvis *et al.* 1986). *R. fredii* is a new species of *Rhizobium* which nodulates soybean (Scholla and Elkan 1984).

The genus *Bradyrhizobium* comprises all the slow-growing strains of the former *Rhizobium* genus, i.e. the cowpea miscellany, and *Bradyrhizobium japonicum* (formerly known as *Rhizobium japonicum*).

Until now, it was generally recognized that most tropical nitrogen-fixing bacteria nodulating legumes were part of the cowpea miscellany. In fact, these bacteria may belong to either genus, *Bradyrhizobium* or *Rhizobium*. Many fast-growing strains have already been isolated from tropical trees belonging to the genera *Acacia*, *Leucaena*, and *Sesbania*. A taxonomic study under way at the ORSTOM laboratory in Dakar has shown that these strains are closely related to temperate fast-growing ones and consequently should be included in the *Rhizobium* group.

1.1.2. Specificity in connection with nodulation and effectiveness

It is relevant to make a distinction between the specificity related to the nodulation process and the specifity which concerns the nitrogen-fixing potential of the system.

The first type of specificity has been recognized for many years. It is well known that host plants exhibit different degrees of specificity, the species that nodulate with many types of strains being designated as promiscuous, in contrast to the specific ones. Probably, the most promiscuous species are to be found in group 1 of the legume trees defined later (section 1.1.3.2.).

The concept of specificity related to effectiveness comes from studies in *Stylosanthes* sp. (Date and Halliday 1980). This concept was found to apply to other legumes including tree legumes, such as *Acacia seyal*. This tree species nodulates both with *Rhizobium* and *Bradyrhizobium* strains, but in general it fixes nitrogen actively only with *Rhizobium* strains.

1.1.3. Examples of host relationships

1.1.3.1. Soybean

As indicated earlier, soybean which generally nodulates with *Bradyrhizobium japonicum*, also nodulates with fast-growing strains of the genus *Rhizobium*, that are now designated as *Rhizobium fredii*, and with *Bradyrhizobium* of the cowpea miscellany. However, it should be noted here that there are differences in the host-relatedness of soybean cultivars, the host range of Asian cultivars being broader than that of American cultivars. The former cultivars can nodulate with *Bradyrhizobium* of the cowpea miscellany, whereas the latter ones cannot (Roughley *et al.* 1980).

1.1.3.2. Tropical trees

Tropical tree legumes can be classified into three groups according to effective nodulation patterns with *Rhizobium* and *Bradyrhizobium* (Dreyfus and Dommergues, 1981).

1. Trees of group 1 nodulate effectively with *Bradyrhizobium*, e.g. *Acacia albida*, *Dalbergia melanoxylon*, *Dalbergia sisso*, *Prosopis africana*, *Pterocarpus erinaceus*, *Gliricidia sepium*.
2. Trees of group 2 nodulate effectively with *Rhizobium*, e.g. *Acacia nilotica* (var. *adansoni* and *tomento*), *Acacia raddiana*, *Acacia senegal*, *Prosopis juliflora*, *Sesbania grandiflora*.
3. Trees of group 3 nodulate with both genera, *Rhizobium* and *Bradyrhizobium*, e.g. *Acacia seyal*, *A. sieberana*, *Erythrophleum guineense*.

This classification is liable to revision since it has already been observed that host plants known to nodulate with strains of *Rhizobium* alone or of *Bradyrhizobium* alone were later found to nodulate with both genera. This has been already reported in *Acacia senegal* (section 1.1.2.), and is also well known in *Leucaena leucocephala*, a species that generally nodulates with *Rhizobium*, but which occasionally nodulates with *Bradyrhizobium* (Dreyfus and Dommergues 1981; Sanginga *et al.* 1986).

1.1.3.3. Sesbania rostrata

Stems of *S. rostrata* nodulate with strains which, in spite of their fast-growing characteristics, are closely related to *Bradyrhizobium*. These strains, which can nodulate the roots, are unique since they can grow on molecular nitrogen as the sole nitrogen in pure culture (Dreyfus *et al.* 1983). They could probably be incorporated in a new species or even a new genus of nitrogen-fixing bacteria (Jarvis *et al.* 1986). Roots of *S. rostrata* nodulate with *Rhizobium* strains that also nodulate the roots of non-stem nodulated *Sesbania* spp. Few of these strains are also able to nodulate the stems of stem-nodulating *S. rostrata*, but they are unable to grow on molecular nitrogen as the sole nitrogen source *in vitro*.

1.1.3.4. *Parasponia*

Parasponia sp. (Ulmaceae) is the only non-legume genus known to nodulate with *Bradyrhizobium* (Trinick 1981). Whereas most *Bradyrhizobium* strains are rather promiscuous, *Bradyrhizobium* strains isolated from *Parasponia* sp. fail to nodulate most of the tropical legumes that usually nodulate with *Bradyrhizobium* strains (Trinick and Galbraith 1980).

1.1.4. Selection of strains for inoculation

In this paragraph, we shall not deal with the methodology involved in the selection of strains, a topic that has been properly addressed in specialized books (e.g. Somasegaran and Hoben 1985) and that is briefly presented hereafter (section 3.2), but shall call attention to the following considerations.

Since many tropical soils have a low pH and often a high aluminium content, it is advisable to screen strains for their tolerance to acidity and high aluminium concentration. However, one should be well aware of the fact that the host plant is usually less tolerant to these soil constraints than the symbiotic bacterium. Thus, it is necessary not only to improve the tolerance of the micro-organisms, but also that of the host plant. This point is well illustrated by the behaviour of *Leucaeoa leucocephala* (2.2.2.2). Moreover, it is recognized that the process of symbiosis, notably nodulation, is about ten times more sensitive to acidity than either bacterial or root growth alone (Evans *et al.* 1985).

In contrast with the established dogma that locally isolated strains are best adapted to the environmental conditions, and consequently the most effective in these conditions, it was found that some introduced strains could markedly improve nitrogen fixation when used for inoculating. Thus, strain TAL 651 isolated from *Psophocarpus* sp. and maintained in NifTAL MIRCEN collection at Hawaii, was found to be the most efficient strain for *Acacia holosericea* grown in Senegal (Cornet and Diem 1982).

1.2. *Frankia*

Apart from legumes (and plants of the genus *Parasponia*), which are nodulated by rhizobia, about 200 plant species covering 19 genera and 8 families are nodulated by nitrogen-fixing micro-organisms known as *Frankia* (section 2.4). The actinomycetal nature of *Frankia* was detected using cytological techniques for many years (Becking 1975; Gardner 1976) and confirmed in 1978 when Torrey's group isolated a strain of *Frankia* and cultivated it *in vitro* for the first time (Callaham *et al.* 1978).

1.2.1. Actinorhizal nodules

Morphologically and anatomically different from legume nodules, actinorhizal nodules are formed from modifications of lateral roots that produce lobes characterized by successive dichotomous branching and, in the next stage, a coralloid mass composed of a cluster of lobes. The nodule continues to grow for several years until it reaches the size of a tennis ball. Larger nodules, up to 20 cm in diameter, have been found on the roots of 20-year-old *Allocasuarina stricta* in Tunisia. The lobes at the outer part of the nodular mass, which are the most recently formed, fix nitrogen actively. Depending on the plant species and/or the environmental conditions, the lobe may or may not develop a determinate nodule root growing more or less vertically upward.

The symbiotic micro-organism, *Frankia*, occupies specific cortical cell layers outside the central vascular cylinder unlike legume nodules which have the symbiotic bacterium *Rhizobium* or *Bradyrhizobium* located within the peripheral vascular bundles.

Cross-sections of actinorhizal nodules usually show two structures: (i) the encapsulated filamentous hyphae which ramifies profusely passing from one cortical cell to another; and (ii) vesicles which exhibit different characteristics according to the host-plant species. Vesicles can be septate (e.g. *Alnus glutinosa* or *Colletia spinosa*) or non-septate (e.g. *Purshia*), spherical (e.g. *Alnus*, *Colletia*, *Ceanothus*, or *Hippophaë*), pear, club-shaped, or filamentous (e.g. *Comptonia*, *Coriaria*, *Myrica*, or *Datisca*). Vesicles are considered as the sites of nitrogen fixation in the nodules (Torrey 1985). However, in some plants, namely plants belonging to the genus *Casuarina*, no vesicles have been detected, which raises the problem of the site of nitrogen fixation in these nodules. A third structure, called sporangia, may exist in actinorhizal nodules. Sporangia have been found in effective nodules of *Alnus glutinosa*, *Myrica gale* and in ineffective nodules of *Elaeagnus umbellata* (Newcomb 1981). Whereas all strains of *Frankia* are able to produce sporangia in most culture media, only some strains can produce sporangia in the nodules of certain host-plants. It is well established that *Alnus* has both spore-positive and spore-negative nodules, the sporulating or non-sporulating character of the nodules being particular to certain strains of *Frankia*. In a recent study, Normand and Lalonde (1982) showed that the most effective strains of *Frankia* of *Alnus* were of the spore-negative type, whereas the spore-positive type strains had an effectiveness that was on the average 70 per cent less than that of the most effective spore-negative strains.

The haemoglobin content in nodules of legumes is correlated with their nitrogen-fixing ability. The haemoglobin of legumes, which is called leghaemoglobin, serves to facilitate the oxygen flux to rhizobia respiring at extremely low, non-toxic, free oxygen concentration. In actinorhizal systems, the presence of a haemoglobin entity had been disputed for many years. Recently, Tjepkema (1983) through the use of sensitive spectrophotometry confirmed the presence of high concentrations of haemoglobin in nodule slices of *Casuarina cunninghamiana* and *Myrica gale*. Lower concentrations of haemoglobin were observed in the nodules of *Comptonia peregrina*, and *Elaeagnus angustifolia*, and negligible to trace amounts were found in *Ceanothus americanus* and *Datisca glomerata* (Tjepkema 1984; Appleby 1984).

1.2.2. Isolation and culture of *Frankia*

The difficulty of isolating *Frankia* from actinorhizal nodules and its subsequent cultivation explains why it took until 1978 to perform the first successful isolation (Callaham *et al.* 1978). The problem of isolating *Frankia* is not dealt with here as it has already been addressed in detail elsewhere (Lalonde and Calvert 1979; Baker and Torrey 1979; Diem *et al.* 1982a, 1983; Carpenter and Robertson 1983)

Frankia are slow-growing actinomycetes; even when cultured in the best adapted media currently known, the doubling time often exceeds 48 hours. Furthermore, the growth requirements of the different strains of *Frankia* are not yet well known.

Frankia cultured *in vitro* usually exhibit structures, i.e. hyphae, vesicles, and sporangia. Vesicles are generally formed in nitrogen-deficient media. Nitrogen fixation, as demonstrated by the acetylene reduction assay, or $^{15}N_2$ incorporation, has been shown to accompany the formation of vesicles, indicating that these structures are the site of nitrogen fixation. A fourth structure has recently been reported in cultures of *Frankia* of *Casuarina equisetifolia* (Diem and Dommergues 1985). This occurs when vegetative hyphae develop into wide torulose hyphae, which are called reproductive torulose hyphae (RTH), on account of their seemingly major role in the propagation of *Frankia* strains of *C. equisetifolia*.

1.2.3. Infectivity and effectivity

Attempting to classify the known *Frankia* isolates according to host specificity groups should be avoided since some strains of *Frankia* can infect two or more specificity groups, such as *Alnus* and *Elaeagnus* (Lalonde and Simon 1985), *Alnus* and *Comptonia* (Becking 1982), or *Casuarina* and *Hippophaë* (Diem *et al.* 1983; Zhang *et al.* 1984), and also because strains isolated from a given host are unable to infect this host, but can nodulate plants of other specificity groups (Diem *et al.* 1982b; Zhang *et al.* 1984). Some strains are known to be highly specific; *Frankia* strain ORS021001, for instance, nodulates only true *Casuarina* and not *Allocasuarina* species (Gauthier *et al.* 1984), whereas other strains have a wider host range, e.g. ORS 022602 (Puppo *et al.* 1885).

After an infective strain has been isolated, its effectiveness has to be evaluated. The procedure adopted for the selection of *Frankia* is similar to that used in the case of rhizobia, but screening strains of *Frankia* takes longer because nodulation is usually slower than in legumes (up to 2 weeks) and again, because the growth of some host plants is also slow at the plantlet stage, e.g. *Casuarina* spp. As in the legume symbiosis, some infective strains may be ineffective (e.g. Baker *et al.* 1980); furthermore, there may be some interaction between the host plant and *Frankia*, a fact that should be considered when selecting strains for their effectivity.

1.2.4. Influence of inoculation

It is well known that leguminous crops introduced in soils without specific *Rhizobium* strains benefit readily from inoculation, provided that the conditions for nodulation and nitrogen fixation are favourable. The situation is the same for actinorhizal plants: both pot and field experiments show that inoculation with the proper strain of *Frankia* usually enhance growth and nodulation of actinorhizal plants grown on soils that previously had a low or zero *Frankia* population.

As part of a large reclamation project in northern Quebec, more than seven million seedlings of actinorhizal plants, especially *Alnus crispa*, were successfully inoculated on an industrial scale with *Frankia* inoculum (Périnet *et al.* 1985).

Similar experiments performed in Senegal have demonstrated that inoculating *Casuarina equisetifolia* not only improved tree growth in the coastal sandy soils, but also contributed efficiently to stabilizing the moving dunes. The effects of inoculation were much greater than those obtained by applying relatively large amounts of nitrogen fertilizer (Table 1.1). Unpublished results by ORSTOM group indicate that inoculated *C. equisetifolia* produced more biomass than trees fed with nitrogen fertilizer. These findings concur with observations made by Sellstedt and Huss-Danell (1985) on *Alnus incana*, indicating that the energy required in nitrogen fixation is probably not great

Table 1.1

Influence of inoculation with *Frankia* ORS021001 on height, dry weight, and N_2 fixation of 11-month-old *C. equisetifolia* (Gauthier *et al.* 1985)

Treatments				
Inoculation	N addition (g/tree)	Height (cm)	Dry weight (g/tree)	N_2 fixed (g N_2/tree)
0	0.5	170 a	295 a	0
0	2.5	192 a	409 a	0
+	0.5	216 b	525 b	3.3–2.3[1]

Figures in same columns followed by same letter do not differ significantly. $P = 0.05$ (Duncan test).

1. First figure calculated from direct isotope dilution method, second figure from *A* value method.

enough to slow down biomass production in alders. One hypothesis suggests that, at least in the case of some actinorhizal plants, the symbiosis with *Frankia* favourably alters the hormonal balance of the plant, thus enhancing its growth.

The slow growth and poor yield of *Frankia* are considered a major impediment in the preparation of inocula for actinorhizal plants. However, technological progress will probably make it possible to produce commercial inocula in the near future (Righetti and Hannaway 1985).

2. The host plants

2.1. Legumes grown for grain, forage, green manuring, and mixed cropping

Leguminous plants other than trees are widely grown in the tropics, providing people and cattle with a protein-rich diet; they are also often used as green manure crops (e.g. Chée 1982) or introduced in mixed cropping systems (e.g. Sprent 1983; Simon 1986). All the plants presented hereafter belong to the subfamily Papilionoideae, whereas nitrogen-fixing trees are found in the three subfamilies of leguminous plants (section 2.2).

2.1.1. Grain legumes

Arachis hypogaea (peanut, groundnut) nodulates freely with *Bradyrhizobium* of the cowpea miscellany. Peanut cultivars exhibit clearcut variations in the nodulation ability, which affects the amount of nitrogen fixed. Nodulation is most sensitive to environmental constraints. In unfavourable conditions, nitrogen fixation may be totally inhibited. On the other hand, when soil has been properly managed, nitrogen fixation can reach 140 kg N_2/ha per crop (Ganry *et al.* 1985).

Cajanus cajan (pigeon pea) nodulates with *Bradyrhizobium* of the cowpea miscellany. Fixation rates of 90–150 kg N_2/ha/year have been reported (Dobereiner and Campelo 1977).

Cicer arietinum (chick-pea) has specific requirements for *Rhizobium*, which explains why inoculation is essential in soils that have never been planted with this plant before.

Glycine max (soybean), like chick-pea, has specific requirements for *Rhizobium* and *Bradyrhizobium* (section 1.1.3.1), so that inoculation is mandatory in areas where this legume is newly introduced. In favourable sites the amount of nitrogen fixed is in the range of 100–200 kg N_2/ha per crop (Gibson *et al.* 1982).

Phaseolus vulgaris has specific requirements so that inoculation is often beneficial. Acidity or nitrogen excess in soil are definitely harmful for the symbiosis. Other species of *Phaseolus*, namely *P. mungo* and *P. aureus*, are perfectly adapted to acid soils, where nitrogen fixation is satisfactory. In *P. aureus*, nitrogen fixation rates of 224 kg N_2 fixed/ha per crop have been reported (Dobereiner and Campelo 1977). Climbing cultivars are consistently superior in nitrogen fixation than most of the bush types. Three factors contribute to the variability encountered, and these are the supply of carbohydrates to the nodules, relative rates of N uptake from soil and time of flowering (Graham 1981).

Vigna unguiculata (cowpea) nodulates readily with *Bradyrhizobium* strains of the cowpea miscellany, which are present in most tropical soils, so that inoculation is not necessary. The amount of nitrogen fixed varies widely with the cultivar used: comparing the nitrogen fixation rates of four cultivars of cowpea grown in identical conditions, Eaglesham *et al.* (1982) found that these rates covered a span from 49 to 101 kg N_2 fixed/ha. Exceptionally high rates of 354 kg N_2-fixed/ha have been reported (Dobereiner and Campelo 1977).

Voandzeia subterranea (bambara groundnut) nodulates readily in many soils, which suggests that this legume is associated with strains of *Bradyrhizobium* of the cowpea miscellany. However, specific strains have been reported to be exceptionally effective (National Research Council 1979). Investigations are under way to screen the cultivars which exhibit the best nitrogen fixing ability.

2.1.2. Forage legumes

Centrosema pubescens does not seem to have very specific rhizobia requirements, so that inoculation is not essential except in some situations.

Desmodium spp has probably moderately specific rhizobia requirements. There are reports of exceptionally high nitrogen fixation rates (up to 360 kh N_2 fixed/ha/year) by *Desmodium intortum* (Dobereiner and Compelo 1977).

Lablab purpureus, ex-*Dolichos lablab* (lablab bean), often mentioned as a grain crop, is more frequently used as a forage legume. It nodulates easily with *Bradyrhizobium* of the cowpea miscellany, so that inoculation is generally not required.

Macroptilium atropurpureum, ex-*Phaseolus atropurpureus*, cv siratro, nodulates with most strains of *Bradyrhizobium*; thus it does not require inoculation. Nitrogen fixation rates are high (Dobereiner and Campelo 1977).

Neonotonia wightii, ex-*Glycine wightii*, nodulates with *Bradyrhizobium*, but, according to Dobereiner and Campelo (1977), this species is seldom well nodulated because it does not seem to tolerate unfavourable environmental factors, such as acidity or P deficiency.

Pueraria phaseoloides (tropical kudzu) generally nodulates profusely and spontaneously.

Stylosanthes spp. (stylo) fall into three groups according to their effectiveness response with rhizobia strains: (1) group PE (promiscuous and effective) comprises species nodulating with a wide range of strains of the cowpea miscellany (like *Arachis*, *Lablab*, *Macroptilium*, *Pueraria*, *Vigna*), (ii) group PI (promiscuous, but often ineffective) comprises species that frequently nodulate with a wide range of rhizobia, but are often ineffective in nitrogen fixation, and (iii) group S (specific) includes species that nodulate with a narrow range of rhizobia strains (Date and Halliday 1980). *Stylosanthes humilis*, which belongs to group PI, was reported to fix 90 kg N_2/ha/year (Dobereiner and Campelo 1977).

2.1.3. green manure and mixed cropping

Canavalia ensiformis (horse bean or jackbean), and *Canavalia gladiata* (swordbean) are not only valuable green manure and cover crops, but also highly productive pulse crops. *Canavalia ensiformis* was reported to fix ca 60 kg N_2/ha/year (Dobereiner and Campelo 1977).

Crotalaria juncea (sunhemp) is cultivated as a source of bast fibre in India. Since it nodulates with strains of *Bradyrhizobium* of the cowpea type, which are present in most soils, inoculation is seldom necessary. If the plant is given adequate phosphate, 1 ha of *C. juncea* can add up to 300 kg nitrogen to the soil (National Research Council 1979). Green manuring with *C. juncea* was reported to increase signficantly the yield of different crops in South America (Dobereiner and Campelo 1977).

Phaseolus javanica and *Pueraria phaseoloides* are successfully used as cover crops, providing nitrogen and humus, and shielding the soil from water erosion. To date, nitrogen fixation rates have not been estimated with precision.

Tephrosia spp. are often used for green manuring, e.g. *Tephrosia candida* in Brazil (Dobereiner and Campelo 1977).

2.2. Tree legumes

2.2.1 Introduction

In many tropical regions gradual loss of soil fertility, insufficient firewood, and shortage of fodder are becoming serious problems especially in the many areas where the human populations are growing. Increasing the use of fertilizers or importing fuel are theoretical, but not realistic solutions to these problems. Alternatives must be identified that are low in cost and in energy demand (Roskoski *et al.* 1982). The introduction or reintroduction of trees in the agro-systems and the development of the use of woody plants with low nutrient requirements could successfully help solve the problems outlined above. Legume trees, together with actinorhizal plants, are good candidates because many fix nitrogen from the atmosphere, and thus can grow in

nitrogen-deficient soils. Furthermore, the common association of root systems with mycorrhizal fungi enables nitrogen-fixing trees to extract various crucial nutrients from infertile soils. The problem is much more complicated than generally thought, not only because of the vast range of variation in the nitrogen-fixing potential of the different species or even individual trees, but also because of the doubtful validity of figures published by experimenters who did not make allowances for the spacial and temporal variations that inevitably occur during biological nitrogen fixation. One fact is clear: the nitrogen-fixing potential of legume trees indeed covers a wide range, viz. from less than 1 to 300 kg N_2 fixed/ha/yr and over. Table 1.2. gives examples of reliable estimates of nitrogen fixation by a few tropical legume trees. Many of them have been obtained using the acetylene reduction method. This method has serious limitations, but since no simple, inexpensive alternatives exist, its widespread use will probably continue (Righetti and Hannaway 1985).

Table 1.2

Nitrogen fixation by trees in the tropics

Species	Method of estimation	N_2 fixed (kg/ha/yr)
Acacia mearnsii	B	200
	I	4–11
Acacia holosericea	A	6
Acacia pennatula	A	34
Gliricidia sepium	A	13
Inga jinicuil	A	35
Leucaena leucocephala	A	110
Prosopis glandulosa	C	25–36
Erythrina poeppigiana	D	57–66
Casuarina equisetifolia	B	58

A: acetylene reduction assay; B: nitrogen balance studies; C: by ^{15}N abundance studies indicating that 50% of total plant N comes from nitrogen fixation; I: isotope method (A value).

2.2.2. Host plants

The family Leguminosae is divided into three sub-families, Mimosoideae, Caesalpinoideae, and Papilionoideae, the best known genera of nitrogen-fixing trees being as follows (National Research Council 1979, 1980; Domingo 1983; Dobereiner 1984; Brewbaker *et al.* 1984; Halliday 1984 *a*, *b*). Caesalpinioideae: *Acrocarpus*. *Cassia*, *Cordeauxia*, *Schizolobium*; Mimosoideae: *Acacia*, *Albizia*, *Calliandra*, *Desmanthus*, *Enterolobium*, *Inga*, *Leucaena*, *Lysiloma*, *Mimosa*, *Parkia*, *Pithecellobium*, *Prosopis*, *Samanea*; Papilionoideae: *Dalbergia*, *Erythrina*, *Flemingia*, *Gliricidia*, *Sesbania*. Data on nodulation ability are available for only some of the species belonging to these different genera (Allen and Allen 1981; Kirkbride 1984).

As a general rule, members of the Caesalpiniodeae subfamily, i.e. mainly trees found in tropic regions, do not nodulate, as is the case with six *Cassia* species: *Cassia fistula*, *Cassia grandis*, *Cassia javanica*, *Cassia leiandra*, *Cassia nodosa*, and *Cassia siamea* (Halliday 1984*b*). However, there are exceptions, and these include *Acrocarpus fraxinifolius*, *Cordeauxia edulis*, and *Schizolobium parahyba*.

The majority of the Mimosoideae, which are also trees or shrubs growing in the humid and dry tropics, nodulate, e.g. many species of the genus *Parkia*. *Parkia biglobosa*, however, does not nodulate.

Nearly all the Papilionoideae, which are mainly shrubs and herbs, found around the world, nodulate.

Table 1.3

Tentative classification of some native and introduced West African woody legumes according to nodulation response patterns (after Dreyfus and Dommergues 1981)

Groups	Species	Specificity[1]
Group 1		
Nodulating with fast-growing rhizobia *(Rhizobium)*	*Acacia farnesiana*	S
	Albizia lebbeck	S
	Acacia nilotica	S
	Acacia raddiana	S
	Acacia senegal	S
	Leucaena leucocephala	S
	Prosopis juliflora	S
	Sesbania sp.	S
Group 2		
Nodulating with fast- and slow-growing rhizobia *(Rhizobium and Bradyrhizobium)*	*Acacia seyal*	S
Group 3		
Nodulating and slow-growing rhizobia *(Bradyrhizobium)*	*Acacia albida*	P
	Acacia holosericea	P
	Acacia sieberana	S
	Prosopis africana	P

[1] S: specific; P: promiscuous.

Table 1.3. is an attempt to classify some native and introduced African nitrogen-fixing trees according to their nodulation patterns. The impression that tropical tree legumes are much more promiscuous than temperate ones, i.e. they nodulate with a wide range of tropical rhizobia, is generally accepted (Halliday 1985). In fact, the promiscuous character of nitrogen-fixing trees varies according to the host plant, some of them being definitely promiscuous (e.g. *Acacia albida* or *A. mearnsii*), whereas others are rather specific like *Sesbania* spp. Usually, hosts nodulating with fast-growing strains of *Rhizobium* appear to be more specific than those nodulating with slow-growers or simultaneously with fast- and slow-growers. Some of the most promising species of nitrogen-fixing trees classified according to the two main climatic zones of the tropics are dealt below.

2.2.2.1. *Dry tropics*

As a preliminary remark, it should be noted that, though nodules are seldom found on nitrogen-fixing trees in the field in the dry tropics, nodulation does effectively exist on the same species grown in greenhouses or nurseries. One probable explanation is that water stress severely inhibits nodulation (Felker 1984).

Acacia albida, *A. raddiana*, and *A. senegal* These *Acacia*, native to Africa, are usually considered as highly valuable legume trees serving especially as soil improvers in agroforestry, and sources of fuel-wood, forage, and gum (*A. senegal*). Their nitrogen potential seems to be rather poor, but could possibly be improved by capitalizing on the great variability of the host plant. The subject has not yet been adequately studied.

Acacia holosericea Native to Australia, this species nodulates readily with *Bradyrhizobium* strains of the cowpea miscellany, but its nitrogen-fixing potential is rather low (Cornet *et al.* 1985). In addition *A. holosericea* is most often attacked by root-knot nematodes, which precludes the use of this species in agroforestry.

Albizia lebbeck Native to Bangladesh, Burma and Pakistan, this tree has been propagated in many tropical and subtropical regions (National Research Council 1980). Large perennial nodules of large size have been observed on adult trees in Senegal, but the potential nitrogen fixation of this species has not yet been evaluated.

Cordeauxia edulis This small bush, native to the semi-desert region bordering Somalia and Ethiopia is remarkable for its unique tolerance to drought (National Research Council 1979). It has been reported to be nodulated.

Prosopis spp. The nitrogen-fixing characteristics of this genus, which is currently receiving the pride of place in semi-arid regions and saline soils, were recently reviewed (Felker *et al.* 1981). Measuring the nitrogen compartmentalization of the biomass, productivity of leaves, branches, trunk and reproductive tissues, Rundel *et al.* (1982) estimated a nitrogen-fixation rate of 23–36 kg N_2/ha/yr with a 33 per cent stand cover. From determinations of natural abundance $^{15}N/^{14}N$ ratios, Shearer *et al.* (1983) estimated that *Prosopis* fixed approximately 43–61 per cent of its nitrogen, this range of figures applying to six of the seven sites they studied in the Sonoran desert, California. *Prosopis juliflora*, a tree native to Central America and northern South America, has been introduced in many arid zones of the world; it grows fast, even on soils very low in nutrients, probably thanks to its good nitrogen-fixing potential. In some places, *P. juliflora* is an agressive invader and thus is considered a hindrance (National Research Council 1980). Other species of *Prosopis*, namely *Prosopis cinerea* and *Prosopis tamarugo* are widely utilized in arid countries; their nitrogen-fixing potential has never been evaluated. An experimental study of the nodulating ability of *Prosopis chilensis*, *P. tamarugo* and *P. alba* has been initiated recently. Among these species, *P. chilense* seems to respond best to inoculation. The effectiveness of the nitrogen fixation process appears to vary greatly with the different *Prosopis* populations (Torres 1985).

2.2.2.2. *Humid tropics*

Acacia auriculiformis Native to Papua New Guinea and northern Australia, *A. auriculiformis* has been successfully introduced into Indonesia, Malaysia, and the Philippines. It produces profuse bundles of nodules and can thrive on soils deficient in nitrogen and organic matter, which suggests a good nitrogen-fixing capacity (Domingo 1983).

Acacia mangium A native of Australia, and southern Papua New Guinea, *A. mangium* is now being tried in several places in South-East Asia (Domingo 1983) and Africa, namely southern Senegal, Benin, Congo, and the Ivory Coast. In the three latter countries it grows exceedingly well (National Research Council 1983).

Acacia mearnsii A highland tree from southern Australia, *A. mearnsii* was introduced in India and Natal more than 100 years ago (Boland, *et al.* 1984), and successfully grown on tropical and subtropical plateaux, for example in Madagascar and Kenya. It has a remarkable nitrogen-fixing potential. However, in some soils, for example highland soils of Burundi in Eastern Africa, acidity and aluminium toxicity are so pronounced that nodulation is severely restricted, thus depriving the host plant of the benefice of nitrogen fixation.

Albizia falcataria This species, native to the eastern islands of the Indonesian archipelago and the west of Irian, has been spread throughout South-East Asia. Domingo (1983) considered it to be one of the fastest growing trees in the world. It is reported to nodulate, but its nitrogen-fixing potential is probably lower than that of other species of the same genus since fertilization may be needed (National Research Council 1979).

Calliandra calothyrsus Native to Central America, this small legume tree was introduced into Java in 1936. Its profuse nodulation, suggesting an active nitrogen fixation potential, and high litter production make it a first class soil improver, and as such, it is often used in rotation schedules and in intercropped systems (Domingo 1983). *Calliandra calothyrsus* has been successfully introduced beneath stands of non-nitrogen fixing trees, such as *Eucalyptus deglupta* and *Pinus merkusii* (National Research Council 1983).

Erythrina spp. More than 100 species are planted as shade trees, windbreaks, living fences, support plant and for alley cropping, wood, food, and medicinal purposes. In Rwanda, *Erythrina abyssinica* nodulates profusely, which suggests that it is associated with *Bradyrhizobium* of the cowpea miscellany. Nitrogen fixation rates of 12–40 kg N_2 fixed/ha/yr have been reported from *Erythrina poeppigiana* in coffee and cacao plantations (Anon. 1986)

Gliricidia sepium This is one of the most common trees of Mexico, Central America, and northern America. Estimates of nitrogen fixation based on nodule biomass and rates of nitrogenase activity are c. 13 kg N_2 fixed/ha/yr in the conditions prevailing in Mexico (Roskoski *et al.* 1982). *Gliricidia sepium* thrives well in the Amazon region. *G. sepium* has been introduced in Western Africa, but its nitrogen-fixing activity is impeded by attacks of root nematodes.

Leucaena leucocephala This tree has been the focus of a great deal of research during the past decade (National Research Council 1977; IDRC 1983). Native to Central America, it has been planted in many tropical countries including South-East Asia (Domingo 1983), Africa (Okigbo 1984), and South America (Dobereiner 1984). *L. leucocephala* is often used in agroforestry. Kang *et al.* (1981*a*, *b*) found that five or six annual prunings of the *Leucaena* hedge rows yielded between 5 and 8 tons of dry tops/ha/yr with N-yield of between 180 and 250 kg/ha/yr and were able to sustain maize grain yield at about 3.8 tons/ha/yr for two consecutive years with no N addition. Another alternative to utilizing *Leucaena* prunings as a N source is to apply them as mulch or to incorporate them in the soil before planting. The prunings as a N source appeared to be more effective when incorporated in the soil than when applied as mulch. High maize grain yield was obtained with application of 10 tons fresh prunings or a combination of 5 tons fresh pruning and N at 50 kg/ha.

Leucaena leucocephala nodulates with fast-growing *Rhizobium*, probably closely related to *R. loti*. *Leucaena leucocephala* was credited with a very high nitrogen-fixing potential, ranging from 600 to 1000 kg N_2 fixed/ha/yr (Guevarra *et al.* 1978). A more realistic figure of 110 kg N_2 fixed/ha/yr has been calculated by Högberg and Kvarnström (1982). Using the isotope technique, K. Mulongoy and N. Sanginga (personal communication) found that *L. leucocephala* inoculated with its specific strain could fix up to 200 kg N_2/ha/yr in the conditions prevailing at the IITA station, Ibadan, Nigeria. Early efforts to grow *L. leucocephala* in acid soils were unsuccessful. Inoculating the host plant with acid-tolerant strains of rhizobia induced nodulation, but did not increase the production of the tree (Halliday 1985). Recently, genotypes of *L. leucocephala* tolerant to acidity were identified to acid Amazonian soils (Hutton 1984), which means that trees tolerant to acidity can probably be selected and planted in acid soils, where they could effectively fix nitrogen, if used in association with acid-tolerant *Rhizobium* strains.

Mimosa scabrella Native to the Parana region of south-eastern Brazil, this species can be recommended for the mid-elevation cool tropics and subtropics (National Research Council 1980). It has been reported to respond positively to inoculation (Dobereiner 1984), but its nitrogen-fixing potential is unknown.

Pierocarpus indicus In many countries of south-eastern Asia (e.g. Papua New Guinea, Indonesia, Malaya, the Philippines), *P. indicus* is reputed as choice timber for furniture. It nodulates, but its nitrogen-fixing potential has not yet been studied (Domingo 1983). Since many genotypes have been identified, it would be worthwhile exploiting the related variability and thus improving tree productivity.

Sesbania bispinosa and *S. grandiflora* Native to many Asian countries, these tree legumes are widespread throughout South-East Asia, where they have a number of uses, including green manuring. Both species nodulate profusely, and are probably active nitrogen fixers, which could explain their extraordinary ability to restore soil fertility (Domingo 1983).

Parasponia spp. The Parasponia genus is the only non-legume genus known to form nitrogen-fixing nodules with rhizobia (Trinick 1975, 1982; Becking 1982; Akkermans and Houvers 1983). Only the *Parasponia* species from the tropical Malay archipelago have been described. Like some legume trees listed above, *Parasponia* can be nodulated both by rhizobia and *Bradyrhizobium*, but it generally prefers specific strains of *Bradyrhizobium* (section 1.1.3.4). Very high nitrogen fixation rates, up to 850 kg N_2 fixed/ha/yr have been reported (Trinick 1981), but are open to question although relatively high figures can be expected.

2.2.3 Improvement of nitrogen fixation

The two approaches recommended for actinorhizal plants—one related to the symbiotic micro-organism, the other to the host plant—should be used concomitantly to improve nitrogen fixation by legume trees.

2.2.3.1. Inoculation with rhizobia

As in annual crops, inoculations on trees can only be expected to have beneficial effects if specific rhizobia are absent or scarce, or if the strain to be introduced is both more competitive and more effective than the native strains. The difference between annual crops and trees is that seedlings are, or should be, grown in a sterile substrate in a nursery to avoid attacks by root pathogens. In this situation, inoculation with specific rhizobia is systematically recommended and the beneficial effect is proportionally greater if the soil's nitrogen content is low. After the seedlings have been transplanted to the field, there are two possibilities. (i) if specific and efficient rhizobia are already present in plantation soils, the difference between uninoculated and inoculated plants tends to decrease within a few years; (ii) if the soil totally lacks the specific rhizobia, the beneficial effect of inoculation may be spectacular. An example of absence of response to inoculation was noted in a trial established in Senegal; 17 months after transplantation uninoculated *Acacia holosericea* were 1.05 m tall, while the inoculated trees were 1.19 m, which is not significantly different (Cornet *et al.* 1985). The reason for this virtually ineffective inoculation was that the soil already contained the *Bradyrhizobium* strains that nodulate *A. holosericea*.

An example of successful inoculation comes from an experiment conducted in Australia, in which, 15 months after transplantation, *Leucaena leucocephala* inoculated with *Rhizobium* strain C381 had reached a mean height of 2.10 m, whereas uninoculated trees only grew to 0.33 m (Diatloff 1973). In the Australian case, the inoculation generated a positive response because the soil did not naturally contain any of the *Rhizobium* strain specific to *L. leucocephala*.

2.2.3.2. Genetic improvement of the host

In symbiotic nitrogen-fixing systems, the host genotype is considered to interact with the infective strain of *Rhizobium* in conditioning the nitrogen-fixing potential. Exploiting the large differences that exist between the genotypes of a given plant species provides us with a promising approach that, for actinorhizal plants, will be discussed later (section 2.4.2). Except for

Leaucaena leucocephala and *Prosopis* spp., genetic variation for tree legumes has not yet been well explored (Koslowski and Huxley 1983).

2.2.3.3. *Controlling the impact of limiting factors*

Though this problem is discussed later in section 4, it is necessary to stress here that management practices such as application of fertilizers, may be good agriculturally, but may not always be suitable for forestry or agroforestry. Some alternatives exist. The best known is inoculation with mycorrhizal fungi. The double inoculation of trees with rhizobia and a vesicular-arbuscular mycorrhizal fungi at the nursery level is more effective than inoculation with rhizobia alone, the remarkable feature of inoculation with a vesicular-arbuscular mycorrhizal fungi being that it improves the uniformity of tree growth after transplantation (Cornet *et al.* 1985).

2.2.4. Discussion

Despite the existence of some very successful systems involving nitrogen-fixing trees in both forestry or in agroforestry, some authors have questioned the economic validity of these systems, since the application of nitrogen fertilizers is very easy, and their acquisition relatively cheap in comparison to the management of nitrogen-fixing systems (Turvey and Smethurst 1983). This outlook is probably correct in many cases occurring in temperate countries. On the other hand, in the tropics, active denitrification and intense leaching often decrease the effectiveness of nitrogen fertilizers to well below the level obtained in temperate climates. This fact, coupled with the high price of nitrogen fertilizers in many developing countries, challenge the wisdom of using the chemical approach in tropical forestry and agroforestry, which means that further efforts to improve biological nitrogen fixation are more than justified. Highly active nitrogen-fixing systems that can withstand the diverse and often harsh conditions of the tropics are not yet available, but by carefully selecting the genotypes of the symbiotic micro-organism and those of the host plant, the nitrogen-fixing potential of trees can be improved. Not so long ago this was a dream. Now it is a tangible objective that can be achieved in the near future.

2.3. *Stem-nodulated legumes*

2.3.1. Introduction

Most nitrogen-fixing legumes bear nodules on their root system. However, some species also form stem nodules and are called stem-nodulated legumes. Three genera have been reported to comprise stem-nodulated species: *Sesbania*, *Aeschynomene*, and *Neptunia*. So far, only two *Sesbania* species have been reported to produce stem nodules: *S. rostrata*, which is native to Western Africa, and *Sesbania punctata*, probably native to Madagascar. The latter is presently being studied at the ORSTOM laboratory, Dakar. The *Aeschynomene* species known to have stem nodules are: *Aeschynomone afraspera*, *Aeschyomene denticulata*, *Aeschynomene elaphroxylon*, *Aeschynomene evenia*, *Aeschynomene filosa*, *Aeschynomene indica*, *Aeschynomene paniculata*, *Aeschynomene pfundii*, *Aeschynomene pratensis*, *Aeschynomene rudis*, *Aeschynomene schimperi*, *Aeschynomene scabra*, *Aeschynomene sensitiva*. The only *Neptunia* known to be stem-nodulated is *Neptunia oleracea*. In view of a recent comprehensive review (Dreyfus *et al.* 1984), attention is drawn mainly to the specific characteristics of these annual nitrogen-fixing systems and their implications for tropical agriculture.

2.3.2. Attributes of stem-nodulated legumes

2.3.2.1. *Nodulation sites and nodule initiation*

Nodulation occurs at predetermined sites on the stems. These sites have been identified as incipient root primordia, and remain dormant as long as they are not infected by specific rhizobia. In some species the root primordia protrude more or less through the stem epidermis; in other species they form a subepidermal dome hidden under the epidermis. In *Sesbania rostrata*, the root primordia always pierce the stem epidermis, forming a fissure that facilitates the penetration of the rhizobia. The genus *Aeschynomene* includes species with nodulation sites ranging from the *Sesbania* type with protruding root primordia (e.g. *Aeschynomene afraspera*), which nodulates readily, to the hidden root primordia type (e.g. *A. crassicaulis* and *A. elaphroxylon*), which is definitely less susceptible to rhizobial infection (Alazard 1985).

In *Sesbania rostrata*, the infection, which starts at the level of the fissure encircling the root primordia, spreads inward as narrow, branched intercellular threads to penetrate the host plant cells, where the rhizobia are finally released (Tsien *et al.* 1983; Duhoux 1984). In other words, the infection does not develop via the root hairs, like in most temperate legumes, but directly between the cells at the base of the root primordia, so that the nodule genesis seems to consist of three phases: intercellular infection, development of infection threads and intracellular infection. This mode of infection is not systematically observed in stem-nodulated legumes. In several *Aeschynomene* species, for instance, the mode of infection does not involve the development of infection threads. Following the stimulation of one or more meristematic zones at the base or within the root primordia by the presence of rhizobia, the nodule develops and become visible 2 (*S. rostrata*) to 8 days (*A. afraspera*) after stem inoculation (D. Alazard and E. Duhoux, personal communications). Like root nodules, stem nodules contain leghaemoglobin. This haemoglobin is present in large quantities, and furthermore has an oxygen affinity which is greater than that of the soybean (Appleby 1984), a plant known for its high nitrogen-fixing potential (Wittenberg *et al.* 1985).

2.3.2.2. *Associated rhizobia*

Strains of rhizobia-infecting stem-nodulated legumes have been classified into three main groups, according to their host relatedness.

The *Sesbania rostrata* group All the strains in this group (type ORS570) are fast-growers. Their unique characteristics have already been presented (section 1.1.3.3). In continuous culture 'under optimum conditions of oxygen supply, nitrogen fixation rates are 300–400 nmol N_2/mg dry weight and nitrogenase activity reaches 2000 nmol/mg dry weight, the highest values yet recorded for any rhizobia species (Gebhardt *et l.* 1984). All rhizobia strains of infecting the stems of *S. rostrata* are highly specific.

The *Aeschynomene* group Three subgroups have been recognized (Alazard 1985); subgroup α comprising fast-growing strains that have a narrow host range (e.g. *Rhizobium* of *A. afraspera*); subgroup ß, intermediate between subgroups α and γ (e.g. *Bradyrhizobium* of *A. indica*), and subgroup γ comprising slow-growing strains typically of the cowpea group (e.g. *Bradyrhizobium of A. elaphroxylon*).

The *Neptunia* group The strains of this last group are fast-growers and are probably closely related to *R. meliloti.*

2.3.2.3. *Stem inoculation*

Spontaneous stem inoculation has often been observed, but is always irregular. The rhizobia are dust-borne, water-borne (by water on lowland soils and rainwater), and also conveyed by other vectors such as insects. Seed inoculation by specific rhizobia will induce excellent root nodulation, but only partial stem nodulation. Thus, the shoots have to be inoculated to ensure adequate infection of all the nodulation sites on the plant. The recommended method consists of spraying a liquid culture of the specific rhizobia on the stems using a standard sprayer. This procedure, together with a more sophisticated one, are described in recently published note by Dreyfus *et al.* (1985).

Considering the differences in the anatomy of nodulation sites (section 2.3.2.1), it is easy to understand that plants with the most highly evolved nodulation sites (e.g. *Sesbania rostrata*) nodulate readily, whereas those with hidden root primordia (e.g. *Aeschynomene elaphroxylon*) only nodulate if the stems are heavily inoculated.

2.3.2.4. *The nitrogen-fixing potential*

Various methods of estimation (isotopic, balance, and difference) used, and more recent unpublished data showed that *Sesbania rostrata* fixes between 100 and 300 kg N_2/ha in a period of *c.* 7 weeks (Rinaudo *et al.* 1983), thus indicating that it has one of the most effective nitrogen-fixing potential known to date. Such high performances can be explained by three sets of characteristics: (i) the unique properties of the associated rhizobia (section 2.3.2.2.) and the leghaemoglobin of *S. rostrata* (section 2.3.2.1); (ii) the fact that the stem nodule is a combination of functionally well integrated photosynthetic and nitrogen-fixing tissues, and as a result, satisfies part of its internal energy requirements (Eardly and Eaglesham 1985); and finally (iii) the tolerance of the whole system to combined nitrogen (section 2.3.2.5).

Stem-nodulated legumes other than *S. rostrata* do not exhibit such a high potential, although some, such as *Aeschynomene afraspera*, can be considered as good nitrogen fixers.

2.3.2.5. *Tolerance to soil combined nitrogen*

One of the dreams of the rhizobiologist has always been to obtain nitrogen-fixing systems that cannot be repressed by combined soil nitrogen. Root-nodulated legumes are repressed, although progress has recently been made through genetic improvement of the host plant (Gresshoff *et al.* 1985; Herridge and Betts 1985). On the other hand, stem-nodulated legumes are beautifully evolved systems, and are therefore capable of nodulating and sustaining nitrogen-fixing activity in the presence of relatively large amounts of mineral nitrogen in the rhizosphere. Thus, for *A. afraspera* grown in hydroponic conditions, the threshold of combined nitrogen is c. 10–15 mM for nodulation and c. 6 mM for nitrogen fixation. In soil, the threshold is ca 200 kg N/ha for nodulation and c. 100 kg N/ha for nitrogen fixation (D. Alazard, personal communication). These results confirm earlier findings with *Sesbania rostrata* (Dreyfus and Dommergues 1980). Exploiting this remarkable tolerance to combined nitrogen should probably be rewarding in the future (section 4).

2.3.3. Implications for agricultural practices

2.3.3.1. *Soil fertility*

Several field-simulation trials carried out in Senegal indicated that, when *S. rostrata* was applied as green manure in rice fields, the first following crop yielded 100 per cent more than the control plot, and the subsequent crop yielded 50 per cent more (Rinaudo *et al.* 1983). Other experiments using *Aeschynomene afraspera* as green manure showed similar beneficial effect on the rice yields (D. Alazard, personal communication). Such results evidence that stem-nodulated legumes can significantly contribute to the maintenance and the restoration of soil fertility in paddy soils.

2.3.3.2. *Current limitations*

We saw earlier that ploughing under stem-nodulated legumes as green manure can improve the soils used in tropical agriculture. Recent investigations carried out in Senegal indicate that these legumes can also be used as

Table 1.5

Comparison of two clones of *Casuarina equisetifolia*[1] (Sougoufara *et al*. 1987)

	Shoot			Nodule	ARA[2]	
Treatment	d.wt. (mg per plant)	N (%)	N total (mg per plant)	d.wt. (mg per plant)	per plant	per g nodule
Uninoculated						
Clone α	130 a	0.73 a	0.95 a	0 a	0 a	NA[4]
Clone ß	90 a	1.02 a	0.92 a	0 a	0 a	NA
Inoculated with *Frankia*[3]						
Clone α	660 b	1.71 b	11.29 b	54 b	2.88 b	54 b
Clone ß	1730 b	2.02 c	34.93 c	88 c	4.58 c	56 b

[1] 7 month-old cuttings, nine replicates.

[2] Acetylene reducing activity, expressed as µmol C_2H_4 per plant or per g nodule, dry weight (d.wt.).

[3] Each cutting was inoculated with 2 ml (20 µg of proteins) of a 4-week-old culture of *Frankia* strain ORS021001. Values in columns followed by the same letter are not significantly different, $P = 0.05$ (nodule dry weight; ARA), $P = 0.01$ (shoot dry weight, total N and N%).

[4] NA—not applicable.

species. This is illustrated in Table 1.5 which reports the results of a preliminary experiment conducted out at the ORSTOM Research Station of Bel Air, Dakar, Senegal. Two clones of *C. equisetifolia* (α and ß) characterized by different nitrogen-fixing potentials were grown in a nitrogen-deficient sandy soil; one set of plants was not inoculated, the other was inoculated with *Frankia* strain ORS021001 (Diem *et al*. 1983). In the former, both clones followed a very poor but not significantly different growth pattern. By contrast, in the inoculated group clone ß produced 2.6 times more biomass (expressed in terms of dry weight and total nitrogen) than clone α. Concomitantly, the nodule weight and the nitrogen-fixing activity of the whole plantlets (measured using the acetylene reduction method) of clone ß were 1.6 times significantly higher than the same characteristics of clone α. Interestingly, the specific nitrogen-fixing activity (that is the activity expressed on the nodule weight basis) of both clones did not differ significantly. Since the characteristic is probably directly conditioned by the genotype of the *Frankia* strain used, the result should not be surprising.

2.4.3. Exploiting the variability of the host plant

The above experiment clearly implies that the nitrogen-fixing potential of *Frankia* systems could be improved not only by selecting the best strains of *Frankia*, but also by selecting the most suitable host plant. In the preceding case, selection was based on the nodulating ability of the plant, since the goal was to produce hypernodulating clones.

The host's role in the symbiosis of crop legumes was recognized many years ago (Caldwell and Vest 1977). However, this characteristic has only been fully exploited by a few research groups (e.g. Attewell and Bliss 1985; Phillips and Teuber 1985; Gresshoff *et al*. 1985). In the case of woody plants, the problem is somewhat easier to solve for two reasons. First, wood plants constitute wild material, which has not yet been submitted to breeding manipulations and which often exhibits an extensive genetic variability. Secondly, perennial plants can be selected through vegetative propagation techniques; it is economically feasible to use these techniques for trees, but not for annual crops with a plant density of 100 000 or more individuals per ha.

In the case of *Alnus* spp. host selection via micropropagation techniques was successfully used by Lalonde's group (Lalonde and Simon 1985). Another example of this approach has already been presented (Table 1.5), which clearly shows that, by screening the clones of *Casuarina equisetifolia* which have the best nodulation, hence the highest nitrogen-fixing potential, nitrogen fixation rates can be markedly increased. The only problem encountered with *Casuarina* in the past was that vegetative propagation based on the use of cuttings was unsatisfactory because of frequent plagiotropism. This problem has been recently solved by Duhoux *et al*. (1986), who devised a most reliable micropropagation technique based on the use of immature female inflorescences. Field experiments are currently under way to check the behaviour of clones selected for their high nitrogen fixation potential and micropropagated to the method just referred to.

3. Inoculation

The purpose of inoculating a legume or an actinorhizal plant is to ensure that an adequate number of cells of the associated strain of rhizobia or *Frankia* is present on the seed of the host plant or in the soil at sowing or planting time, so that a quick infection of the root system occurs, leading to effective nitrogen fixation. In this section, we focus our attention mostly on the often overlooked problem of the need for inoculation, the other aspects of the topic being only briefly reviewed since they have been developed in detail in many publications (e.g. Brockwell 1977; Date and Roughley 1977; Burton 1979; Meisner and Cross 1980; Thompson 1980; Williams 1984).

3.1 The need for inoculation

The following rules could serve as a general guide:

1. When dealing with nursery soils, which should systematically be sterilized to eliminate pathogens, inoculation with the nitrogen-fixing micro-organism is always required (in this case, rhizobia or *Frankia* inoculation should be complemented by the introduction of mycorrhizal fungi).

forage, which may be most interesting for the future. Consequently, we recommend developing the use of the stem-nodulated species mentioned previously. However, pending further genetic improvement, the culture of those legumes is subject to certain constraints. The main limitations that have been detected are: the high water requirements of most species, the photoperiodic response of some of them, especially *S. rostrata*, and this latter's sensitivity to root nematodes in well drained soils.

2.4. *Actinorhizal plants*

There are far fewer nitrogen-fixing actinorhizal plants than nitrogen-fixing legumes, but their numeric inferiority is offset by their generally excellent capacity to regenerate poor soils and, at the same time, produce not only timber and wood for fuel, but also shelter for cattle and crops.

2.4.1. The host range of *Frankia* systems

Because of their ability to form nodules with *Frankia*, which is an actinomycete, in 1978 the non-leguminous nitrogen-fixing plants became known as 'actinorhizal plants,' (Torrey and Tjepkema 1979), a name now used world-wide. Table 1.4 gives an updated synopsis of the tropical actinorhizal genera. The list will probably be expanded and/or revised as new species or genera are discovered. Recently, the nodulating ability of *Rubus ellipticus*, a plant hitherto recognized as actinorhizal, was questioned by Stowers (1985), in contradiction to earlier reports (Becking 1982) and this now casts doubt on the actinorhizal character of some species of the *Rubus* genus. New actinorhizal plants will probably be engineered in the future (section 5). It is clear that, despite the taxonomic affinities between certain of the actinorhizal genera, overall disparities are far too great to even consider that the nodulating ability may have evolved from a common ancestor (Bond 1983). The wide taxonomic host range of actinorhizal plants gives us reason for optimism (Righetti and Hannaway 1985), because this characteristic suggests the possibility of introducing nitrogen fixation into additional hosts.

2.4.2. Inter- and intra-specific variability in the nitrogen-fixing potential of actinorhizal plants

Lack of reliable estimates makes it difficult to rank the nitrogen-fixing potential of actinorhizal plants correctly. Some preliminary data indicate the existence of a rather wide range of potentials from good to rather poor. Consequently, the nitrogen-fixing potential of each individual species must always be carefully considered when choosing species for reforestation programmes. In addition, attention should be given to intra-specific variability within the nitrogen-fixing potential; it may be impressive within a single

Table 1.4

Genera and distribution of tropical and subtropical species of actinorrhizal plants

Family	Genus	Main known species	Distribution
Casuarinaceae	*Casuarina*		Tropical and subtropical Australia; Indo-Pacific area from India to Polynesia
	Allocasuarina		
	Gymnostoma		
Myricaceae	*Myrica*	*M. javanica*	Mountains of Indonesia and Philippines
		M. cacuminis	Mountains of Cuba
		M. punctata	Mountains of Cuba
		M. shaferi	Mountains of Cuba
Betulaceae	*Alnus*	*A. jorullensis*	South America
		A. nepalensis	Nepal
		A. japonica	Mountains, Asia
		A. maritima	Mountains, Asia
Elaeagnaceae	*Elaeagnus*	*E. latifolia*	Indonesia
		E. conferta	Indonesia
		E. philippensis	Philippines
Rhamnaceae	*Ceanothus*	*C. prostratus*	Pacific North American Coast to Mexico
	Discaria	*D. americana*	South America
		D. serratifolia	Argentina
		D. trinervis	
		D. nana	
	Colletia	*C. paradoxa*	South America
		C. spinosissima	
		C. armata (= *spinosa*)	
	Trevoa	*T. trinervis*	Chile
	Talguenese		Chile
	Kentrothamus		Chile
Coriariaceae	*Coriaria*	*C. japonica*	Mountains of Asia
		C. nepalensis	Mountains of Asia
		C. sinica	China (Hunan)
Rosaceae	*Rubus*	*R. ellipticus*	Continental Asia, Sri Lanka, Luzon, Indonesia
	Cowania	*C. mexicana*	California, Mexico
	Dryas	*Dryas* sp.	Mountains of Asia
	Cercocarpus	*C. ledifolius*	California
Datiscaceae	*Datisca*	*D. cannabina*	Mediterranean area to Central Asia
		D. glomerata	SW and NW Mexico

2. When planning the introduction of a new species of nitrogen-fixing plant in a site where the species has never been grown before, one should make a distinction between promiscuous and specific host plants. If the legume plant is promiscuous, that is capable to nodulate with many types of rhizobia, inoculation is generally unnecessary. By contrast, if the legume has specific rhizobia requirements, inoculation should usually be recommended. In the latter situation, a beneficial effect of inoculation would be expected, provided no limiting factor interfere. Actinorhizal plants are rather specific, so that inoculation is mandatory.

3. When growing a nitrogen-fixing plant in a site where it had already been cultivated before, inoculation is often useless, provided that the associated strains of rhizobia or *Frankia* have survived. One should be well aware of the fact that there are many exceptions to these general rules so that, before starting any inoculation programme, it is necessary to determine whether the associated symbiotic strains of rhizobia or *Frankia* are present or not in the soil to be planted, and establish inoculation need and requisite management practices.

3.1.1. Simple methods of diagnosis

The occurrence of rhizobia or *Frankia* in the field can be simply observed by examining the legumes or actinorhizal plants whose inoculation group is similar to that of the species to be introduced at the site. The presence of nodules is at least *prima facie* evidence of the presence of the symbiont. In the case of rhizobia, it is possible to have an idea of the effectiveness of the symbiosis by splitting the nodules open and examining the colour of the section. Only effective nodules have a pink or red interior, whereas green or black nodules usually indicate ineffective nodulation. The physical appearance of the plant is a good indicator of an effective symbiosis for legumes (Meisner and Gross 1980) and actinorhizal plants. When host plants are green and healthy, the nodules are probably effective, a conclusion that could be erroneous when the nitrogen content of the soil is high enough to sustain the plant growth.

The presence, abundance and effectiveness of naturally occurring rhizobia or *Frankia* can also be assessed in the greenhouse, using different types of devices such a tubes or Leonard jars (Williams 1984).

3.1.2. Field experiments

A precise evaluation of a plant's response to inoculation should be based on the comparison of inoculated and uninoculated plants grown in the field. Date (1977) devised a simple experimental device with three treatments: (i) no inoculation, (ii) inoculation with the associated strain, and (iii) addition of nitrogen fertilizer (applied at the rate of 100–150 N/ha). Table 1.6 shows the possible responses of this type of trial. Since responses to inoculation are highly site-specific, it is advisable to test a maximum number of locations.

More refined trials have been suggested (Meisner and Gross 1980; FAO 1983; Halliday 1984 *a*, *b*), but they are complex and time-consuming. The most elaborate approaches involve the use of isotope techniques (LaRue and Patterson 1981). These last methods have the advantage of indicating the amount of nitrogen that has been effectively fixed by the inoculated plants. This information is most important, especially when the yield of inoculated and uninoculated plants are similar, but when part of the total nitrogen of the inoculated and hence nitrogen-fixing plant is derived from nitrogen fixation. In such a situation, the beneficial effect of inoculation can be detected only when using the isotope method.

3.2. *Strain selection and maintenance of cultures*

The selection of strains for inoculants should be based on two main criteria: (i) infectivity and effectiveness in nitrogen fixation, and (ii) tolerance to environmental constraints (e.g. acidity, soil toxicity, salinity, combined nitrogen, drought, pesticides). Other characteristics may also be desirable: (i) competitive ability, a property required when a superior strain is to be introduced in a soil where native, but less effective, strains are already present; (ii) ability to nodulate a large number of host plants (a trait which

Table 1.6

Possible outcomes of simple three-treatment inoculation trial for determining need to inoculate legume seed (from Date 1977)

	Uninoculated		Inoculated		Inoculated + nitrogen	
Outcome	Nodulation	Plant growth	Nodulation	Plant growth	Nodulation	Plant growth
1	—	poor	—	poor	—	poor
2	—	poor	—	poor	—	good
3	—	poor	+ E	good	+ or —	good
4	—	poor	+ I	poor	+ or —	good
5	+ I	poor	+ E	good	+ or —	good
6	+ I	poor	+ I	poor	+ or —	good
7	+ E	good	+ E	good	+ or —	good

— = Plants not nodulated.
+ = Plants nodulated.
+ or — = Plants may or may not be nodulated depending on whether the applied nitrogen inhibits nodule formation.
E = Effective in nitrogen fixation.
I = Ineffective in nitrogen fixation.

has the advantage of simplifying the distribution of inoculant); and (iii) ability to survive during distribution, storage, and use by farmers.

Multistrain inoculants have been successfully used despite findings suggesting that they should be avoided because of possible antagonistic and competitive effects (Williams 1984).

Methods to select superior strains of rhizobia in the laboratory and in the field have been described elsewhere (Date and Roughley 1977; Stowers and Elkan 1980; Halliday 1984*a*). These methods can be used to select *Frankia* strains. 'Even though the screening procedure is lengthy, attempts to shorten the sequence are ill-advised' (Halliday 1984).

Several methods of culture maintenance are successful: agar cultures in screw-cap tubes or bottles, dried soil, freeze-drying, and drying on porcelaine beads (Date and Roughley 1977; Stowers and Elkan 1980). In addition to freeze-drying the best method is to keep the liquid culture mixed with the same amount of glycerol in a freezer at −80°C. Preference should always be given to methods that minimize subculturing of cultures, thus reducing chances of variation by mutation.

3.3. *Types of inoculant and methods of application*

The quality of inoculants partly depends upon the suitability of the carrier, with best results usually obtained with peat. Many substitutes for this carrier have been proposed, e.g. bentonite, lignite, cellulose powder, various powdered crop residues. Peat has proved generally superior to other carriers. However, the composition of peat is highly variable and thus there are advantages in using a synthetic carrier of constant quality. This is achieved with inoculants obtained by entrapping rhizobia in a polyacrylamide gel or alginate instead of polyacrylamide. Field trials have proved that this last type of inoculant could be successfully used at the farm level (Ganry *et al.* 1985). When possible, peat is sterilized by heating, autoclaving or γ-radiation, and care should be taken to adjust both the moisture level and pH if high rhizobia populations are to be maintained during prolonged storage (Gibson *et al.* 1982). Further details on the preparation of inoculants are given in different reviews, such as those of Brockwell (1977) or Williams (1984).

Three procedures for inoculating are currently used: (i) mixing the inoculant with the seed immediately before sowing: (ii) suspending the inoculant in an adhesive, e.g. gum arabic or substituted methylethyl cellulose, moistening the seed with this and then pelleting the seeds by rolling them in finely ground coating materials at near neutral pH values, such as lime or rock phosphate; and (iii) inoculating the soil itself with the inoculant, by placing it below or alongside the seed bed.

When introducing rhizobia into soil by either the seed or soil inoculation technique, consideration should be given to the factors relevant to the establishment of an organism in an alien environment.

Detrimental factors, in many cases can be overcome through direct control measures, i.e. liming or application of fertilizers, use of inoculants with a high content of rhizobia or *Frankia* cells, protection of the micro-organisms used, and by introduction of the inoculant well before the legumes are planted.

The beneficial effect of high inoculum rates to overcome stress factors has been observed by many authors (Freire 1977; Selbach *et al.* 1978), mainly at the first planting of a legume in an area or as a way of introducing a new rhizobia strain into soil with a native population. A high number of cells around the germinating seed signifies a high number of nodules at the primary roots and inoculation success considering that the native rhizobia are dispersed in the soil. However, success in introducing a strain is dependent on the rate between the number of cells in the inoculum, in the native population, and in the competitive ability of the introduced strain in relation to the native ones. An important point is that nearly all of the experimental work for the introduction of rhizobia strains into a populated soil has been performed for one planting or for a short period of time. Long-term experiments with repeated introduction of the desired rhizobia strain need to be carried out.

As indicated earlier, protection of the symbiotic micro-organisms can be achieved through pelleting of the seed with calcium carbonate or rock phosphate, which have high beneficial effects on nodulation and yield. This technique developed in Australia and disseminated throughout the world has indeed proved to be of high value for the introduction and establishment of forage legumes in pastures in acid soils with no Al and Mn toxicities. The process also serves as an alternative to liming.

Introduction of rhizobia prior to the legume has been applied with success in the 'cerrado' or savannah soils of central Brazil as a means of overcoming the problem of deficient nodulation in the first planting of soybeans (Vargas *et al.* 1982). These areas after opening are usually seeded with upland (not irrigated) rice for 2–3 years. Now farmers are inoculating the rice seed, and when soybean is planted, already, find an established rhizobial population. This technique is much more economical than using large amounts of inoculant. Five or more repetitions are recommended for the first planting when not preceded by the inoculated rice.

Since *Frankia* is probably not motile in the soil, like vesicular-arbuscular mycorrhizae, we suggest to mix thoroughly the *Frankia* inoculant with the soil of the container where the plant is grown.

In the case of stem-nodulated legumes, inoculation is easily achieved by spraying the stem with a liquid culture of specific rhizobia, when the plants are 20–30 days old; inoculating the roots is achieved using any of the procedures described just above.

4. Limitations

Assuming that the plant selected is capable of a high level of production in a particular environment and that the inoculant strain is capable of forming an effective symbiosis with the host, it is then necessary to ensure that all other major limitations are minimized by the adoption of appropriate management practices. Here we shall briefly present some of the major limiting factors that can be encountered in the tropics.

4.1. Soil pH

Most legumes show reduced nodulation with a pH less than 5.0, not only because of the high hydrogen-ion concentration, but also because of the resulting toxicity of aluminium and manganese. Aluminium ions damage the roots of the host plant, which restricts nodulation. Liming is often proposed to alleviate the direct and indirect effects of acidity, but caution must be exercised in the use of this amendment. Ill-managed liming can induce deficiencies in magnesium, copper, zinc, and boron. Mulching has been recommended to stimulate nodulation as it controls manganese toxicity (Granhall 1986).

Some tropical legumes are most tolerant to acidity, nodulating well at pH as low as 4.5–4.7, e.g. *Arachis hypogaea*, *Macroptilium lathyroides*, *Desmodium uncinatum*, *Vigna sinensis*, and *Stylosanthes guyanensis*. *Stylosanthes capitata*, a legume with high tolerance to soil acidity, can nodulate in Leonard jars only when the growth medium is acidified to a pH lower than 5.0, and the calcium and phosphorus levels are lowered 10-fold (Halliday 1984*a*). In contrast with what was claimed earlier, the optimum pH of *Glycine max* is not in the 6.5–7.0 range. Actually, soil acidity *per se* is not the limiting factor for this legume, but if acidity induces aluminium and/or manganese toxicity, nodulation together with nitrogen fixation and yield may be seriously affected (Freire 1984). The effect of Al seems to be indirect on the root growth, on nodulation initiation and on the physiology of the host in the uptake and transport of calcium. A close interrelation exists between Ca, Al, and Mn. Tolerance to Al is related to the uptake and utilization of phosphorus. There is also some indication that uptake of Mn is higher in a soil with higher exchangeable Al (Cabeda *et al.* Cabeda and Freire 1986) than at soil lower in Al.

Legumes show large inter- and intraspecific differences in tolerance to soil acidity. Similarly, rhizobia strains also demonstrate wide variation in acid tolerance. This genetic diversity of the host plants and rhizobia should be exploited for better plant growth and nitrogen fixation (Graham and Chatel 1983).

Plant growth, nodulation, and nitrogen fixation are impaired at high pH by induced deficiencies in manganese, iron, boron, soil salinity, and anionic imbalances (Graham and Chatel 1983). In alkaline or sodic soils, recommended management practices are: addition of gypsum or organic matter. As with soil acidity, an appropriate strategy could be the selection of both host plants and associated *Rhizobium* but in this situation, the goal is to obtain plants and bacteria that are able to tolerate alkaline conditions.

4.2. Mineral influences

After nitrogen, phosphorus is most generally the limiting nutrient in tropical soils. Among tropical legumes, some species are more efficient in using phosphorus than the others, e.g. *Acacia holosericea* (Cornet *et al.* 1985), *Stylosanthes* and *Lupinus* (Gibson *et al.* 1982). Differences also occur between cultivars.

Both calcium and magnesium are essential for the host plant and the symbiotic bacterium. A change in the Ca/Mg ratio can reduce the accessibility of either nutrient. An imbalance is generally induced by high applications of lime or potassium fertilizers (Bergersen 1977; Munns 1977; Andrew 1978).

The effect of potassium on N_2 fixation is indirect, through the host physiology (Andrew 1977). In soils, low in productivity in the tropics, it is common to find high levels of available K and no response to fertilization. This may mislead the farmer for not using the fertilizer. When, however, productivity is increased, extraction of K is also increased and deficiency may appear. Potassium may also be detrimental to nodulation and N_2 fixation. In non-limed soils high in available Mn, the application of K as KCl increases the availability of Mn in the soil and in plant tissue and reduces nodulation (Borkert, *et al.* 1974).

Sulphur and some micronutrients are common limiting factors to N_2 fixation and legume productivity in the poor acid soils of the tropics and subtropics e.g. at the 'cerrado' or savannah soils of South America (Franco 1977). In high pH or limed soils zinc, and boron deficiencies may be a problem specially in high levels of productivity. For alfalfa, boron is a common limiting factor. Molybdenum as part of the nitrogenase molecule plays an essential function in the N_2 fixation. High expectations were witnessed some years ago on the 'miracle' effects of molybdenum. Deficiency in Mo may occur in some acid soils and disappear when soil is limed in most of the cases. In soils with toxic Al and/or Mn, the isolated application of Mo has no beneficial effect.

4.3. Combined nitrogen

The effect of application of nitrogen fertilizers on nodulation and nitrogen fixation is complex, and vary with the form and rate of application, the plant species or cultivar, the strain of rhizobia, the environmental conditions, and the amount of available nitrogen already present in the soil.

Mineral nitrogen in small amounts is beneficial to starting or nodulation and N_2 fixation specially in severely deficient soils (Franco 1977; Freire 1984). This is clearly shown in Leonard jars with sand and nutrient solution. However, in field conditions severe deficiencies very seldom occur.

It has been the classical recommendation of use as 'starter' nitrogen for the growth of legumes. However, field evidence of beneficial effects are not common at least for grain legumes. In the absolute majority of the experimental work conducted with soybeans in Brazil there was initially no economical responses to the use of mineral nitrogen, either at low or high application rates at different times of the growth cycle given that the plants were well nodulated (Barni *et al.* 1978; Freire 1984). In some experiments or crop fields the observer may be confused by taller and greener plants where N is applied at planting time. However, well nodulated plants without N will rapidly compensate and at harvest time the yield will be the same. It must, however, be mentioned that positive responses may be obtained in fields where nodulation and/or N_2 fixation has been inhibited, e.g. inefficient rhizobia strains, temperature, or moisture stress, etc. From a practical point of view it would be an uneconomical general recommendation for farmers to apply N fertilizers for soybeans. For some time in Brazil there was a general practice in the use of a 'starter' of 10–20 kg N for soybeans. Campo and Sfredo (1981) estimated that there was a wasting of 87 000 tons of N at the fertilizers formulas commonly used in the country. For other legumes similar results have been obtained (Kolling *et al.* 1985). For the short cycle with field beans (*P. vulgaris*) it seems that the N_2 fixation cannot supply the needs of the plants and that supplementary N would be beneficial.

In sandy soils, when moisture is adequate nitrification may yield high levels of nitrates that in some cases could even inhibit nodule formation.

It is generally recognized that large additions of nitrogen fertilizer almost always inhibit nodulation and nitrogen fixation. A promising approach to overcome this inhibition is to select lines of plants for good nodulation in the presence of nitrate nitrogen (Gresshoff *et al.* 1985), and then to inoculate such plants with rhizobia strains effective in nitrogen fixation in the presence of combined nitrogen. One should note here that stem-nodulated legumes possess the ability to simultaneously use combined nitrogen from the soil and atmospheric nitrogen (section 2.3.). Other interesting approaches include deep-banding or the use of slow-release fertilizers. Caution should be adopted in the use of nitrification inhibitors (Gibson *et al.* 1982).

4.4. Pathogens

Viral, fungal, and bacterial pathogens, together with insects and nematodes, may interfere with the plant growth and its ability to fix nitrogen. The deleterious effect of nematodes on nodulation and nitrogen fixation by legumes has been reported by many authors (Gibson 1977; Gibson *et al.* 1982). Controlling nematode attacks by a nematicide was shown to significantly improve nitrogen fixation of peanut (Germani *et al.* 1980).

4.5. Importance of management practices

Availability of inoculants and research capability are not sufficient for the diffusion of the practice of inoculation and for obtaining higher productivity from N_2 fixation. The use of rhizobia is just one of the items in a package of practices for legume growth and production. Thus, it must be promoted together with a long series of other inputs and agronomic factors. In many places it is of little use to promote the isolated application of rhizobia inoculant if, for example, lime and/or phosphorus are not applied on account of a lack of knowledge or because prices are too high. Here comes the role of the extension services, showing to the farmers that they can obtain more from their land. After the farmers are convinced, they will make the necessary efforts for the availability of the necessary inputs, credit, etc. This is what happened in southern Brazil 20 years ago. A research/extension integrated programme for the improvement of soil fertility changed completely the poverty situation of the farmers in millions of hectares of poor acidic soils high in exchangeable Al and Mn (Beaty *et al.* 1972). The expansion of soybeans had started some years behind and the effect of inoculation was usually poor. After the diffusion of new management practices for high applications of lime and fertilizers then the farmers started to see the beneficial effects of the rhizobia N_2 fixation. If it is assumed that N_2 fixation now provides an average of 50 kg/N/ha/year the contribution of the rhizobia-soybean symbiosis in Brazil is about 600 thousand tons of N for the 12 million hectares cropped to this legume in 1984.

5. Future

In the last decades a tremendous amount of work has been devoted to the study of biological nitrogen fixation. Exciting results have been achieved at the level of genetics, physiology, and ecology of symbiotic systems, but agriculture and forestry have only recently fully benefited from these remarkable advances in our basic knowledge. Fortunately, some approaches are progressively emerging that will probably give way to practical applications in the near- or mid-term (e.g. host selection via micropropagation techniques). Other research strategies will involve much more effort and time before fundamental results be transferred to the field.

5.1. Approaches for increasing the nitrogen-fixing potential of existing systems

Increased nitrogen fixation can be achieved by acting upon one, or better, both components of the symbiosis. Rhizobia and *Frankia* strains can now be genetically engineered by molecular techniques. Thus, one can foresee, in the short term, that the remarkably high nitrogenase activity of *Rhizobium* strain ORS 571 (from *Sesbania rostrata*) will be transferred to strains of *Rhizobium* nodulating other legumes, probably endowing the latter plants with an increased nitrogen-fixing potential.

The improvement of the host plant should be carried out in parallel to any genetic improvement of rhizobia or *Frankia* strains. Adapted technologies are now at hand for exploiting the variability of the host plant. Exploiting the spontaneous genetic heterogeneity of trees is an elegant means to improve nitrogen fixation by these plants, provided that vegetative propagation techniques are available that would allow mass production of selected clones. The variability of the host plant can be broadened via tissue culture, which offers new exciting possibilities (Lalonde and Simon 1985).

5.2. Improving tolerance to environmental constraints

Very actively nitrogen fixing systems would be useless if they were not tolerant to environmental constraints that limit their activity (section 4). Consequently, it is mandatory to select or engineer nitrogen-fixing systems whose activity is not, or only slightly, reduced by limiting factors. On the one hand, one can use the techniques of genetic manipulation of micro-organisms, which have improved so dramatically in the past few years. On the other hand, plant cell and tissue culture techniques are advanced enough to be successfully used to improve the host performances in hostile environments. The following examples will illustrate the benefit that could result from the manipulation of the host. One major limiting factor is soil salinity, which generally affects the host plant more than the symbiont. Salt-tolerant variant plants can now be produced using somaclonal variation. This approach is based on the fact that by culturing a callus on a salt-enriched medium, some cells of the callus may exhibit salt tolerance. Through continuous subculture on salt-enriched medium, the callus appear to 'gain' a substantial degree of tolerance (Nabors *et al.* 1980). The next step is to regenerate the plant from cultured calluses, a goal that is sometimes difficult to achieve. Nitrate and, to a lesser extent, other forms of combined nitrogen, retard nodulation and nitrogen fixation. Such an inhibition is obviously most detrimental for the economy of nitrogen especially in intensive agriculture. To circumvent this inhibition the best approach is to use nitrogen-fixing systems that are capable of continuing to fix nitrogen even in the presence of large amounts of combined nitrogen. Nitrate tolerant soybeans have been isolated from a soybean cultivar by Gresshoff's group in Australia (Delves *et al.* 1985). As indicated earlier, *Sesbania rostrata* , a stem-nodulated legume, has the unique ability to absorb combined nitrogen with roots and simultaneously fix atmospheric nitrogen with its stem-nodules, even when the amount of soil mineral nitrogen is as high as 200 kg nitrogen/ha. It has been suggested that transferring the stem nodulation characteristic from this plant to non-stem nodulated legumes would be a way to develop new, uninhibited nitrogen-fixing systems (Dreyfus and Dommergues 1980).

5.3. Transfer of nitrogen-fixing ability to non-nitrogen-fixing plants

The following strategies have been envisaged or are already at hand.

5.3.1. Genetic transfer of nitrogen fixation ability to the plant cells

The transfer of cloned genes between nitrogen-fixing micro-organisms and plants should be considered as a long-term project since the stability of the genes required for nitrogen fixation following their incorporation, expression and inheritance by the whole plant after their introduction into the plant cells is not yet known. However, if one take into account the development of our knowledge in plant molecular biology (Evans *et al.* 1985), several possibilities are offered by this strategy.

5.3.2. Hybridization of nitrogen-fixing and non-nitrogen-fixing plants

Gene transfer by wide hybridization has not yet been exploited to obtain new nitrogen-fixing systems. Fortunately, sophisticated technologies such somatic hybridization by protoplast fusion or embryo rescue (National Research Council 1982) are now available, which could hopefully be most helpful in this unexplored field of research.

5.3.3. Micro-grafting

Recently, Kyle and Righetti (1985) showed that the possibility exists of introducing nitrogen-fixing capability of actinorhizal Rosaceae into non-nitrogen-fixing plants of the same family by grafting the former plants onto nitrogen-fixing root stocks. Adopting a similar approach Lalonde's group, at Laval University, is exploring the possibility of grafting *Betulus* sp., a non-nitrogen-fixing tree, on *Alnus* sp., an actively nitrogen-fixing actinorhizal plant.

5.3.4. Indirect transfer of nitrogen-fixing capability through mycorrhizal fungi

The transfer of the whole set of genes required to fix nitrogen from a bacterium to a mycorrhizal fungus is probably feasible and easier than the transfer to a plant. However, a number of difficulties lies ahead, one prerequisite to genetical engineering of nitrogen-fixing endomycorrhizal fungi being the availability of a reliable method to grow this fungus *in vitro*.

5.4. Conclusion

To conclude we would like to stress two points. The first one, which has been already expressed by J. Postgate at the Corvallis meeting (Evans *et al.* 1985), is that 'for a strategic research topic such as the fixation of nitrogen, an open interdisciplinary approach is mandatory', uniting the efforts of chemists, biochemists, geneticists, physiologists, microbiologists, ecologists, and plant molecular biologists. The second message is that the more logical current and future application of biological nitrogen fixation is in land reclamation, forestry, and agroforestry. In such situations, biological nitrogen fixation by itself can meet totally or, at least, largely the nitrogen requirements of the plants. By contrast, in intensive agriculture, maintenance of crop yields cannot be obtained solely through nitrogen fixation. Then it is tempting to use exclusively nitrogen fertilizers. In fact, it would be much wiser to develop management practices based on the integrated use of industrial and biological nitrogen.

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