

## 2. Nitrogen fixation by legumes in the tropics

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### Introduction

Many tropical soils are limited in their ability to produce crops due to severe deficiency in soil nitrogen. Agronomists readily agree that the legumes, with their ability to reduce atmospheric nitrogen to a usable form of nitrogen, should be an important component of tropical agrosystems. Although some nodulated legumes have an outstanding potential for fixing  $N_2$  (*Sesbania cannabina* and *Leucaena leucocephala* can fix up to  $500 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ , Table 1), many of them fail to achieve their potential in the field. Thus when biological or environmental conditions are not favorable, the  $N_2$ -fixing activity of *Leucaena leucocephala* may be as low as 13% of that found in a more favorable environment (Table 1). Other examples given in Table 1 confirm that the range of  $N_2$ -fixing rates of legumes is

Table 1. Examples of  $N_2$  fixation rates ( $\text{kg ha}^{-1} \text{ crop}^{-1}$ ) by some tropical legumes grown for grains, forage or wood

Plant	Location	$N_2$ fixation rate	Method of estimation	References
<i>Glycine max</i> (soybean)	Senegal	165	Difference	[60]
	Senegal	200	Fertilizer equivalent	Mugnier (unpub.)
	India	102	A value	[56]
	n.i.	54–369	n.i.	[70]
<i>Arachis hypogaea</i> (groundnut)	Senegal	25–56	A value	Table 8
	n.i.	84–297	n.i.	[70]
	Israel	87–220	Acetylene technique	[137]
<i>Vigna unguiculata</i> (cowpea)	n.i.	24–240	n.i.	[70]
<i>Centrosema pubescens</i> (centro)	Africa	126–395		[115, 128]
<i>Sesbania cannabina</i>		$542 \text{ yr}^{-1}$		[128]
<i>Leucaena leucocephala</i> (= glauca)		$74\text{--}584 \text{ yr}^{-1}$		[4, 128]
<i>Macroptilium atropurpureum</i>		291		[128]

n.i. = not indicated.

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very large. Such variations are attributable to the impact of limiting factors that occur in the field in such a way that  $N_2$  fixation is often seriously depressed.

The main objectives of this chapter are: (1) to identify the parameters that determine the  $N_2$ -fixing potential of the legumes; (2) to indicate the key limiting factors that operate in the tropics; (3) to suggest methods to reduce or eliminate the effect of these limiting factors.

## 1. Potential Symbiotic Nitrogen Fixation

The potential symbiotic  $N_2$  fixation of a given legume is defined as the maximum activity of that legume when nodulated with the most effective *Rhizobium* strain and grown under the most favorable environmental conditions.

Total  $N_2$  fixation is dependent upon two parameters, nodule weight and the specific  $N_2$ -fixing activity ( $N_2$  fixed per gram nodule). While nodule weight shows a general increase during the growing season, there can be major fluctuations (Fig. 1). Furthermore, specific  $N_2$ -fixing activity, as estimated with the acetylene reduction technique, shows wide fluctuations (Fig. 2). These may be due to short-term changes in temperature, light intensity, moisture, or longer term effects involving these variables or stage of physiological development of the plants. It should be recognized that the acetylene reduction assay measures activity only during the course of the assay, and many observations are required to estimate overall rates of  $N_2$  fixation [154]; in addition, it is necessary to know the appropriate  $C_2H_2:N_2$  ratio to apply to convert the assay results to  $N_2$  fixed.

One important feature of the nodulation pattern is the period taken for nodules to appear and commence  $N_2$  fixation. This lag depends on a number of factors. One is the *Rhizobium* strain involved (e.g. Law and Strijdom [101]); those strains which nodulate first are active competitors against strains which nodulate later. The size of the population of rhizobia, the availability of soil moisture, the soil temperature, and the level of soil nitrogen are other important determinants affecting the speed of nodulation. The nodule pattern usually exhibits a maximum which occurs during the second half of the plant's growth. This maximum is followed by a decrease, often roughly sinusoidal, which indicates successive nodule turnovers. Such processes are most difficult to observe and consequently poorly documented.

The pattern of  $N_2$  fixation by a legume nodule exhibits three phases – lag (during nodule initiation and early development), exponential (rapid nodule growth and development of nitrogenase activity), and senescence (breakdown of nodule tissue and decline in nitrogenase activity, which may be rapid or extend over a considerable period). With a growing plant, nodule initiation and development is occurring continually, but the overall pattern of  $N_2$  fixation by the population of nodules on a plant resembles that of individual nodules, but over a longer time span. Droughting, and excess moisture, are two environmental factors known to promote nodule senescence. But of greater significance is the stage of physiological development. For example, numerous people have attributed the decline in nodule

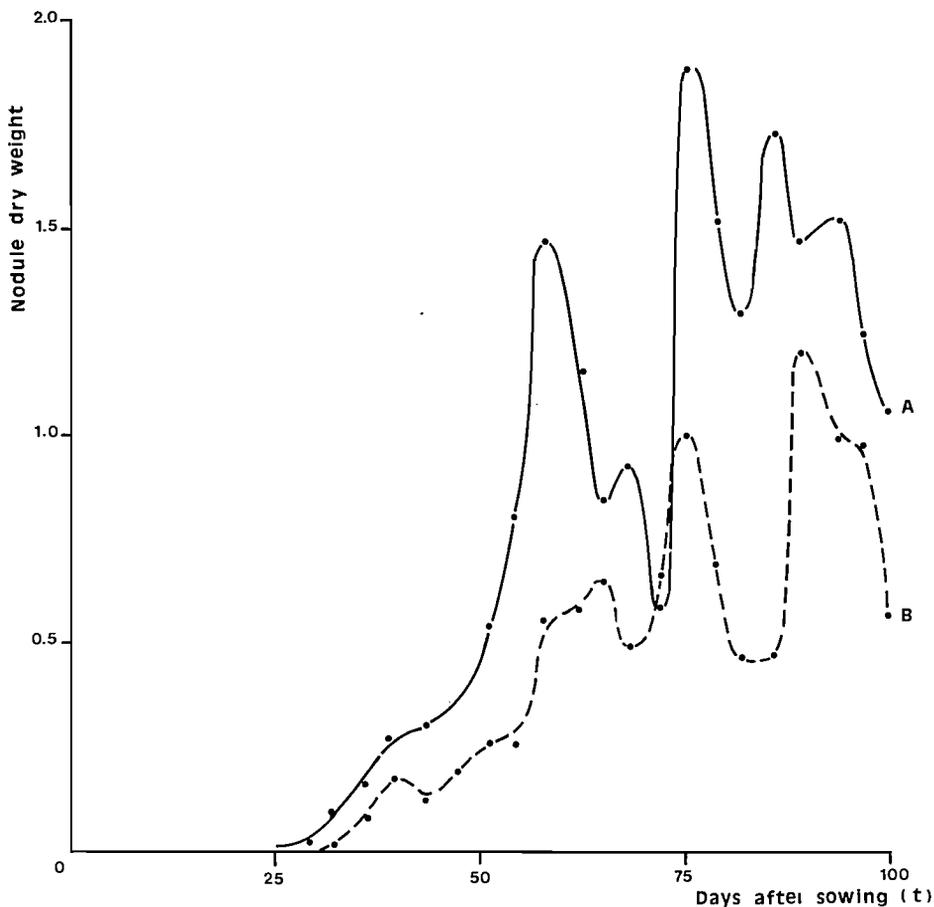


Fig. 1. Nodule weight of *Glycine max* cv. Jupiter grown at the ORSTOM station, Senegal during summer, the most favorable period for soybean growth. Plot A: soybean irrigated during the whole cycle; Plot B: soybean grown in an adjacent plot (same soil) but infested by nematodes; irrigation was interrupted between day 56 and 68. Each point is the mean of 10 replicates (unpublished data).

activity to the diversion of photosynthate supplies from the nodules to developing pods. In an attempt to define this more precisely, Lawn and Brun [102] suggested that the decline occurs when the growth rate of the pods equals that of the total plant shoot. Unfortunately, few other attempts have been made to examine this hypothesis, which could provide a basis for selecting the best species or varieties for an area. The strain of rhizobia has also been implicated as a factor determining the duration of  $N_2$  fixation [30]. One of the major gaps in our knowledge of the symbiosis between legumes and rhizobia is that relating to host and bacterial factors responsible for normal nodule senescence, i.e. not induced by moisture excess or deficiency, high temperature etc.

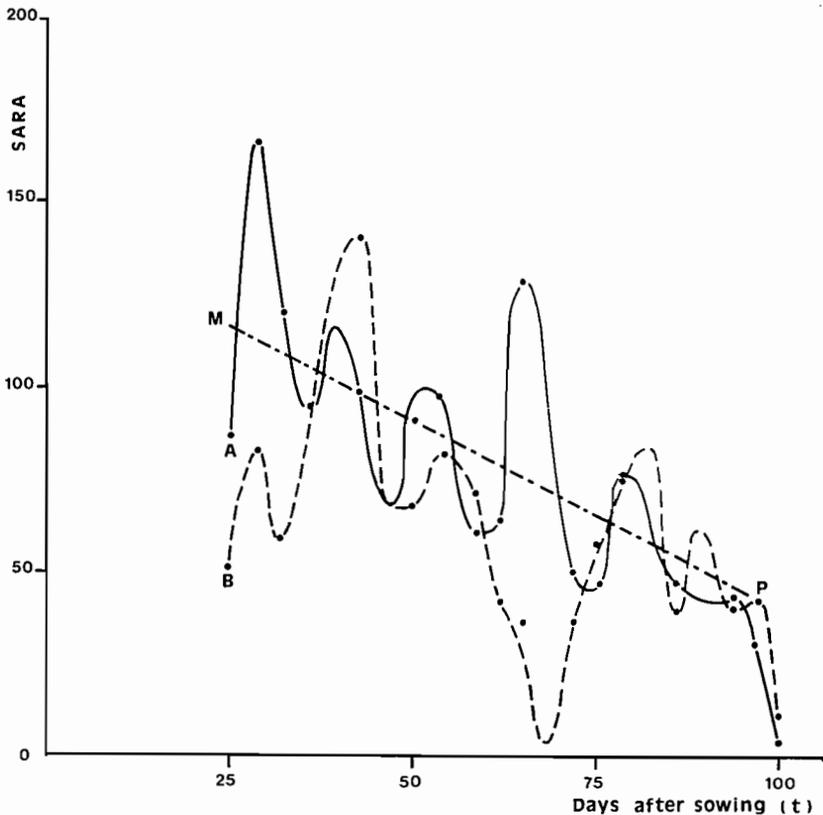


Fig. 2. Pattern of specific  $N_2$ -fixing activity (measured as  $\mu\text{moles } C_2H_4$  per g nodule (d.w.) per h, or SARA) of the soybean plants from plots A and B whose nodule pattern is represented in Fig. 1. The SARA of soybean from plot B exhibited a dramatic decrease when irrigation was interrupted (unpublished data).

The maximum specific  $N_2$ -fixing activity is an interesting feature of legumes. As direct methods of measurement are cumbersome, most determinations utilize the acetylene reduction assay. In general, meristematic-type nodules (e.g. *Trifolium*, *Pisum* and *Phaseolus* species) show significantly higher specific nitrogenase activities than the spherical or determinate-type nodules found on many tropical legumes (Table 2). An interesting exception is *Arachis hypogaea* [149] (Table 2) which has high specific activity. *Vigna* species have also shown high specific activity as young plants ( $> 300 \mu\text{moles/g}$  nodule dry wt/hr) although the levels decline rapidly from two weeks after germination (Gibson, unpublished). An interesting observation in this program was that the rate of N increase in these plants growing in N-free media was constant, even though specific nitrogenase activity, determined by acetylene reduction assays, was declining. This indicates that caution should be exercised in extrapolating from acetylene reduction assays to the determination of  $N_2$  fixation.

Under field conditions, many biological and environmental factors affect the

Table 2. Parameters of N<sub>2</sub> (C<sub>2</sub>H<sub>2</sub>) fixation in field-grown legumes

Plant	Location	Maximum nodule wt. (g plant)		Maximum specific activity (SARA) μmole C <sub>2</sub> H <sub>4</sub> h <sup>-1</sup> g <sup>-1</sup>		References
		fresh	dry	fresh wt. basis	dry wt. basis	
<i>Glycine max</i> (soybean)						
cv. Jupiter	Senegal	—	1.2	—	200	[112]
cv. Clay	Minnesota	4.0	—	80	—	[102, 103]
cv. Chippewa	—id—	4.8	—	26	—	[102, 103]
<i>Arachis hypogaea</i> (groundnut)						
cv. 71-234	Israel	—	0.6–0.9	—	140–975	[137]
cv. 57-422	Senegal	—	0.27	—	400	[112]
cv. 28-206	—id—	—	0.15	—	400	[112]
cv. 55-437	—id—	—	0.09	—	400	[112]
cv. Kediri 71-1	India	—	—	—	600	[3]
Cowpea	Nigeria	—	0.3	60	—	[7]
<i>Sesbania rostrata</i>						
stem	Senegal	40.0	—	53	—	[44]
root		15.0	—	17	—	[44]
<i>Acacia bivenosa</i>	Senegal	—	—	—	40	[43]
<i>Phaseolus vulgaris</i>	Colombia	—	—	45	—	[73, 75]

N<sub>2</sub>-fixing potential of legumes, through an effect on the nodulation pattern or on the specific nitrogenase activity of the nodules. The objective of the following sections is to attempt to define the major factors limiting N<sub>2</sub> fixation by legumes grown in the tropics.

## 2. Limiting Factors

### 2.1. Physical Factors

Relatively little is known of the effect of high soil temperatures on the survival of rhizobia under field conditions. Heating under moist conditions promotes death more rapidly than under dry conditions, and a 'wet' inoculant is more susceptible to higher temperature than a 'dry' inoculant [15]. The protection provided to *R. trifolii* and *R. leguminosarum* by montmorillonite clay against desiccation and heating, is not found within the slow-growing *Rhizobium* sp. [24], although this latter group appear capable of growth at higher temperatures than the fast-growers [15]. Adaptation to high soil temperatures has been advocated [178]. High temperature-tolerant strains of *Rhizobium* sp. have been isolated recently [47]; these isolates are also able to form effective nodules under high temperature conditions. The observation that moderate temperatures (35 °C) can induce plasmid loss, with

consequent loss of nodulating ability [182] could have serious implications regarding the effectiveness and nodulating ability of strains of rhizobia under tropical conditions, although Eaglesham *et al.* [47] found no evidence for 'curing' in their study.

High temperatures reduce the longevity of commercial inoculants, and refrigeration is recommended during transportation and storage. The lack of adequate refrigeration in many tropical and sub-tropical countries can create severe problems in maintaining reasonable populations of rhizobia in inoculants.

Both nodulation and  $N_2$  fixation are adversely affected by higher soil temperatures [64]. In general, temperatures above  $30^\circ\text{C}$  reduce the level of nodulation, although species such as *Acacia mellifera* [78], *Stylosanthes gracilis* and *Pueraria javanica* [157] make good growth up to  $35^\circ\text{C}$ . As with the fast-growing *R. trifolii*, some *Rhizobium japonicum* strains, and those nodulating *Cicer arietinum*, are able to maintain high rates of  $N_2$  fixation at temperatures under which many strains show low nitrogenase activity [30].

Moisture stresses, due to either deficiency or excess, are detrimental to the legume-Rhizobium symbiosis. Rhizobia show a rapid decline in viability under drying conditions, and this is exacerbated by cycles of wetting and drying [132, 135]. Nodulation does not occur when the soil is dry [10, 39] due to the failure of infection [180]. Desiccation severely depresses  $N_2$  fixation by nodulated plants, due either to moisture loss from the nodules [158, 159] or the cessation of photosynthesis [85]. Prolonged desiccation leads to nodule loss (Fig. 1), with a consequent reduction in the level of  $N_2$  fixation while new nodules form. Although the soil in the vicinity of the nodules may be dry, provided adequate moisture is supplied from the region below the nodule zone, reasonable rates of  $N_2$  fixation will be maintained [86]. Soil moisture deficits are frequently associated with high soil temperatures, and the distinction between effects due to moisture stress and temperature is difficult in the field.

Waterlogging, especially when associated with poor soil structure, retards both nodulation and  $N_2$  fixation even though some degree of adaptation is possible [141]. These effects are thought to be associated with low oxygen levels [28, 113, 134]. Excess water has pronounced effects on the growth of *Vigna unguiculata*, the effects being greater when such stress occurs early in plant growth [84, 114]. Legarda and Forsythe [104] found that production by *Phaseolus vulgaris* was retarded where the oxygen diffusion rate was less than  $24\text{--}28\text{ g} \times 10^{-8}\text{ cm}^{-2}\text{ min}^{-1}$ , a value higher than that considered by Loveday [107] and Gradwell [72] to retard the nodulation of Trifolium species. Some years ago, Masefield [110] made the interesting observation that the nodulation of a number of tropical legumes was vastly improved where the water table was high. Recent work in Australia has shown that soybeans grown with a water table maintained at 15 cm were highly productive, raising the possibility of 'paddy soybeans' [87]. From a study of survival of rhizobia during the paddy phase of rice cultivation, Rerkasem and Tongkumdee [138] concluded that introduced *R. japonicum* were more sensitive to flooding than naturally-occurring strains nodulating *Leucaena leucocephala*.

Both photoperiod and light intensity have been reported to affect nodulation and  $N_2$ -fixing activity [64]. Such an effect should not be overlooked with leguminous crops established under the canopy of trees, such as rubber, since in comparable situations nodulation and  $N_2$  fixation are reduced under shaded conditions [161]. Similarly increasing plant density decreases  $N_2$  fixation [74, 160]. Thus  $N_2$  fixation of *Phaseolus vulgaris* cultivar P590 reached a maximum when the density was 8.5 plants per  $m^2$  and rapidly decreased at higher densities (Fig. 3). One of the three cultivars studied was less sensitive to the harmful effect of increasing density. Expressing  $N_2$  fixation on an area basis,  $N_2$  fixation was shown to increase with density for all three cultivars.

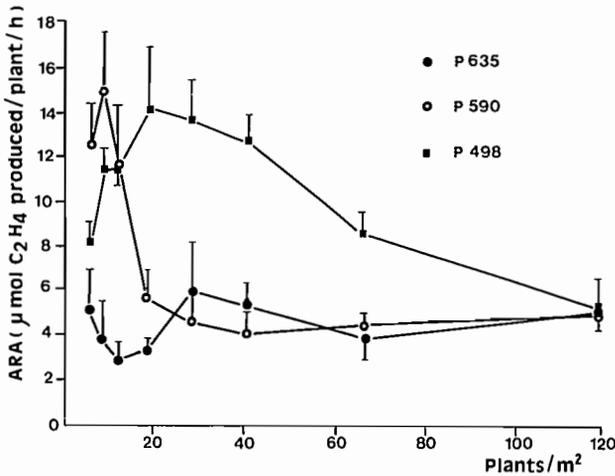


Fig. 3. Influence of planting density on  $N_2$  fixation expressed as  $\mu\text{moles } C_2H_4$  produced per plant per h in three cultivars of *Phaseolus vulgaris*, 39 days after planting [74].

## 2.2. Nutritional factors

Nutritional factors may affect  $N_2$  fixation by legumes by a direct effect on the infection and nodulation process, by influencing the effectiveness of the legume–Rhizobium symbiosis, or through an effect on the metabolism and growth of the plant independent of any effect on the symbiosis. Many plant nutrients have multiple functions, such that it is difficult to distinguish at which level they are acting. Among the nutritional factors which play a role in different stages of the symbiosis, soil acidity, phosphorus, molybdenum and combined N appear to be the most important in tropical conditions.

**2.2.1. Soil acidity.** Tropical legumes and their rhizobia are reputedly able to tolerate low pH better than the temperate legumes [125, 126]; however the useful-

ness of this generalization is now compromised by numerous exceptions [118, 119, 120, 121]. In West African soils, *Vigna unguiculata* and *Arachis hypogaea* are relatively tolerant of low pH (4–5) but as shown by Date [32], *Leucaena leucocephala* does not grow or nodulate in the low pH soils; it responds strongly to liming [88]. The complexity of the situation is indicated by a recent study with *Vigna radiata* [122] and *V. unguiculata* [93] in which strains of *Rhizobium* sp. demonstrated wide variation in acid tolerance, and their performance was influenced by the host cultivar. With soybeans growing in an acid soil in Nigeria, Bromfield and Ayanaba [19] observed that inoculation with strains of *Rhizobium japonicum* selected for symbiotic competence in acid soils resulted in yields up to  $2 \text{ t ha}^{-1}$ , and that liming had no effect on the response.

Acidity, calcium deficiency, and both manganese and aluminium toxicity tend to occur together, and determination of the precise cause of inadequate nodulation and  $\text{N}_2$  fixation is frequently difficult. Toxicity by Mn and Al generally affects plant growth and the effect on nodulation is indirect [48] whereas low pH and calcium deficiency effects are more directed towards symbiotic performance.

**2.2.2. Phosphorus.** In many tropical soils, particularly acid soils, available phosphate is an important limiting factor for both growth and nodulation [118, 119]. Nodules often contain 2–3 times more phosphorus per unit dry matter than the roots [117]. Phosphorus has a beneficial effect on the nodulation of *Stylosanthes humilis*, nodule number and dry weight being increased [57]. It is well known that phosphate fertilization improves nodulation and  $\text{N}_2$  fixation of legumes [2], but equally important is the maintenance of a well-balanced nutrient supply [58]. The effects of vesicular-arbuscular mycorrhizae on phosphate supply of the host plant are discussed in Chapter 8.

**2.2.3. Combined nitrogen.** The inhibition of nodulation and  $\text{N}_2$  fixation by combined nitrogen has been long recognized [54, 173] but the nature of the inhibition is not clearly understood. Three phases are affected — root hair infection, nodule growth and nitrogenase activity. Current evidence suggests that an effect of nitrate on lectins, or specific recognition glycoproteins, on the root hair surface may be responsible for the lowered level of infection [37, 38]. Reduced nodule growth and nitrogenase activity appear to be a consequence of a lower supply of photosynthate to the nodules, due to the diversion of the photosynthate to assimilate the combined nitrogen [62, 67, 155]. Recent evidence for a localized effect of combined nitrogen was obtained with *Sesbania rostrata*, which shows profuse stem nodulation; these nodules are unaffected by supplying the roots with 3 mM nitrate, but root nodulation is severely retarded [42].

Due to fluctuations in nitrate and ammonium levels in soil, it is difficult to assess the significance of combined nitrogen under natural conditions. Tropical soils are frequently low in nitrogen [92], but high levels of nitrate may exist in non-fertilized fields for short periods following the dry season [76]. This could delay nodulation and hence the onset of  $\text{N}_2$  fixation, as we have observed with *Arachis*

*hypogaea* and cowpeas in Senegal, and be an important factor in limiting  $N_2$  fixation. Against this, low levels of combined nitrogen can enhance nodulation and  $N_2$  fixation [62, 63] after an initial delay. Obviously further work is required to determine the precise effects of combined nitrogen under field conditions and to seek ways of reducing any inhibition of symbiotic activity. The suggestion that strains of rhizobia vary in their response to the effects of combined nitrogen [31] should be explored in more detail.

**2.2.4. Trace elements.** Of the various trace elements shown to be essential for successful nodulation (boron, copper, molybdenum, cobalt [118]), molybdenum is most likely to be deficient in acidic tropical soils. In a survey of 41 Brazilian soils, 36 were found to have Mo deficiency [52]. In Australia, the incorporation of Mo in the superphosphate has meant the difference between success and failure in the establishment of legumes in many acidic soils. Under certain conditions, liming will alleviate Mo deficiency by releasing Mo sorbed to clay particles at low pH.

### 2.3. Biological factors

Biological factors directly limiting  $N_2$  fixation by legumes may be broadly divided into two categories, namely those in the host plants and the bacteria affecting the specificity of nodule formation and symbiotic  $N_2$  fixation, and those affecting the survival and activity of the *Rhizobium* in the soil.

**2.3.1. Symbiotic compatibility between *Rhizobium* and host.** Specificity between host legumes and rhizobia occurs at three levels, namely the ability to form nodules, the ability to fix  $N_2$  and the degree to which symbiotic  $N_2$  fixation meets the plant's nitrogen requirements. Despite this, there are groups of plants that tend to nodulate with groups of strains of rhizobia (the cross-inoculation group concept of Fred *et al.* [54]). Although most soils throughout the world possess rhizobia, many do not contain rhizobia for particular hosts. For example, the vast area of Cerrado soils in Brazil generally lacks strains of rhizobia capable of nodulating soybeans, while some soils in Australia contain strains that nodulate soybeans but only ineffectively. Similarly the very strain-specific legumes *Leucaena leucocephala*, *Lotononis bainesii*, *Trifolium semipilosum* and *T. ambiguum* required inoculation when introduced into Australia [32]. It is interesting to note, however, that in West Africa, Asian cultivars of soybeans are effectively nodulated by some native strains of *Rhizobium* belonging to the cowpea miscellany [20, 89].

Many tropical legumes belong to the cowpea cross-inoculation group, and nodulation failure is rare. Due to their ability to form nodules with a wide range of *Rhizobium* sp. strains, the cowpea group is often considered promiscuous. However this generalization can be dangerous as strong specificity often exists with regard to the symbiotic effectiveness of the associations formed [34]. Figure 4 provides examples of all three forms of specificity referred to above, such examples

**HOST SPECIES x BACTERIAL STRAIN SPECIFICITY  
IN NITROGEN FIXATION**

SPECIES	STRAINS									
	32H1									
<i>Macroptilium atropurpureum</i>										
<i>Stylosanthes humilis</i>							X	X	X	
<i>Vigna unguiculata</i>										
<i>Vigna radiata</i>					X		X	X		
<i>Vigna mungo</i>										
<i>Lab-lab purpureus</i>										

RELATIVE TO STRAIN 32H1: >110    81-110    51-80    21-50    1-20

Non-nodulating    Not tested

Fig. 4. Host species x bacterial strain specificity in  $N_2$  fixation [65].

occurring among 10 strains chosen for their symbiotic effectiveness with one or more hosts and six common legumes [65].

Hence it is important to exercise great care when introducing a species to a new area. A previous record of ready nodulation is no guarantee that the species will nodulate in the new environment. More importantly, appropriate nitrogen control treatments should be included with the introduced material in order to ascertain the extent to which the local population of rhizobia, and the inoculant bacteria, are able to develop symbiotic associations that will meet the plant's nitrogen requirements [66].

**2.3.2. Ecology of *Rhizobium* in soil.** Studies under the broad heading of the ecology of rhizobia are generally involved with two aspects, survival, or persistence, of both native and introduced populations, and competition between strains in forming nodules. Most of the biological limitations on the persistence of *Rhizobium* in the soil, including predation, parasitism and lysis, have been previously reviewed [133].

We shall here deal mainly with the survival of both native and newly introduced strains.

Surprisingly, relatively little information is available regarding changes in the populations of rhizobia in tropical soils, or in changes of individual strains in these populations. Recent work in Senegal has shown important fluctuations in the number of rhizobia nodulating *Acacia senegal*, a tree growing in sandy soils in the semi-arid region of the Sahel. During the dry season, when surface temperatures exceed 50 °C, very few rhizobia are found, but the numbers increase rapidly once the rainy season commences (Dreyfus, unpublished). While this may favor the introduction of improved strains, unless they are selected for their ability to withstand the extreme conditions, the benefits of inoculation are unlikely to extend beyond the year in which they are introduced. A major problem in studying individual strains is their identification, although the development of ELISA (enzyme-linked immunosorbent assay) and associated techniques [11, 97, 116, 179] and the use of antibiotic resistance markers in selected strains [23, 98] opens the way for the required detailed investigations without having to use host plants to 'sample' the soil population. Even so, such studies will be restricted to the measurement of changes in the population and further development of techniques will be required to study critical nutritional and biological factors influencing survival of strains, and of populations [133].

Possibly of greater significance is the competition between strains in forming nodules. The factors responsible for competition are not understood [146], although relative numbers of the component strains in a population are obviously important. While it appears that the more effective strains have a competitive advantage in forming nodules on some legumes [100, 108, 139], both the host plant and environmental factors can influence the relative competitiveness of strains on such species (e.g. Jones and Morley [91]). With tropical legumes, Franco and Vincent [51] concluded that competitiveness was a strain characteristic, and was not necessarily related to symbiotic effectiveness with the selected host.

Regarding inoculant strains, the extensive root systems of many tropical legumes pose the additional problem of achieving movement of the introduced strain along the lateral roots from the point of sowing. With soybeans, the one species studied in any detail, both host cultivar [25] and root temperature [175] have a marked effect on competition for nodule formation. Until the factors affecting competitiveness are more clearly understood, and methods devised to screen for appropriate attributes, it is likely that introduced strains will not persist for a significant period, at least as a component of the soil population that is forming the nodules, due to greater competitiveness of the naturally-occurring strains [32].

*2.3.3. Attack by nematodes and nodule-feeding insects.* Root-knot and cyst nematodes interfere with the nodulation of many legumes, including *Glycine max*, *Arachis hypogaea* and *Vigna unguiculata* [140, 164]. Soybeans are infected by both *Meloidogyne* species [8] and *Heterodera javanica* races [105]. Nematodes

tend to prefer infecting nodules to roots, but in some cases, nodules have been found on the nematode-induced galls.

Root-lesion nematodes — such as *Pratylenchus* — have been shown to dramatically impede nodulation in many soils of Western Africa. By controlling the population of *P. sefaensis* by soil fumigation with 1,2-dibromo-3-chloropropane, the nodule weight on soybean increased from 1.7 to 2.8 g plant<sup>-1</sup>, and the grain yield from 1.28 to 2.16 t ha<sup>-1</sup> [60]. Similar effects have been found for groundnuts [59]. Besides their harmful effect upon nodulation, nematodes can also affect the specific N<sub>2</sub>-fixing activity of the nodules (Table 3). Interestingly whereas the nematode infestation similarly affects nodulation of the three cultivars, its effect upon SARA significantly varies according to the cultivar.

Table 3. Specific nitrogenase activity (SARA) and nodule weight of three groundnut cultivars grown in nematode-infested soils in Senegal, expressed as percentage of the same characteristics measured on plants grown in a fumigated, nematode-free soil [112]

	cv. 55-437	cv. 28-206	cv. GH119-20
SARA	36%	90%	60%
Nodule weight	42%	32%	44%

The nodules on legumes are a favored place for *Sitona* sp. and *Rivellia* sp. to lay their eggs, with most of the damage arising from the feeding larvae. Diatloff [40] reported *Rivellia* sp. larvae in nodules on *Glycine wightii*, *Centrosema pubescens* and cowpeas, with up to 70% of the *G. wightii* nodules being infested. *Colaspis brunnea* is reported to attack soybean nodules [167] while at ICRISAT in India, chickpea nodules are reputedly attacked by insects [3].

### 3. Increasing N<sub>2</sub> Fixation by the Legumes

The foregoing observations and comments indicate that the N<sub>2</sub>-fixing potential of legumes is far from being exploited. In this section, various approaches to increase symbiotic N<sub>2</sub> fixation by legumes will be discussed. These approaches range from the survey of hitherto unexploited legumes and the breeding of new cultivars better adapted to specific environments, through the selection of the most effective, and competitive, strains of rhizobia for those plants in their particular environment, to the use of management practices designed to minimize the impact of nutritional and environmental limitations on production by nodulated legumes.

#### 3.1. Survey and utilization of unexploited N<sub>2</sub>-fixing symbiotic systems

Up to now, the beneficial Rhizobium–legume symbiosis has only been partially explored. Of the 13,000 known species of legumes, only 100 are commercially grown. Most of the new genera and species which remain unstudied are tropical

legumes. Already some species appear to have outstanding potential, such as *Leucaena leucocephala*, which not only shows a high N<sub>2</sub>-fixing activity, but also offers one of the widest assortment of uses of all tropical legumes [4]. This legume tree can be used as forage for cattle, firewood, timber, reforestation, wind breaks and organic fertilizer. The winged bean, *Psophocarpus tetragonolobus*, a climbing legume with an active N<sub>2</sub>-fixing capacity, has many uses [94]. Its leaves, flowers, pods, seeds and tubers have high nutritional values with high protein and vitamin contents. A recent publication by the National Academy of Sciences [5] lists numerous species with interesting nutritional characteristics and/or abilities to grow in harsh environments (Table 4). With few exceptions, little attempt has been made to domesticate these species. Little is known of their nodulation or N<sub>2</sub>-fixing capabilities, although several belong to the subfamily Caesalpinioideae, a subfamily remarkable for the proportion of component species on which nodules have not been found. Growing such plants in the tropics should be of great value to meet the need of new sources of nitrogen and protein provided their nitrogen requirements can be obtained through effective nodulation.

Table 4. Interesting legumes with potential as food crops in tropical regions [5]

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Root crops

*Pachyrhizus erosus* and *P. tuberosus* (yam bean)

*Sphenostylis stenocarpa* (African yam bean)

*Vigna vexillata*

Pulse crops

*Voandzeia subterranea* (Bambara groundnut)

*Canavalia ensiformis* (Jackbean), *C. gladiata* (Swordbean)

*Lablab purpureus* (Lablab bean)

*Tylosema esculentum* (or *Bauhinia esculenta*)<sup>a</sup> (Marama bean)

*Vigna aconitifolia* (moth bean), *V. umbellata* (Rice bean)

*Lupinus mutabilis* (Tarwi)

*Phaseolus acutifolius* (Temporary bean), *P. lunatus* (Lima bean)

Fruits

*Ceratonia siliqua* (Carob)

*Tamarindus indicus*<sup>a</sup> (Jamarina)

Forages

*Acacia* sp., *Prosopis* spp.,

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<sup>a</sup> Sub-family Caesalpinioideae

Permanently or temporarily waterlogged soils, regarded in some areas as being favorable to legumes, are common in some tropical areas, and tropical aquatic legumes provide an opportunity to increase protein production in these soils. Genera such as *Aeschynomene*, *Sesbania* and *Neptunia* are known to grow in waterlogged conditions, but only few attempts have been made to adapt them to agricultural usage. In Vietnam, planting *Sesbania aculeata*, *S. cannabina* and *S.*

*grandiflora* between flowering rice has produced 10 t ha<sup>-1</sup> green matter, equivalent to 50 kg ha<sup>-1</sup> fertilizer N (Dao The Tuan, personal communication). Similarly *Sesbania aculeata*, *Crotalaria juncea*, *C. usaramoensis* and *Aeschynomene americana* were found to be a suitable green manure incorporated to flooded rice soils within 4 to 6 weeks after sowing [168]. The incorporation of these last four species was equivalent to 20 to 40 kg N ha<sup>-1</sup> applied as ammonium sulfate. Aquatic legumes, used as green manure in flooded paddy fields could thus favorably compare with the N<sub>2</sub>-fixing fern, *Azolla*.

Within the aquatic legumes, stem-nodulated plants such as *Sesbania rostrata* [44, 45], and to a lesser degree *Aeschynomene indica* [181] and *Neptunia oleracea* [144], represent a further step in the adaptation of legumes to waterlogging. Due to its profuse stem nodulation, *Sesbania rostrata* has five to ten times more nodules than the best nodulated crops, which should confer on this species an outstanding potential for N<sub>2</sub> fixation in flooded soils (Fig. 5). Unlike most other legumes, *Sesbania rostrata* can grow on saline and alkaline waterlogged soils, and it is able to nodulate profusely in the presence of high levels of combined nitrogen in the soil. The occurrence of stem nodules has only been reported rarely, presumably due to the lack of interest by taxonomic botanists in N<sub>2</sub> fixation, and their possible belief that they were finding insect galls. Of interest, another stem-nodulated tree legume, *Aeschynomene elaphroxylon* (Dreyfus, unpublished) has been found growing in waterlogged soils in Senegal. Should the use of these species prove significant in raising the productivity of tropical agrosystems, the possibility of transferring the ability to produce dormant stem meristems (the site of infection by rhizobia, erroneously described as lenticels [44]) from such species to other legumes, or even non-legumes, should be explored.

### 3.2. Plant breeding

There is an increasing awareness of the importance of host genetics in the symbiosis between legumes and their rhizobia. Three levels of interest may be defined, although they are not mutually exclusive: (i) breeding to improve N<sub>2</sub> fixation; (ii) studies to describe genes responsible for the absence of nodules, or for ineffective nodulation; (iii) studies at the molecular biology level to understand and define genes and gene products responsible for various steps in nodule formation and development, and for the induction and maintenance of nitrogenase activity by the rhizobia.

These aspects have been reviewed recently [26, 65, 77, 129, 130, 169, 170], and will not be considered in detail in this section.

The most outstanding example of selection and breeding for improved N<sub>2</sub> fixation involves the heterogeneous *Trifolium pratense* [129]. Yield increases up to 40% over the parental population were achieved. In small part only, this was due to improved plant vigour independent of N<sub>2</sub> fixation. The selected material nodulated earlier, and with a greater volume of nodule tissue, but the efficiency expressed as C<sub>2</sub>H<sub>4</sub> produced per g nodule dry wt in C<sub>2</sub>H<sub>2</sub> reduction assays, was unaffected. Breeding for earlier, and improved, nodulation of *Trifolium ambiguum* led to the release of commercial material suitable for Australian conditions [82].



Fig. 5. (a) Three-month old stand of *Sesbania rostrata*; (b) with stem nodules; (c) stem-nodule section.

Selection and breeding for attributes other than  $N_2$  fixation can also enhance symbiotic  $N_2$  fixation. For example, *Phaseolus vulgaris* lines selected at CIAT, Colombia for later flowering and rapid pod development, fixed 2–3 times as much  $N_2$  as earlier flowering lines, presumably due to the competitive effects of pod development on  $N_2$  fixation (D.R. Laing, personal communication). This is an aspect of plant breeding that should be foremost in the minds of breeders and agronomists selecting unadapted material for use under tropical conditions.

Another aspect of selection and breeding is that many plant breeders depend on local populations of rhizobia, or even nitrogen fertilization, during their programmes. There is a distinct danger that the bred material, when tested in a different location, will produce poorly solely because the appropriate rhizobia are not present. This is demonstrated by the comparative failure of some *Glycine max* lines bred in certain US regions to yield as well when tested in a similar climatic region but with a different population of rhizobia in the soil. Hence it is vital that any breeding or selection programme involving legumes be undertaken in conjunction with trained legume bacteriology scientists.

Attributes that may be amenable for selection, with a view to improving symbiotic performance, include ability to nodulate and to fix  $N_2$  in the presence of moderate levels of combined nitrogen, early nodulation, continued  $N_2$  fixation during pod development, and the ability to maintain reasonable rates of  $N_2$  fixation during moderate drought and/or temperature stress [83]. Vesicular-arbuscular mycorrhizae infection is another characteristic worthy of study, with the possibility that genetic variation within host material could be exploited to improve symbiotic performance.

Much publicity has been given to the possibility of transferring the *nif* (nitrogen fixation) genes to the plant genome. Consideration of the problems involved, particularly in locating the genes such that nitrogenase can function [68, 131], suggests that this approach to increasing  $N_2$  fixation will be very difficult. More feasible could be the transfer of those genes in legumes, or the nodulating non-legumes, responsible for the establishment of symbiotic associations with *Rhizobium* or *Frankia* [36, 68, 169]. Such an approach will require a closer understanding of the physiological processes involved in the development and function of nodules than we presently possess. However, it is possible that many species already possess some of the necessary genetic information and the transfer of only three or four genes may be adequate to confer nodule-forming ability on such species.

### 3.3. Microbial approach

**3.3.1. Strain selection.** As indicated in 2.3.2, a successful inoculant strain of *Rhizobium* must be effective in  $N_2$  fixation with the selected hosts, competitive in nodule formation on these hosts, and in many situations, show indefinite persistence as a significant component of the *Rhizobium* population. Effectiveness studies, made under a range of environmental and nutritional conditions, and involving different species and cultivars, are most efficiently done under laboratory or greenhouse conditions [66]. Most of the strains now included in commercial inoculants around the world have been selected from laboratory or greenhouse trials, albeit under optimal conditions in most instances. Date [32] and Ayanaba [6] each record successful strain selections for inoculant use on forage legumes and soybeans, respectively, growing under tropical conditions. In other work, Silvestre [152] observed an increase in soybean yield from 240 to 1440 kg ha<sup>-1</sup> following

inoculation, while in Senegal, inoculation of *Vigna unguiculata* with CB756 significantly hastened and increased nodulation when the soil population of *Rhizobium* sp. was low.

The need to check inoculant strains against all species for which it is recommended is paramount. Although the cowpea cross-inoculation group is diverse, there are a number of species with specific requirements (Table 5). For example, 13 *Acacia* species were shown to fall into three groups according to their effectiveness response patterns with a large group of fast- and slow-growing strains [43]. Further-

Table 5. Promiscuous and specific cross-inoculation groups of tropical legume species

Nodulation group	Rhizobia growth rate Characteristics	Legume species
Promiscuous	Slow-growing strains	<i>Acacia albida</i>
		<i>Arachis hypogaea</i>
		<i>Cajanus cajan</i>
		<i>Clitoria</i> sp.
		<i>Crotalaria</i> sp.
		<i>Glycine wightii</i>
		<i>Indigofera</i> sp.
		<i>Lablab purpureus</i>
		<i>Macroptilium atropurpureum</i>
		<i>Phaseolus lunatus</i>
		<i>Psophocarpus tetragonolobus</i>
		<i>Pueraria</i> sp.
		<i>Stylosanthes guyanensis</i> (most cultivars)
Specific	Slow-growing strains	<i>Stylosanthes humilis</i>
		<i>Teramnus</i> sp.
		<i>Vigna radiata</i>
		<i>Vigna unguiculata</i>
		<i>Voandzeia subterranea</i>
		<i>Zornia</i> sp.
		Specific
<i>Centrosema</i> spp.		
<i>Coronilla</i> spp.		
<i>Desmodium intortum</i>		
<i>Lotononis</i> spp.		
<i>Lotus</i> sp.		
<i>Stylosanthes</i> spp.		
Specific	Fast-growing strains	<i>Acacia raddiana</i>
		<i>Acacia senegal</i>
		<i>Albizia julibrissin</i>
		<i>Andura</i> sp.
		<i>Leucaena leucocephala</i>
		<i>Psoralea</i> sp.
		<i>Samanea</i> sp.
		<i>Sesbania sesban</i>
<i>Sesbania rostrata</i>		

more, the strain selected for an inoculant should be the most effective strain available for that species (Fig. 4).

There are many situations in which there is no response to inoculation, particularly where legumes in the cowpea cross-inoculation groups are involved [6]. While this may be due to the presence of highly effective and competitive strains in the soil, it may be due to (i) the failure of the inoculant strains to form nodules, (ii) nutritional or environmental factors limiting symbiotic development, and (iii) nutritional or environmental factors limiting plant production *per se*. For all field studies, it is important that control treatments with adequate fertilizer nitrogen be included, in order to help define any major limitations and determine potential productivity of that crop. However, in order to make the assessment realistic, N fertilizer should not be applied until the inoculated treatments have nodulated [66]. It is also important to determine whether the inoculant strains have formed nodules, either by visual inspection or, more usually, by serological or other procedures used to identify particular strains [46, 148, 172].

The significance of competition between strains, and the lack of knowledge of the fundamental basis of strain competition, was discussed above (2.3.2). An example of host effects on strain competition, and its significance with regard to N<sub>2</sub> fixation and plant growth, is shown in Table 6. Whereas strain 61B9 forms a more effective symbiosis on both *Macroptilium atropurpureum* and *Vigna unguiculata* than does strain QA922, in a mixed inoculant, QA922 dominates the response on *V. unguiculata*, whereas 61B9 has the stronger effect with *M. atropurpureum*.

Table 6. Shoot weight of *Macroptilium atropurpureum* and *Vigna unguiculata* when inoculated with three strains of *Rhizobium* sp., either singly or in pairs, with equal numbers of each strain – 10 replicates (B.L. Dreyfus and A.H. Gibson, unpublished)

	<i>Macroptilium atropurpureum</i> (mg plant <sup>-1</sup> ) <sup>a</sup>			<i>Vigna unguiculata</i> (g plant <sup>-1</sup> ) <sup>b</sup>		
	CB756	61B9	QA922	CB756	61B9	QA922
CB756	98	110	87	3.0	3.3	2.9
61B9	–	107	108	–	2.7	0.6
QA922	–	–	82	–	–	0.4

<sup>a</sup>Uninoculated, 15 mg. LSD = 15.7, *P* = 0.05.

<sup>b</sup>Uninoculated, 0.4 g. LSD = 0.5, *P* = 0.05

The selection of strains of rhizobia able to persist in soils remains a major problem, especially for the harsh conditions frequently encountered in tropical soils. Date and Halliday [33] have shown that special procedures are required to isolate some acid-tolerant strains for *Stylosanthes* spp. while Eaglesham *et al.* [47] have shown that selection of high temperature-tolerant strains of *Rhizobium* sp. is possible. Numerous studies from Alexander's laboratory in Cornell University (e.g. Ramirez and Alexander [136]) have indicated that, in general, predation is not a

serious problem. Nor have studies on bacteriophage action consistently shown that this significantly affects field populations of rhizobia, although lysogenic phages may have a significant effect in mixed strain inoculants [9, 109, 147]. Of potential interest is the possibility of conferring on inoculant strains characteristics enabling them to survive in hostile environments. However, before the application of such bacterial breeding techniques, it will be necessary to identify the genetic basis of the attributes.

*3.3.2. Inoculation techniques.* The preparation of inoculants prior to inoculation involves different steps (strain selection, culture, preparation of carriers, the mixing of culture broth and carrier, maturation, and storage) which have been reviewed in detail recently [22, 35, 165]. In this section we shall focus our attention (1) on the form of carriers that can be used, and (2) on techniques of inoculation.

Legume inoculants have been prepared commercially since late in the 19th century. Their quality partly depends upon the suitability of the carrier, with the best results usually obtained with peat or soil high in organic matter [163]. Many substitutes for peat have been proposed, such as bentonite, lignite, cellulose powder, various powdered crops residues, etc., but peat has proved generally superior to these carriers. However the composition of peat is highly variable, and rigorous testing of new deposits is essential before the material is used commercially. Due to variation in peat quality, even from a single source [162], there is appeal in having a synthetic carrier of constant quality. Initial tests with entrapping rhizobia in a polyacrylamide gel have been promising [41] and current efforts are directed to adapting this approach for use at the farm level. Where possible, peats should be sterilized by  $\gamma$ -radiation or autoclaving [142] or partially sterilized by heating [22], and care should be taken to adjust both the moisture level and pH if high counts are to be maintained during prolonged storage. Frozen inoculants are prepared for soybeans in the USA, but difficulties in providing adequate storage conditions in many tropical countries precludes consideration of this type of commercial inoculant.

The classical procedure for inoculating seed is to prepare a slurry containing an adhesive, such as gum arabic [17]; coating the inoculated seed with finely divided  $\text{CaCO}_3$ , rock phosphate, or bentonite, confers a degree of protection from immediate desiccation. Other techniques involve the use of peat granules [13, 53], or other inoculant material (e.g. marble chips), sown with the seed. Alternatively 'drenching', or spraying suspended peat inoculant into the furrow below the seed, has achieved a degree of popularity for large-scale sowings, especially as there are considerable labour savings compared with preparing inoculated seed [18, 145]. This and the granule method have other advantages over conventional inoculation methods in that the bacteria are protected from potentially-damaging fungicide or insecticide seed dressings, the bacteria are not lifted above the soil during (epigeal) germination, and it is possible to achieve high rates of nodulation.

*3.3.3. Vesicular-arbuscular mycorrhizae.* Since legumes have a restricted root

system, they are 'relatively poor foragers for phosphate' [123] and since many tropical soils are phosphorus deficient, a positive response to root infection by vesicular-arbuscular mycorrhizae (VAM) can be expected. Thus, it is not surprising that VAM and phosphorus application have similar favorable effects not only on plant growth but also on nodulation and specific  $N_2$ -fixing activity in many tropical soils. Besides beneficial nutritional effects, VAM may be of importance in semi-arid conditions, such as providing increased resistance to drought, or in alleviating some forms of soil toxicity (see Chapter 8).

### 3.4. Soil management

Assuming that the crop 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 that host, it is then necessary to ensure that all other major limitations are minimized by the adoption of appropriate management practices (Table 7).

Table 7. Major factors limiting symbiotic  $N_2$  fixation in West Africa; proposed means of controlling their effect

Limiting factor	Control
1. Moisture stress	<ul style="list-style-type: none"> <li>– irrigation, mulching</li> <li>– search for drought resisting cultivars and <i>Rhizobium</i> strains</li> <li>– stimulating VAM infection</li> </ul>
2. Nematodes <sup>a</sup>	<ul style="list-style-type: none"> <li>– fumigation by nematicides</li> <li>– biological and integrated control</li> </ul>
3. Soil acidity and toxicity	<ul style="list-style-type: none"> <li>– liming</li> <li>– addition of organic matter (FYM; green manure; compost)</li> <li>– selections of acid-resistant strains</li> </ul>
4. Mineral deficiencies, especially phosphorus	<ul style="list-style-type: none"> <li>– addition of phosphorus</li> <li>– stimulating VA mycorrhizal infection</li> <li>– determination of other deficiencies and their rectification</li> </ul>
5. Inadequacy of native <i>Rhizobium</i> populations and competition between native and introduced strains	<ul style="list-style-type: none"> <li>– inoculation with strains selected for effectiveness, competitiveness and persistence</li> </ul>

<sup>a</sup> Some pests and diseases may become serious in some circumstances

**3.4.1. Mulching.** Surface mulches of straw, sugarcane 'bagasse', or even grass-clippings, are recommended to conserve soil moisture, reduce soil temperature,

decrease the nitrate concentration in the soil and to control competing weeds. Masfield [110, 111] reported a 3-fold increase in the nodulation of cowpeas following mulching. Significant increases in  $N_2$  fixation by soybeans were found when straw was incorporated into soil, with the effects being comparable to those achieved with  $CO_2$  fertilization of the canopy [151]. However, results obtained using 'bagasse' in Brazil were variable, with the yield and nodulation of *Glycine wightii* and *Teramnus uncinatus* being increased and that of *Medicago sativa* decreased; *Phaseolus vulgaris* showed improved nodulation but severely depressed grain yield [127]. It is obvious that mulching can be beneficial but careful experimentation is required to determine the most effective forms and rates of incorporation.

**3.4.2. Irrigation and drainage.** As previously discussed (2.1), both drought and waterlogging reduce nodulation and  $N_2$  fixation. Irrigation can provide marked yield improvement where the soil is not waterlogged for long periods after application [150]. Care should be taken to ensure that the irrigation water is not alkaline. The poor nodulation of *Arachis hypogaea* at Bambey, Senegal was attributed to water with pH 8.0, due to a high Mg-salts level. The need to drain crop land should be obvious from poor nodulation and plant growth, but less obvious will be waterlogging effects in pastures where the growth of associated grasses will tend to obscure, and accentuate, the poor growth of the legume component due to waterlogging.

**3.4.3. Liming.** Tropical legumes vary in their response to liming [118, 119, 120, 121] and caution must be exercised in the use of this practice. While it raises pH, and alleviates Mn and Al toxicities, ill-managed liming can induce deficiencies of Mg, Cu, Zn and B [1]. Due to the high cost of liming, a more appropriate strategy could be the selection of legumes, and their rhizobia, that are able to tolerate acid conditions [143].

**3.4.4. Nitrogen fertilizer.** The effects of nitrogen fertilizer on the nodulation and  $N_2$  fixation by legumes are complex, and vary with the form and concentration of the fertilizer, the species (and possibly the strains of rhizobia), the environmental conditions, and the level of available N in the soil [31, 171]. The data in Table 8 show depression of  $N_2$  fixation at high rates of application, and either no effect or stimulation at an intermediate rate. Low initial levels of fertilizer N can promote extensive nodulation and  $N_2$  fixation by overcoming any N deficiency induced before nodulation occurs [63]; they may also enable less effective strains to form a more effective symbiosis than they would in the absence of combined N.

As a generalization, well-nodulated legumes grow and yield at a similar level to plants supplied with adequate mineral nitrogen, provided they do not suffer N deficiency in the early stages of growth. Above a certain level of supplied N, further increases reduce the level of  $N_2$  fixed; below that level, the full  $N_2$ -fixing potential may not be achieved. The aim, therefore, should be to supplement soil N with the

Table 8. Effect of nitrogen application on  $N_2$  fixation (estimated by *A* value method) by groundnut

Rates of application of nitrogen fertilizer at seeding ( $kg\ ha^{-1}$ )	kg $N_2$ fixed per ha		
	Senegal <sup>a</sup>		Ghana [99]
	(1974)	(1975)	(1975)
15	52.0	67.5	60.7
30	56.0	75.1	69.3
60	25.0	—	—

<sup>a</sup> Ganry (1976), unpublished data

required dose of 'starter' N. As an example, trials in southern Brazil at a number of centers have indicated the benefits of 4–8  $kg\ N\ ha^{-1}$  as a starter dose for soybeans (Jardim Freire and Kolling, personal communication). Similar results have been obtained in Minnesota, USA (Ham, personal communication), but in Illinois, USA, only one response to fertilizer N was reported from 132 trials [176]. By using different forms of N fertilizer, the response was enhanced, or depressed [81]. Although it is generally recognized that combined N retards nodulation and  $N_2$  fixation, it is obvious that by correct application of fertilizer N,  $N_2$  fixation may be promoted. Only through a series of trials on different soils, with different crops, and in different agricultural cropping systems, will the most appropriate level of application be determined [64]. Other interesting approaches include deepbanding [177] or the use of slow-release fertilizers [50]; caution should be adopted in the use of nitrification inhibitors [156].

**3.4.5. Other fertilizers.** After nitrogen, phosphorus is most likely to be the limiting nutrient in tropical soils. A striking example of the effect of P fertilization was reported by Graham and Rosas [75], who observed a linear increase in nodule mass (9-fold), and specific nitrogenase activity of *Phaseolus vulgaris* over the range 0–315  $kg$  triple superphosphate  $ha^{-1}$ . None of the 30 cultivars showed reasonable nodulation or  $N_2$  fixation at low P. However, some *Stylosanthes* [21] and *Lupinus* [69] species make better growth with low P levels and it would be of interest to know the physiological basis of this characteristic. Sulphur, and less commonly potassium, may also be limiting in acidic, well-leached tropical soils. As indicated by Munns [119] and Gates and Muller [58], a balanced nutrition is essential to maximum production, and suitable trials should be undertaken to ensure that nutrients are not limiting production.

**3.4.6. Micronutrients.** In alkaline soils, Mn, Fe, B and Zn are less available than in acid soils, while in acid soils, Mo availability is reduced [79]. In many tropical regions, trace elements have received little attention, although it is possible that the deficiency of any one, but particularly Mo, B, Fe or Co, could severely limit productivity.

**3.4.7. Organic matter.** It is unfortunate that many tropical soils have very low organic matter levels in view of the generally beneficial effects of organic matter on the physical structure, and the chemical nature, of soil. Another attribute of organic matter is that it has been reported to enhance legume nodulation and  $N_2$  fixation [14, 30, 90, 124]. Various reasons may be advanced for these effects. They could be providing nutrients for the rhizobia or protecting them against harmful environmental factors. They could be promoting plant growth [49], or they could be exerting their effect through their influence on soil structure, by helping retain moisture in the soil, and by reducing phosphate fixation to clays [27]. Regardless of the actual effect(s), and this is most worthy of investigation, agronomic practices should be directed towards increasing the organic matter content of those tropical soils low in organic matter.

An interesting effect of different types of organic matter on nitrogen fixation by *Arachis hypogaea* has been observed (Table 9). The amount of  $N_2$  fixed was determined by the 'isotope dilution' method and from the differences in total N of nodulated and non-nodulated legumes (see Appendix). Compost and farmyard manure (FYM) increased  $N_2$  fixation 30–50%, as also found by Masefield [111], whereas millet straw had no effect. Under some conditions, green manuring may depress  $N_2$  fixation (Ganry, unpublished).

Table 9. Aftereffects of different types of organic matter on  $N_2$  fixation in peanuts, expressed in mg  $N_2$  fixed per plant (Ganry, Guiraud and Dommergues, unpublished)

Treatments	Estimation of $N_2$ fixation by 2 methods <sup>a</sup>	
	<sup>15</sup> N	Difference
Control	101	97
Incorporation of pearl millet straw	111	101
Incorporation of compost	142	158
Incorporation of FYM	136	146

<sup>a</sup> See Appendix

**3.4.8. Multiple cropping systems involving legumes.** These systems involve two or more crops grown on the same field in one year. The cropping may be sequential, or concurrent (i.e. intercropping).

Sequential cropping involving legumes and rice is common in paddy rice fields in Asia. In China and Vietnam, milk vetch (*Astragalus sinensis*) and *Sesbania* species respectively are commonly used, and in Thailand, soybeans are being investigated [138]. The legume may be used as a green manure crop, or as a grain crop, although in this latter case, the benefits are likely to be reduced as the grain is removed.

Intercropping is very popular in Africa and India. The most important legume in Africa, *Vigna unguiculata*, is grown with other crops in 98% of its cultivated area, while in Nigeria 95% of the groundnuts are intercropped [61, 70]. Although the yield of individual crops is usually decreased, the overall yield is often higher.

Furthermore,  $N_2$  fixation by the legume may be enhanced compared with the single-cropped legume, either in absolute amounts, or when expressed as a proportion of total plant N (Table 10). These experiments were done in lysimeters containing 78 kg soil uniformly labelled with  $^{15}N$ . Presumably the cereal has utilized most of the available N with the consequence that  $N_2$  fixation by the legume is favored. Another example of intercropping involves maize (*Zea mays*) and the climbing bean (*Phaseolus vulgaris*) which uses the maize for support [73].

Table 10. Percent of total  $N_2$  fixed symbiotically in two leguminous plants grown as pure crops or in association with pearl millet (Ganry, Guiraud and Dommergues, unpublished data)

Plant species	Pure crop	Intercropping
Soybean	88	97
Groundnut	75	90

#### 4. Concluding Remarks

The legume is not so much a plant as it is a symbiotic association, and it will be as a symbiotic association that the legumes will realize their potential in helping to overcome the severe nitrogen deficiency limiting production in many tropical soils. Hence it is important that as much attention be given to the rhizobia, both as free-living components of the soil microflora and as partners in the  $N_2$ -fixing symbiosis, as is given to the agronomy, selection, breeding, pathology and physiology of the more obvious partner in the symbiosis. In addition to selecting effective, competitive and persistent strains for particular hosts, and to devising appropriate procedures for producing inoculants, and applying them, consideration must be given to management practices that minimize the effect of nutrient deficiencies and toxicities, environmental stresses, and biological agents that affect the association. It is obvious from the limited data in Table 1, and the wide range of species listed in Tables 4 and 5, that the legumes possess an exciting potential to help overcome nitrogen deficiency in many types of agrosystems. However it will only be through the combined efforts of scientists in many disciplines that this potential will be achieved.

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## Appendix. Principles of the Main Methods for Estimating Nitrogen Fixation Under Field Conditions

In the field, legumes receive their nitrogen from two sources, soil (+ fertilizer) N, and through N<sub>2</sub> fixation. Two of the methods to differentiate between these sources depend on total N determination in the nodulated plant, and the comparison of this value with that for non-nodulated plants, or with plants in different N-fertilizer rate treatments. The third method depends on <sup>15</sup>N, either added to the soil or measured as a change in natural abundance, while the fourth utilizes the ability of nitrogenase to reduce the surrogate substrate, acetylene, to ethylene, which is then determined by gas chromatographic procedures.

### 1. The difference method

During, or at the end of, the growing season, the total plant N in a non-nodulated legume, or a non-legume, is subtracted from that in the nodulated legume [174], the assumption being that both sets of plants remove the same amount of soil nitrogen. This method is most effective when the control is of the same species as the test plant. Non-nodulating isolines are available for a number of soybean cultivars [106] while non-nodulating *Arachis hypogaea* lines have been developed recently [29, 71]. The alternatives are to use soils free of the appropriate rhizobia (these are often very difficult to find, and maintain) or to use a non-legume, which may be criticized because the rooting pattern and growth habit is unlikely to be the same as that of the legume.

## 2. Fertilizer equivalent method

The growth of the nodulated plant is compared with a series of N-fertilizer 'rate of application' treatments applied to non-nodulated plants of the same species, or such plants very heavily inoculated with a known ineffective strain. This approach was used successfully during the International Biological Programme to determine  $N_2$  fixation by a number of legumes in a wide range of environments [128].

## 3. $^{15}N$ -tracer methods

$^{15}N$ -tracer methods are regarded as the most reliable as the measure of  $N_2$  fixation is direct [16]. The principal method used involves adding  $^{15}N$ -labelled fertilizer and comparing the dilution of the label in nodulated and non-nodulated plants (isotope dilution method; [56]) although attention is being given also to the 'natural abundance method'.

Non-nodulated plants assimilate soil ( $S_1$ ) and fertilizer ( $F_1$ ) nitrogen, whereas nodulated plants assimilate these forms ( $S_2$  and  $F_2$ ) plus atmospheric  $N_2$  ( $Y$ ). The  $^{15}N$ -labelled fertilizer is added to soil at a rate not exceeding  $30 \text{ kg N ha}^{-1}$  (and preferably less) in order to minimize effects on  $N_2$  fixation. If  $s_1$  and  $s_2$  are the fractions of total plant N derived from the soil N, and  $f_1$  and  $f_2$  the percentage derived from fertilizer N, and  $y$  the percentage originating from  $N_2$  fixation, then:

$$S_1 + F_1 = 1 \quad (1)$$

and

$$S_2 + F_2 + Y = 1 \quad (2)$$

The assumption is that both nodulated and non-nodulated plants will take up nutrient from each source in proportion to the amount available. Thus

$$F_1/S_1 = F_2/S_2 \quad (3)$$

Introducing equation (1) into equation (3) gives

$$S_2 = \frac{F_2}{F_1} (1 - F_1) \quad (4)$$

$$Y = 1 - \frac{F_2}{F_1} \quad (5)$$

As the percentages  $f_1$  and  $f_2$  have the same denominator (atom %  $^{15}N$  excess in the labelled fertilizer,  $a\%e$ ):

$$y = \frac{a\%e \text{ in nodulated plant}}{a\%e \text{ in non-}N_2\text{-fixing plant}} \quad (6)$$

The amount of  $N_2$  fixed is then calculated by multiplying total N in the plants by  $Y(\Sigma N \cdot Y)$ ; it can also be calculated by using the 'A' value transformation proposed by Fried and Broeshart [55].

A variation on this method involves incorporating  $^{15}N$  into the soil organic matter ( $^{15}N$  fertilizer plus glucose, sucrose or cellulose incubated in the soil), with all the  $^{15}N$  then being available following mineralization. As Ham [80] points out, the assumption made is that inorganic nitrogen available to the plant by mineralization of the labelled organic matter represents the mineralizable fraction of soil nitrogen.

The 'natural abundance method' depends on the difference in the level of  $^{15}N$  in atmospheric  $N_2$  and that in the pool of soil nitrogen. Modern mass spectrometers are highly sensitive and are able to detect the small differences in the  $^{15}N$  constitution of plants fixing  $N_2$  and those assimilating their nitrogen in a combined ( $NO_3$ ,  $NH_3$ ) form, which has a slightly higher level of  $^{15}N$  than atmospheric  $N_2$ . This and the isotope dilution method are described in greater detail by Knowles [95], and the methods for determining  $^{15}N$  are described by Bergersen [12].

#### 4. *The acetylene reduction technique*

This indirect assay of measuring  $N_2$  fixation is simple to use, relatively cheap to establish and operate, and has been a major factor facilitating the large increase in  $N_2$  fixation research in recent years. The assay only measures activity during the assay period. While this is generally satisfactory for comparative purposes (but see below), it requires that frequent assays must be made to construct daily and seasonal profiles for estimating nitrogen fixation over a long period [7, 154]. It is also essential to determine a conversion ( $C_2H_2:N_2$ ) ratio by doing other measurements (e.g.,  $^{15}N$ , total N). Many regard the theoretical ratio as 3:1, as the reduction of acetylene to ethylene requires one pair of electrons whereas  $N_2$  to  $2NH_3$  requires 3 pairs of electrons. However this ignores  $H_2$  evolution, the electrons for which are also used to reduce acetylene, and should raise the theoretical ratio to 4:1, or higher. While published values average around 3.8–4.0:1 [81], the range is 0.6–6.2:1, and it is not possible to specify a suitable conversion factor for any situation without doing the essential calibrations for those conditions. Recent work has shown that strains of rhizobia may differ in the  $C_2H_2:N_2$  ratio, while the level of desiccation of the nodules also affects the ratio (Gibson and Sprent, unpublished), requiring that care must be exercised in comparisons of treatments in which these factors are variables.

Other considerations in the use of the technique were discussed recently by Ham [80], Knowles [95, 96], by Turner and Gibson [166], and by Silvester [153]. In brief, the technique is valuable, but great caution must be exercised, particularly in the interpretation of the data.

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