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Future directions for biological nitrogen fixation research

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Introduction

Enormous progress has been made in the last decade in understanding the physiology and genetics of nitrogen-fixing microorganisms, but relatively little has been done to translate theory into practice. The object of this note is to define strategies for research on applied nitrogen fixation and thus to help increase food and energy production and contribute to the restoration of soil fertility.

Attention is given here to the symbiotic nitrogen-fixing systems, legumes and actinorhizal plants, because of their unquestionable importance in tropical agriculture, agroforestry and forestry (National Research Council, 1979; Dawson, 1986).

No attempt is made to evaluate the priorities for research on Azolla, non-symbiotic Cyanobacteria or non-symbiotic nitrogen-fixing bacteria in plant litter (Hill *et al.*, 1988) or in the rhizosphere of cereals and pasture grasses (associative nitrogen fixation). Problems related to nitrogen fixation by Cyanobacteria have already been extensively explored and carefully reviewed (IRRI, 1979; Lumpkin and Plucknett, 1980; Roger and Kulasooriya, 1980; Roger and Reynaud, 1982; Roger and Watanabe, 1986). Generally the ecosystem received little nitrogen from non-symbiotic rhizospheric nitrogen-fixing microorganisms because they do not get enough energy to fix appreciable amounts of nitrogen and are often hindered in their activity in the field (Alexander, 1982; Barraquio *et al.*, 1984; Beringer and Hirsch, 1984). However recent experiments based on the use of ^{15}N suggest that the nitrogen-fixing bacteria associated with the roots of several tropical crops, including wetland rice, sugar cane and some forage grasses, can fix significant amounts of nitrogen (Boddey and Dobereiner, 1987). Rhizospheric microorganisms can also contribute to plant growth through other processes, *e.g.* the production of growth factors, facilitating mineral uptake or cancelling the inhibitory effect of chemicals or pathogens (Kapulnik *et al.*, 1987; Nayak *et ak.*, 1986; Okon, 1984).

This paper first considers research priorities for Rhizobium and *Frankia* and their host plants and then discusses the methodology used to evaluate the amount of nitrogen fixed *in situ*. This methodology must be made adequate (i) to test the behaviour of the different nitrogen-fixing systems and their adaptability to field conditions, and (ii) to generate information needed in designing management practices that eliminate major environmental constraints. The conclusion contains suggestions intended for use in developing countries.

Research on the symbiotic microorganism

Improvement of the symbiotic microorganism

In work to improve symbiotic microorganisms the usual approach is to start by evaluating the effectiveness, competitiveness and performance of Rhizobium and *Frankia* in the field. In this evaluation careful attention must be given to the effect of environmental stresses on the establishment and nitrogen-fixing activity of the strains that are used as inoculants. The environmental constraints are the result of physical and chemical factors such as soil pH, salinity, moisture, temperature, combined nitrogen, pesticides, and especially fungicides (Alexander, 1982; Halliday, 1984; Schmidt, 1978). Biological stresses include competition and predation, problems which have been addressed extensively by Alexander (1982) and also a wide range of other biological interactions involving antibiotics, bacteriophages and bacteriocins (Schmidt, 1978). Recent investigations carried out in our laboratory

showed that antibiosis might significantly limit Rhizobium infection of legume trees grown in some tropical soils (O Diagne and H G Diem, unpublished data). But since 'accumulating data indicate that site variations in performance of selected strains is common' (Halliday, 1984), in addition to preliminary screenings in the laboratory or glasshouse, the response to inoculation with a given strain must also be tested in field trials.

Many strains of Rhizobium are currently available for use in inoculating annual legume crops like soybean, chickpea, cowpea, peanut, lupine, alfalfa, clover. But relative few effective strains of Rhizobium nodulating nitrogen-fixing trees have been isolated; some of the best known are strains for *Leucaena leucocephala*, *e.g.* TAL 1145 from NifTAL (Roskoski, 1986) and IRc 1045 and 1050 from IITA (Sanginga *et al.*, 1985; 1986). There is still much work ahead to collect Rhizobium and *Frankia* strains for leguminous nitrogen-fixing trees and then screen them for genetic compatibility, nitrogen-fixation effectiveness, and tolerance to environmental stresses, especially soil acidity (Dommergues, 1987).

Using molecular techniques (molecular cloning and recombination), new strains of Rhizobium and *Frankia* will probably be engineered to contain multiple copies of the major genes involved in the symbiosis: genes of nitrogen fixation and nodulation, and genes involved in interstrain competition. Genes that control the conversion of tryptophan to indolacetic acid may prove useful in stimulating nodulation since tryptophan-catabolizing mutants of Bradyrhizobium have been shown to enhance the nodulation of *Glycine max* (Kaneshiro and Kwolek, 1985).

Stress-tolerance strains of rhizobia will probably be engineered in the near future. In this respect, recent progress towards identifying the genes controlling tolerance to soil acidity shows promise (P Graham, personal communication). Another example is that of strains synthesizing siderophores. Strains with this characteristic actively fix nitrogen in groundnuts grown in iron-deficient soils, while strains lacking this characteristic are ineffective (M J Dilworth, personal communication). To obtain highly effective and stress-tolerant strains it may be possible to use 'bacteria whose genetic libraries already contain adaptation traits to prevailing environmental stresses' and engineer them for better nodulation, nitrogen fixation and competitiveness (Roskoski, 1986).

Inoculation technology

The adaptation characteristic should always be established through field trials, as was indicated above, but the choice should not be limited to strains of Rhizobium or *Frankia* isolated locally on the ground that native strains are systematically better adapted to the environment. As a matter of fact, a strain that appears to be the most effective and best adapted to a given plant species may have been isolated from nodules of a plant belonging to another genus or species and grown under quite different conditions. The best strain for inoculating *Acacia holocericea* in Senegalese soils, for instance, appeared to be strain Tal 651 (Cornet and Diem, 1982), a strain isolated from *Calopogonium mucunoides* in Malaysia (Halliday and Somasegaran, 1984). The famous strain of *Bradyrhizobium* CB756 (syn: TAL 309) from Zimbabwe has been successfully used to inoculate legumes of the cowpea group grown in soils far away from its native habitat.

Inoculating the host plant with its specific symbiotic microorganism is usually achieved by mixing a culture of the selected or improved strain of Rhizobium with presterilized peat, the usual inoculant carrier. But since the composition of peat is highly variable, there are advantages in using a synthetic carrier of constant quality such as polyacrylamide or alginate (Jung *et al.*, 1982). Recent progress has been made in our laboratory in the preparation of this new type of inoculant.

Turning to the actinorhizal plants, pure cultures of *Frankia* have not often been used in the past because of the difficulty in isolating and cultivating the strains, especially those of Casuarina (Diem *et al.*, 1982, 1983) and consequently in obtaining the inoculants. However, thanks to recent progress in our knowledge of *Frankia* physiology and culture, we are able to produce inoculants whose effectiveness was demonstrated in preliminary field experiments in Senegal where inoculation of *Casuarina equisetifolia* seedlings in the nursery markedly increased the growth, nodulation and nitrogen content of the trees after their transplantation to the field (Sougoufara, unpublished data). Similar results demonstrating increased growth of *Casuarina*

cunninghamiana plantings following inoculation with *Frankia* have been reported from Australia and Zimbabwe (Reddell *et al.*, 1987). We hope that soon actinorhizal plants will be systematically inoculated with pure cultures of *Frankia* in large areas. Inoculating the host plant with soil or crushed nodules is a technique that is still recommended, but should be discouraged because of the high risk of contaminating seedlings or cuttings with root pathogenic agents, like *Rhizoctonia solani* or *Pseudomonas solanacearum* in the case of *Casuarina equisetifolia* (Liang Zichao, 1986), or nematodes in the case of the Australian acacias introduced in western Africa.

When the inoculum is applied directly on the seeds, it may be necessary to protect the symbiotic microorganisms against acidity by pelleting the seeds with calcium carbonate or rock phosphate. This is a widely used technique developed in Australia (Williams, 1984).

In the case of trees raised in containers, inoculation with Rhizobium is best achieved by spraying or drilling the inoculum directly into the container at the time of seeding or planting. For *Frankia* it is advisable to mix the soil or subtratum of the container with the inoculum because *Frankia*, like vesicular-arbuscular mycorrhizal fungi (VAM), is not mobile in the soil (Dommergues, 1987). After the containerized plants have been transplanted to the field, the effect of inoculation in the sterile nursery soil will remain only if the soil does not contain specific and effective, or non competitive native strains.

Thanks to their capacity to fix nitrogen, legumes and actinorhizal plants can grow on nitrogen-deficient soils, which are also often deficient in phosphorus and other elements. In these soils the effects of mycorrhizal infection on nitrogen fixation are about as beneficial as the addition of phosphorus. Virtually nothing is known about the direct interaction between the nitrogen-fixing microorganism and the mycorrhizal fungus (Hayman, 1986). In nature, roots of most legumes and actinorhizal plants are infected with VAM fungi, whereas ectomycorrhizal infection of the roots is restricted to a lower number of perennial plant species, which are either legumes, for example species of genera such as Afzelia, Inga, Acacia (Malloch *et al.*, 1980) or actinorhizal species of general such as Alnus, Elaeagnus, Casuarina (Gardner, 1986). Nitrogen-fixing plants only need to be inoculated with endo- or ectomycorrhizal fungi in soils where these microorganisms are absent or rare, *e.g.* sterilized nursery soils. In those soils, dual inoculation with the specific Rhizobium and VAM fungi has significantly increased the growth, nodulation and nitrogen fixation in legumes (*e.g.* Ganry *et al.*, 1985; Roskoski *et al.*, 1986) and in actinorhizal plants (Diem and Gauthier, 1982). Obviously the response to the dual inoculation may vary with site and plant species, and more field trials are needed to evaluate the actual effect of these treatments on nitrogen fixation.

With a few exceptions, the technology of inoculation with ectomycorrhizal fungi is now applicable (Schenck, 1982). More investigations are needed to improve the production of VAM fungi inoculants. New procedures for the multiplication of VAM fungi may facilitate the mass production of inoculum (Mosse and Thompson, 1984). Methods for processing the endomycorrhizal inoculum by entrapping fungal structures in alginate seem widely applicable (Ganry *et al.*, 1985).

Research on the host plant

The amount of nitrogen fixed by any nitrogen-fixing plant is related to its Nitrogen-Fixing Potential (NFP), *i.e.* its ability to fix nitrogen in a constraint-free environment. The NFP is not only conditioned by the associated symbiont but also by the host plant. Consequently, to provide the maximum nitrogen input for a given ecosystem, host plants with a high NFP are required.

Another essential characteristic for the host plant is its good tolerance of environmental stresses, be they physical (*e.g.* acidity, excessive temperature, drought), chemical (*e.g.* excess of combined nitrogen), or biological (*e.g.* root pathogens). Because of these stresses, however, even the ideal nitrogen-fixing system cannot attain its full potential under field conditions. The amount of nitrogen that is really fixed in the field is called the Actual Nitrogen Fixation (ANF). The ANF of stress-sensitive nitrogen-fixing plants is expected to be much lower than their NFP; conversely the ANF of stress-tolerant species is expected to be much closer to their NFP.

Annual legumes

A number of currently cultivated annual legumes have been bred for agronomic traits such as high yield, diseasee and pest resistance and stress tolerance. Selecting and breeding crop legumes for improved nitrogen fixation is just beginning but is already generating considerable interest. Three possible approaches need to be studied:

Improving the NFP of the host plant. The first step is to evaluating nitrogen fixation through parameters such as nodule weight (the simplest criterium), dry weight or total nitrogen content of plants grown on a nitrogen-free substrate, or the acetylene reduction activity.

Controlling the specificity of the host plant, i.e. attempting to obtain plants which nodulate effectively with most of the native soil rhizobia (promiscuous nodulation) or, on the contrary, plants which nodulate only with specific rhizobia (selective receptivity), but not with native strains (Devine, 1984; Hobbs, 1985). The former strategy was tested by Nagju (1980) in Africa, where high-yielding US-bred cultivars of soybeans have to be inoculated with specific *Bradyrhizobium japonicum* strains. Instead of using the required specific inoculum, Nangju proposed another solution, based on the fact that soybeans cultivars from southeast Asia nodulated effectively with native African rhizobia; hybridization of the Asian and US cultivars had resulted in lines combining the desirable agronomic characteristics of the US cultivars and the symbiotic potential for nitrogen fixation with the native rhizobia characteristic of southeast Asian germplasm. The value of promiscuously nodulating plants is questionable because of the risk of competition, and, as a result, nodulation with ineffective or poorly effective native strains of Rhizobium.

Developing host plants whose NFP is not affected by climate or soil constraints, such as acidity or combined (mineral) nitrogen. This research could be conducted using the following methods:

(i) *Screening existing genotypes for desired traits.* This simple method based on the exploitation of the natural intraspecific variability was successfully used by Herridge (1987) to identify 32 elite genotypes of soybean tolerant to combined nitrogen from an original collection of 489 lines. Similar results had been obtained before on a smaller scale by Hardarson *et al.* (1984) who compared eight cultivars of soybean and observed in one of them that nitrogen fixation was not affected by high levels of soil combined nitrogen.

(ii) *Conventional plant breeding.* Legume crops such as pea, bean, alfalfa, soybean have sufficient genetic variability in symbiotic nitrogen fixation to serve as the basis for a classical breeding program in which the goal is to improve nitrogen fixation in the absence or presence of soil combined nitrogen or in response to a specific Rhizobium inoculant or to the indigenous Rhizobium populations (Devine, 1984). This approach has been successfully used with soybean and bean at CIAT, Colombia (Graham and Temple, 1984).

(iii) *Mutagenesis breeding.* Improved lines of soybean that can fix nitrogen even in the presence of high levels of nitrate in the soil have already been obtained as a result of mutagenesis breeding (Carroll *et al.*, 1985). Similar procedures could be used to generate improved lines of other nitrogen-fixing plants.

(iv) *Hybridization of nitrogen-fixing and non-nitrogen-fixing plants.* Gene transfer by wide hybridization to obtain new nitrogen-fixing systems has not yet been exploited. Sophisticated new methods, like somatic hybridization by protoplast fusion or embryo rescue, are now available, and could be appropriately used in this promising field of research.

(v) *Genetic transfer of nitrogen fixation ability to plant cells.* The transfer of cloned genes from nitrogen-fixing microorganisms to plants requires lengthy research since it has not yet been determined whether, after being introduced into a plant, all the genes required for nitrogen fixation are permanently incorporated, expressed, and inherited by the whole plant.

Stem-nodulated legumes

Most nitrogen-fixing legumes have nodules on their root system. However, some species also form nodules on the stems: they are called stem-nodulated legumes. Three genera have been reported to comprise stem-nodulated species: *Sesbania*, *Aeschynomene* and *Neptunia*. As yet, only two species of *Sesbania* have been reported to produce stem nodules: *S. rostrata*, which is native to Western Africa and *S. punctata*, probably native to

Madagascar. About 15 species of Aeschynomene are now known to have stem nodules, *e.g. A. afraspera, A. indica, A. nilotica, A. elaphroxylon* (Alazard, 1985).

Several of these legumes (*Sesbania rostrata, Aeschynomene afraspera, A. nilotica*) are not only characterized by an unusually good NFP but also have the unique capacity to absorb combined nitrogen through their roots while fixing atmospheric nitrogen through their stem nodules, even in the presence of high soil mineral nitrogen (Becker *et al.*, 1986; Dreyfus *et al.*, 1984). Transferring the stem nodulation character from these plants to non-stem nodulated legumes could be used to develop uninhibited nitrogen-fixing plants. The introduction of such plants into intensifying agrosystems would allow substantial yield increase, using less nitrogen fertilizers than is presently recommended.

Developing the use of the stem-nodulated legumes as soil improvers through green manuring or as a source of fodder is to be encouraged.

However, genetic improvement is still needed for species of stem-nodulated legumes that have very high water requirements, unsatisfactory photoperiodic responses, *e.g. Sesbania rostrata*, and great sensitivity to nematodes in drained soils, again, *e.g. S. rostrata.*

Nitrogen-fixing trees including actinorhizal plants

Improving the NFP of perennial legumes or actinorhizal plants is a research priority.

A simple but slighted approach relies on the spontaneous variations in a tree population within a provenance[1]. Exploiting spontaneous variations requires two operations. After the best provenances have been identified the whole population in the provenance must be screened to identify the more actively nitrogen-fixing individuals. This procedure should be based on non-destructive assays such as the enumeration of nodules combined with the measurement of the acetylene reducing activity of the different individuals. Thereafter, the superior phenotypes must be vegetatively propagated (*e.g.* Datta and Datta, 1984; Duhoux *et al.*, 1986; Leaky, 1986).

Sougoufara *et al.* (1987) recently used this two-step approach successfully to increase the NFP of *Casuarina equisetifolia.* These authors identified a clone of *C. equisetifolia* (called clone β) with a much higher NFP than that of a reference clone (clone α), *i.e.* a clone with a potential similar to that of the commonly grown seedlings. Two sets of clones (α and β) were grown in a sterile nitrogen-deficient soil. One set was inoculated with a *Frankia* strain, the other was not. After 7 months, the uninoculated clones had grown poorly, while the inoculated clones had grown satisfactorily, but their response to inoculation differed markedly. Inoculated clone β produced 2.6 times more biomass (expressed in terms of dry weight and total nitrogen) than inoculated clone α, and its nodule weight and NFP (expressed as acetylene reducing activity per plant) were significantly higher (1.6 times). The difference in the NFP of the clones appeared to be correlated to their nodule weight. Recent field trials carried out in Senegal show that, after 2 years of growth, clone β exhibited a significantly higher NFP than clone α. These results suggest that the NFP can be measured in very young trees; in the example above the first screening was made at 7 months.

In vitro micrografting of a non-nodulating species onto a nodulating (and nitrogen-fixing) rootstock may be used to introduce the ability to nodulate into non-nodulating species of the same genus or family. Preliminary results obtained by Kyle and Righetti (1985) with actinorhizal Rosaceae are most promising.

The transfer of the whole set of genes required to fix nitrogen, from a bacterium to a mycorrhizal fungus could also be considered. However, many difficulties must be overcome first, one prerequisite to genetic engineering of nitrogen-fixing endomycorrhizal fungi being the availability of a reliable method to grow this fungus *in vitro.*

Improving the NFP of trees could probably also be achieved using any of the methods mentioned above for annual legumes.

Evaluation of the amount of nitrogen fixed in the field

How much symbiotically fixed nitrogen enters

[1] In the field of forestry the term provenance refers to natural populations of trees originating in a specific geographic location.

the different ecosystems (agrosystems, agroforestry systems and forests)? Without an answer to this question it is virtually impossible to develop management practices that improve the process *per se* and the transfer of fixed nitrogen to non-nitrogen-fixing plants, be they food crops or trees. Reliable estimates of nitrogen fixation can be made using the following methods: 'nitrogen difference', '^{15}N enrichment' (improperly called 'isotope dilution'), '^{15}N depleted material', 'natural ^{15}N abundance', 'acetylene reduction', 'analysis of nitrogen solutes in the xylem sap'. These methods have been described and discussed in a number of recent publication (*e.g.* Bergersen, 1980; Herridge, 1982a; LaRue and Patterson, 1981; Silvester, 1983). Under carefully controlled conditions each can give reasonable estimates (*e.g.* Bergersen, 1986, Herridge, 1982a). Whenever possible at least two methods should be used simultaneously.

Annual plants

All these methods can be used rather easily to estimate the amount of nitrogen that is fixed in the case of annual plants grown in containers or in the field.

The analysis of nitrogen solutes in the xylem sap is a recently proposed method, which appears to be appealing because it does not require much equipment, but it can only be used on certain legumes that are called ureide exporters. In ureide exporters, much of the nitrate absorbed by the roots is passed to the shoot as free unreduced nitrate because of the low nitrate reductase activity of their roots. In non-nitrogen-fixing plants, the xylem-nitrogen is found mainly in the form of nitrate and amino acids, whereas in nitrogen-fixing plants it contains mainly ureide nitrogen, and the relative abundance of ureides in sap can be used as an indication of nitrogen fixation activity. This method has been very successfully used in the case of soybean (Herridge, 1982b; 1984) and cowpea (Pate *et al.*, 1980). On the other hand, in amide exporters, only a small proportion of the nitrate absorbed by the roots escapes the reductase system of the roots, whose sap therefore contains mainly amides regardless of whether nitrogen is fixed. This makes it impossible to use sap analysis for estimating nitrogen fixation in amide exporting legumes (Bergersen, 1986).

Perennial plants

In these plants there are special difficulties, *e.g.* logistic and sampling problems, variations in the nitrogen-fixing activity at different ages, or interference by different processes such as losses and redistribution of nitrogen in the different horizons or compartments of the agroforestry system.

In the old and simple 'difference method', the amount of nitrogen fixed is considered to the difference between the total nitrogen yield of the nodulated (nitrogen-fixing) plant and the total nitrogen yield of a non-nodulated (non-nitrogen-fixing) companion plant, preferably of the same species, that serves as a control. Estimates can only be accurate if structure and function of the root systems of both plants are similar. Despite its shortcomings, this method and related methods based on nitrogen balance studies, often provide applicable evaluations of nitrogen fixation when used in nitrogen-deficient soils.

The ^{15}N enrichment method can only be used in the case of trees less than 2–3 m high grown containers of relatively large volume, *i.e.* about 1 m^3 (*e.g.* Gauthier *et al.*, 1985).

The most promising method is probably the natural ^{15}N abundance method wherein small differences between the natural abundance of ^{15}N in non-nitrogen-fixing and nitrogen-fixing plants are studied. Soil nitrogen frequently contains slightly more ^{15}N than atmospheric nitrogen. Furthermore, in most biological reactions, as a result of isotope discrimination, the lighter of two isotopes is favored slightly. Because of these two phenomena, nitrogen derived from nitrogen fixation has a minutely lower ^{15}N content than nitrogen from the soil. Studies of the δ^{15}N values (parts per thousand difference in % ^{15}N compared with an appropriate air standard) show that nitrogen-fixing plants generally have low δ^{15}N values (Knowles, 1983). Using the δ^{15}N values of the non-nitrogen-fixing and nitrogen-fixing plants it is possible to calculate the fraction of the plant nitrogen that is derived from fixation. This method requires an isotope ratio mass spectrometer and scrupulously careful manipulations, but the results are as reliable as those obtained from the ^{15}N enrichment method (Bergersen, 1986). One of the first studies using this method was carried out in the Sonoran desert on Prosopis trees that had a δ^{15}N of +3.0

(soil = + 6.0), which suggests that they fixed nitrogen. Since no nodules were visible the tree was presumed to develop nodules on deep roots which are not normally sampled (Virginia *et al.*, 1981).

In spite of its limitations (Bergersen, 1980; Hansen *et al.*, 1987; LaRue and Patterson, 1981), the acetylene reduction method, when used in the field, gave relatively exact estimations of the amount of nitrogen fixed (*e.g.* Roskoski, 1981 and 1982).

Preliminary studies on the composition of the sap of 35 nitrogen-fixing leguminous trees have been carried out at NifTAL by Kessel *et al.* (1987). Only two species, *Acacia mearnsii* and *Sesbania grandiflora*, showed a high relative abundance of ureides in the xylem sap (81.5% in *A. mearnsii* and 78.8% in *S. grandiflora*). For the two species, ureides are the major nitrogen compounds in the sap, and the ureide method could probably be used for measuring their NFP.

Conclusions

Highest priority should be given to research designed to increase nitrogen fixation in symbiotic systems, legumes and actinorhizal plants, especially Casuarinaceae (Dommergues, 1987).

In the past most efforts have been directed to improving the associated microorganism (Rhizobium and more recently *Frankia*). Marked progress has been achieved by selecting the most efficient Rhizobium or *Frankia* strains, and by improving the inoculant technology, which includes the concept of synthetic carrier.

However, it is now clear that a substantial gain in the NFP level can be obtained by exploiting the variability of the host plant. In the case of trees, a very significant improvement of the NFP can easily be obtained through selection of elite individuals. In the case of annual crops, a number of other methods are available, including screening lines for their NFP.

Research should be aimed at improving the NFP of plants together with their tolerance to a number of environmental constraints, especially the excess of combined nitrogen in the soil, the ultimate goal being high crop yields. Since high yields cannot be obtained through the biological nitrogen fixation process alone without chemical fertilizers, the ideal solution is to combine biological and chemical means. This combination can only be effective when nitrogen-fixing systems are uninhibited by combined soil nitrogen. Probably the development of nitrogen-fixing plants that continue fixing significant amounts of atmospheric nitrogen even while nitrogen fertilizer is being applied would be a step forward in symbiotic nitrogen fixation (Herridge, 1987). Such plants have already been obtained through systematic screening of lines of annual crops. More sophisticated methods, like mutagenesis breeding or the transfer of the stem-nodulating character to non-stem nodulated legumes, should also be utilized.

The nitrogen-fixing systems which have been improved in the laboratory, glasshouse or nursery are not necessarily able to reach their maximum potential in the field. They must be fitted to the field situation. Thus, before deciding to use a new nitrogen-fixing system, it is mandatory to systematically test its nitrogen-fixing ability in the field. This type of evaluation obviously presupposes the availability of accurate, simple methods to measure nitrogen fixation *in situ*. Progress has been made recently in developing such methods for annual crops, but more investigations are urgently needed to overcome the difficulties specific to perennial plants.

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