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Measuring plant-associated nitrogen fixation in agricultural systems

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2008

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Published by the Australian Centre for International Agricultural Research (ACIAR)
GPO Box 1571, Canberra ACT 2601, Australia
Telephone: 61 2 6217 0500
aciar@aciar.gov.au

Unkovich M., Herridge D., Peoples M., Cadisch G., Boddey R., Giller K., Alves B. and Chalk P. 2008. Measuring plant-associated nitrogen fixation in agricultural systems. ACIAR Monograph No. 136, 258 pp.

This report may be downloaded and printed from <www.aciar.gov.au>.

ISBN 978 1 921531 26 2 (print)
ISBN 978 1 921531 27 9 (online)

Editing and design by Clarus Design Pty Ltd
Printing by Paragon Printers Australasia

Foreword

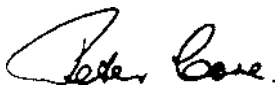
Nitrogen (N) is one of the key drivers of global agricultural production. Between 150 and 200 million tonnes of mineral N are required each year by plants in agricultural systems to produce the world's food, animal feed and industrial products. To meet those requirements, close to 100 million tonnes of N are fixed annually via the industrial Haber Bosch process. Many have argued that improving the efficiency with which fertiliser N, the major product of industrial dinitrogen (N_2) fixation, is used in world agriculture is vital to the long-term sustainability of the planet. That would appear to be a reasonable goal, given the often low efficiency of fertiliser-N use, with gaseous losses contributing to global warming, and leaching and erosion losses to the degradation of watercourses and storages.

An equally important goal is the more effective exploitation and utilisation of biologically fixed N in agricultural systems. It would, at the least, complement fertiliser-N use and may ease the long-term pressure for expanded industrial production. It is also possible that particular systems within the global framework could become far more reliant on biologically fixed N, rather than fertiliser N, for N inputs. Thus, plant-associated N_2 fixation currently contributes 50–70 million tonnes annually to the global agricultural N budget. Increasing that level of input—in some cases, just maintaining it—requires a substantial investment in fundamental research to optimise the various N_2-fixing systems and have them applied. However, it is not possible to undertake experiments to identify treatment effects on N_2 fixation, nor on-farm surveys to determine activity at a regional or country level, unless the process can be accurately and reliably quantified. It also follows that the development of plant varieties and farming practices to optimise N_2 fixation is, and will continue to be, linked to the degree to which scientists have access to measurement methodologies.

ACIAR recognised the need for such methodologies through the funding of projects to develop them and by publishing, in 1989, 'Methods for evaluating nitrogen fixation by nodulated legumes in the field', by M.B. Peoples, A.W. Faizah, B. Rerkasem and D.F. Herridge (ACIAR Monograph No. 11). That practical handbook appears to have been widely used, with about 700

copies distributed, more than 100 citations in refereed journals and positive feedback from overseas and Australian scientists. In the 20 years since 1989, significant advances have been made in the development and application of techniques for measuring N_2 fixation, prompting the need for revision of the handbook. In late 2006, during the months leading up to the 15th International Congress on Nitrogen Fixation in Cape Town, South Africa, that need turned into reality through an evolving discussion between the authors on book content, contentious issues, target audience, sources of funding and other matters.

ACIAR is pleased to publish this very timely update of the original handbook. The authors are to be complimented on the thoroughness with which they have incorporated the exciting advances made in the science during the past 20 years. It will be of great benefit to scientists and students in all countries that are engaged in the study of N_2 fixation. ACIAR hopes also that it might stimulate others to quantify this very important process, not only to add value to their own research but also to add to the global pool of knowledge on plant-associated N_2 fixation.



Peter Core
Chief Executive Officer
ACIAR

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Acknowledgments

Many thanks to Graeme Batten for the section on analysis of plant nitrogen by near infrared spectrometry (NIR). The Grains Research and Development Corporation of Australia (GRDC) provided invaluable financial support to bring the authors together for a workshop at Byron Bay, Australia, in August 2007 to thrash out details of the measurement methodologies and to debate and finally agree on many contentious issues.

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Glossary

diazotroph	a N_2-fixing bacteria
endophyte	an organism that lives in or on another organism without causing disease or harm
eukaryote	organisms (like animals, plants or fungi) that have highly organised cell structures and internal cell membranes
heterotroph	an organism that needs to obtain organic C for growth and development (i.e. non-photosynthetic)
nitrogenase	the enzyme complex found only in N_2-fixing bacteria and which is responsible for fixing N_2 into NH_3
prokaryote	mostly single-celled organisms (bacteria) without a nucleus
rhizobia	a general term given to N_2-fixing bacteria in symbioses with legumes
Rhizobium	one of the most common of many genera of N_2-fixing bacteria
symbiosis	an organised association between two organisms in which both organisms directly benefit from the association
%Ndfa	percentage of N derived from the atmosphere (percentage dependence on N_2 fixation for growth)
^{15}N enrichment	increase in ^{15}N above background as a result of the addition of ^{15}N-labelled materials

1

Biological nitrogen fixation

Although dinitrogen (N_2) gas represents almost 80% of the earth's atmosphere, it is not directly available to plants. Biological N_2 fixation is the process whereby a number of species of bacteria use the enzyme nitrogenase to convert atmospheric N_2 into ammonia (NH_3), a form of nitrogen (N) that can then be incorporated into organic components, e.g. protein and nucleic acids, of the bacteria and associated plants. In this way, unreactive N_2 enters the biologically active part of the global N cycle.

After photosynthesis, N_2 fixation is probably the most important biologically mediated process on earth. There is a wide diversity of N_2-fixing organisms, called diazotrophs. Some can fix N_2 in the free-living state, while others fix N_2 in association with plants (Table 1). Relationships between diazotrophs and plants range from the rather loose associations of heterotrophic bacteria around plant roots, to endophytic bacteria residing in the vascular tissues of tropical grasses, to highly evolved, complex symbioses involving morphological differentiation of both microbe and plant in specialised root structures.

Farmers in Vietnam inspecting N_2-fixing groundnut crops. In agricultural systems across the globe, crops such as these can fix 3–10 kg shoot N/ha per day, with potential annual inputs of several hundred kg N/ha.

Table 1. Examples of the range of bacterial genera that fix N_2 in agricultural systems (modified from Ledgard and Giller 1995)

Status of organism	N_2-fixing organism	Plant host
	Heterotrophs	
Free-living		
Anaerobic	*Clostridium, Methanosarcina*	
Microaerophilic	*Frankia, Azospirillum, Bradyrhizobium*	
Aerobic	*Azotobacter, Derxia*	
Root-associated		
Microaerophilic	*Azospirillum, Paenibacillus*	
Endophytic	*Herbaspirillum, Acetobacter*	Sugarcane, tropical grasses
Symbiotic	*Frankia*	*Alnus, Myrica, Casuarina*
	Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium	Many legumes, *Parasponia*
	Ochrobactrum	*Acacia* sp.
	Blastobacter	*Aeschynomene* sp.
	Methylobacterium	*Crotalaria* sp.
	Burkholderia, Ralstonia, Cupriavidus?	*Mimosa* sp.
	Allorhizobium, Devosia	*Neptunia* sp.
	Herbaspirillum	*Phaseolus* sp.
	Azorhizobium	*Sesbania rostrata*
	Phyllobacterium	*Trifolium* sp.
	Autotrophs	
Free-living		
Microaerophilic	*Rhodospirillum, Bradyrhizobium*	
Aerobic	*Cyanobacteria*	
Symbiotic	*Anabaena azollae*	*Azolla* sp.
	Cyanobacteria, Bradyrhizobium	Fungi (lichens), cycads *Gunnera*
		Aeschynomene sp.

Symbioses between N_2-fixing bacteria and eukaryotes include the cyanobacteria (often referred to as blue–green algae) with fungi in lichens, cycads and *Gunnera*; actinomycetes (generally placed in the genus *Frankia*) with a range of angiosperms such as *Alnus* and *Casuarina*; and rhizobia with legumes (Figure 1). Examples of the last include soybean, pea, lentil and common bean.

The ability to measure (quantify) amounts of N_2 fixed in agricultural and natural ecosystems is critical to our goals of meeting world food-protein demand, of protecting and enhancing the earth's natural capital, and of managing the N cycle for environmental protection. This manual focuses directly on plant-associated biological N_2 fixation, both symbiotic and associative. Practical advice on the measurement of free-living N_2 fixation can be found in Bergersen (1980b) and Warembourg (1993).

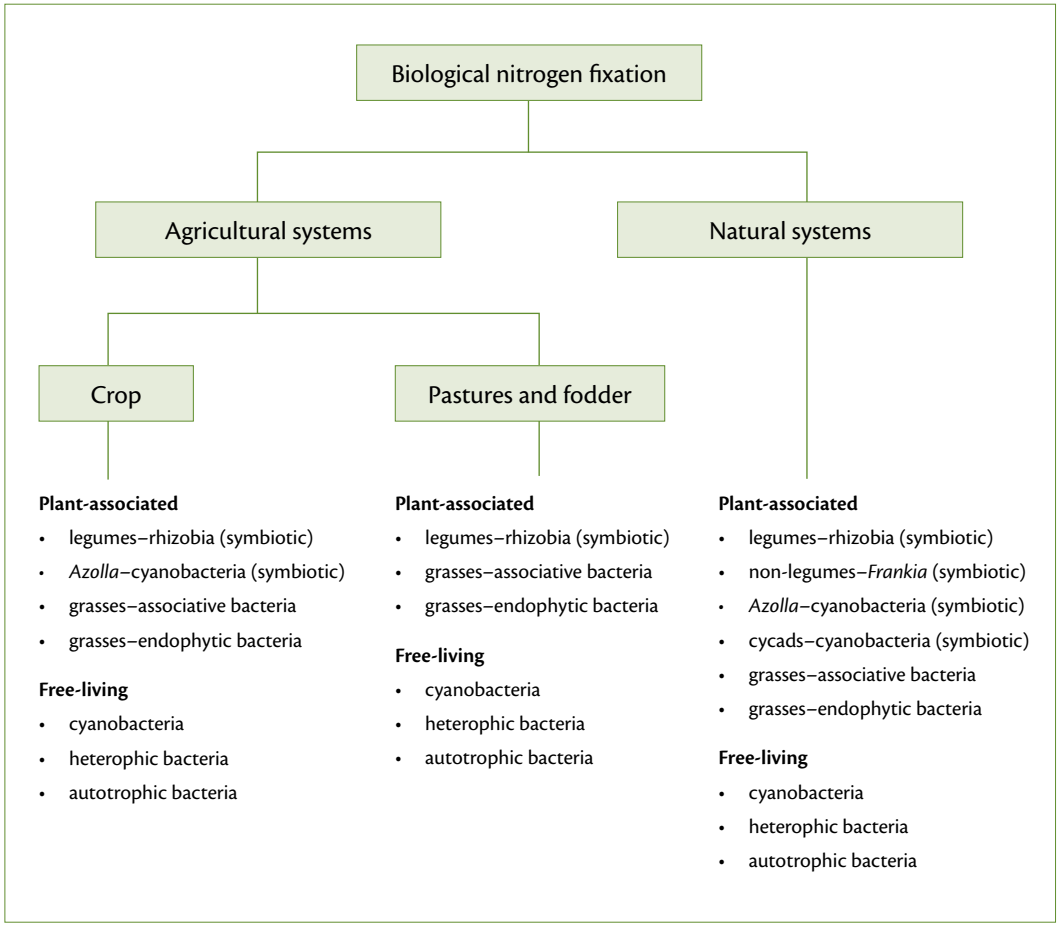

Figure 1. The biological N_2-fixing agents in agricultural and natural systems

What do we mean by these terms? With **symbiotic N_2 fixation,** significant net transfer of photosynthetically fixed plant carbon (C) to the N_2-fixing bacteria occurs, concurrently with net transfer of biologically fixed N directly from the bacteria to the host plant. With legumes, this all happens in highly specialised structures called nodules, which contain the bacteria and are formed on the roots or stems of the plants.

The area of soil immediately surrounding the roots of plants (i.e. rhizosphere) provides an ideal environment for the growth of a range of micro-organisms. Within this soil environment, many N_2-fixing bacteria may proliferate due to energy, nutrients and other compounds released by, or derived from, the plant roots. Nitrogen fixation in this situation is referred to as **associative N_2 fixation**. The term is also used to describe N_2 fixation by bacteria that may also live in association with plant leaves, stems or leaf sheaths.

Asymbiotic N_2 fixation is N_2 fixation by bacteria that does not involve direct, controlled exchange of N and C between bacteria and plants.

Free-living N_2 fixation is N_2 fixation by bacteria living freely in soils or water and not associated with any other plants or animals. Free-living N_2 fixation is a form of asymbiotic N_2 fixation.

Biological N_2 fixation in agriculture

1.1

With 3.4 billion ha of pastures, 1.4 billion ha of arable land and 136 million ha of permanent crops, agriculture covers nearly 40% of the world's land surface. Nitrogen fixation can contribute directly to agricultural production by providing the N of the leafy vegetative parts, pods, seeds and tubers of plants used as feed for livestock or harvested for human consumption. Nitrogen fixation is also a major source of N for agricultural soils via the N-rich residues that remain following plant harvest or grazing.

Many more measurements of N_2 fixation have been made in agricultural environments than in natural ecosystems, and these are summarised in Table 2. Further detailed information on specific N_2-fixing associations can be found in reviews by Ledgard and Giller (1995), Peoples et al. (1995b), Unkovich and Pate (2000), and Giller (2001).

Heterotrophic free-living N_2 fixers utilising plant residues such as straw and leaf litter appear to contribute only small amounts of N to dryland agriculture (mostly <5 kg N/ha per year). Nitrogen fixation by cyanobacteria

and photosynthetic bacteria inhabiting the floodwater and soil surface of rice fields might contribute as much as 30 kg N/ha (Firth et al. 1973). There are few conclusive data to indicate that agronomically significant amounts of N are fixed by bacteria associated with non-legumes in temperate agriculture, but studies have demonstrated measurable inputs of fixed N with tropical grasses such as sugarcane, in the order of 10–65 kg N/ha per year (Boddey et al. 1995b). Symbiotic relationships between legumes and rhizobia are responsible for the largest contributions of fixed N to farming systems (Table 2), and the identification of new niches for the production of legumes is arguably the most important means of increasing N_2 fixation in agriculture (Giller 2001).

Establishment of effective N_2-fixing symbioses between legumes and their N_2-fixing bacteria (rhizobia) is dependent upon many environmental factors, and can be greatly influenced by farm management practices (Peoples et al. 1995a). As a consequence, N_2 fixation cannot be assumed to occur as a matter of course, a fact reflected in the large range of values presented in Table 2. One of the most common factors limiting a legume's ability to fix N_2 is the absence of sufficient numbers of effective rhizobia in the soil. Fortunately, strains of rhizobia can be introduced into soil relatively simply by inoculation and, in many countries, this has been practised successfully on a commercial scale for

Table 2. Amounts of N_2 fixed by different agents in agricultural systems (modified from Ledgard and Giller 1995). Not all agricultural systems have all of these components.

N_2-fixing agent	Range measured (kg N/ha per crop or year)	Range commonly observed (kg N/ha per crop or year)
Free-living		
Heterotrophic bacteria	1–39	<5
Cyanobacteria	10–80	10–30
Associative		
Tropical grasses	0–45	10–20
Crops	0–240	5–65
Symbiotic		
Azolla	10–150	10–50
Green manure legumes	5–325	50–150
Pasture/forage legumes	1–680	50–250
Crop legumes	0–450	30–150
Trees/shrubs	5–470	100–200

Cultivation of land in Myanmar in preparation for legume sowing. Myanmar is one of the world's major pulse (i.e. food legume) producing countries, with the grain either consumed domestically or exported.

many years. Legumes that most often need inoculation are those that require specific rhizobial strains to form an effective symbiosis. They may need to be inoculated only when they are grown in regions outside their centres of diversity, or where they have not traditionally been grown or have not been grown for a number of years (Brockwell et al. 1995).

Rates of N_2 fixation tend to be highest when plant-available mineral N in the soil is limiting but water and other nutrients are plentiful. There is an effective feedback mechanism on N_2 fixation whereby rates progressively decline with increasing availability of mineral N. Even so, high rates of N_2 fixation are commonly achieved because most cropping systems are dominated by cereals that utilise large quantities of soil mineral N. Thus, mineral N in farmers' fields is often relatively low, but not always. The wide variability in measured N_2 fixation by legumes in Table 2 makes it difficult to generalise how much N is fixed by particular legume species in different regions of the world. Collectively, the data suggest maximum rates of N_2 fixation of 3–10 kg shoot N/ha per day (Unkovich and Pate 2000), and potential annual inputs of several hundred kg N/ha (Table 2). Provided other factors are not limiting, the amount of N_2 fixed by legumes is primarily influenced by plant growth and dry matter production.

As a general rule, legumes fix 15–25 kg shoot N for every tonne (t) of shoot dry matter accumulated (Figure 2), with an average of about 20 kg shoot N per t shoot dry matter. Similar relationships seem to hold for both crop and pasture legumes in both experimental plots and farmers' fields. Common bean is the major exception to this generalisation, fixing only about 10 kg shoot N per t dry matter. Since below-ground N represents 25–50% of total plant N (see section 3.1.1), the general rule becomes 30 kg total N fixed per t of shoot dry matter for most legumes, and 15 kg total N fixed per t of shoot dry matter for common bean. These values are gross generalisations and tell us nothing about specific effects of crop genotype, farming practice or environment on legume N_2 fixation. Such information can come only from the measurement of N_2 fixation using appropriate methodology.

Global inputs of biologically fixed N into agroecosystems by the range of agents in Table 2 have been calculated to be in the order of 50 million t of N annually, which is about half the annual application of mineral fertiliser N to agricultural lands (e.g. Smil 1999; Herridge et al. 2008).

1.2 Biological N_2 fixation in natural ecosystems

In natural ecosystems, N_2 fixation is most important in early successional phases following disturbance such as fire, but may become less important as soil N fertility improves. A resurgence of the importance of N_2 fixation at later stages of succession can be supported when the ecosystem becomes more abundant in C and limited in N.

There are specific difficulties in attempting to quantify inputs from N_2 fixation in natural ecosystems. Apart from limitations in N_2 fixation methodologies (see later chapters), estimates of N_2 fixation are constrained by the wide variability in the distribution of specific N_2-fixing agents and their N_2-fixing intensities in response to gradients of soil water and nutrient availability, and/or competition with other species. Further complications arise due to the transience of N_2 fixation. For example, lichens and cyanobacterial mats may fix N_2 for only a short period immediately after rain. In other environments, there may be large effects of wet/dry or cold/warm seasons resulting in seasonality of nodulation, dry matter and N accumulation (Boddey et al. 2000). However, low rates and seasonal activities of N_2 fixation across large areas of forest, grassland, savanna, shrubland and desert can still result in substantial inputs of N at regional or global scales. Such inputs may be in the order of 100 million t annually (Cleveland et al. 1999).

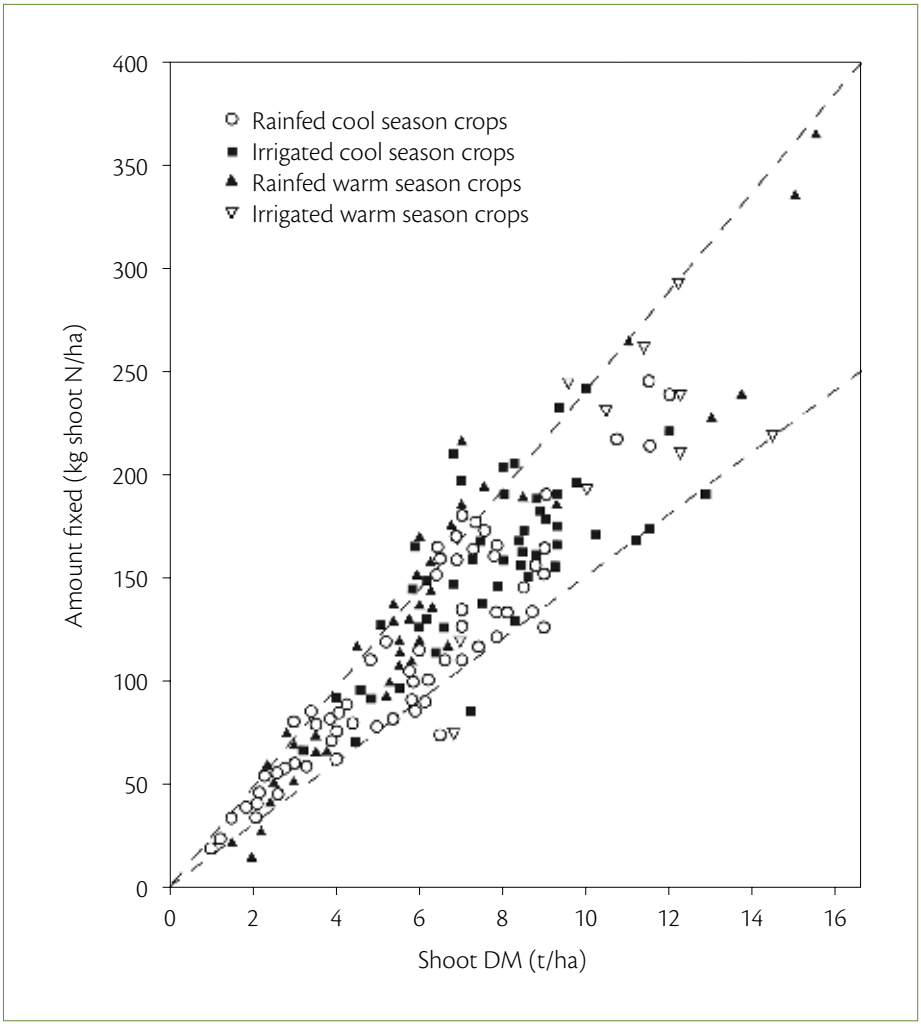

Figure 2. Relationship between amounts of shoot N fixed (kg N/ha) and shoot dry matter (DM, tonne/ha) for rainfed and irrigated warm season (black gram, green gram, cowpea, soybean) and cool season legumes (chickpea, faba bean, pea, lentil, grasspea, lupin) growing in different geographic regions. The upper dashed line indicates the 25 kg N fixed per tonne DM relationship, while the lower dashed line shows 15 kg N fixed per tonne DM.

Bagged winged-bean tubers in Myanmar. Winged bean is one of a number of food and oilseed legumes grown in Myanmar, but is the only one to produce an edible tuber.

1.3 The need for measurement

Much has been written on the desirability of harnessing biological N_2 fixation to provide a renewable source of N for human and animal protein, to develop more sustainable farming systems and to maintain natural ecosystems. It is not possible to undertake experimentation to identify and understand the principal factors regulating N_2 fixation, nor can it be managed for the benefit of the environment or agricultural productivity, unless it can be reliably measured. This handbook provides practical advice on the application of current methodologies for measuring plant-associated N_2 fixation in different situations. The handbook highlights potential sources of error and pitfalls in the use of the various measurement techniques, and provides step-by-step guidance on the application of the techniques and in the interpretation of the resulting data. For most of the techniques, three things need to be measured to quantify plant-associated N_2 fixation: plant dry matter, the concentration of N in the dry matter (%N) and the percentage of total plant N derived from N_2 fixation (%Ndfa).

2

Why, when and how to measure nitrogen fixation

There is no single 'correct' way to measure N_2 fixation, and since all current methodologies have limitations, measuring the exact amount of N_2 fixed continues to be a challenge. Ideally, several different methods should be used simultaneously, particularly if they are complementary, i.e. not relying on the same underlying assumptions. This book will provide you with critical information to help you choose and implement an appropriate methodology and to interpret the resultant data correctly. However, before selecting a particular method, a number of questions should be answered.

2.1 Why do you want to measure N_2 fixation?

While it is tempting to aim for the most sophisticated methodology, this approach may not necessarily provide results in the most cost- and time-efficient way. As in all research, it is important to formulate clear objectives and a hypothesis that will guide experimental design and choice of measurement method. Reasons for wanting to quantify N_2 fixation include (a) to determine if the plants in question have any capability to fix N_2, (b) to determine if management practices affect N_2 fixation, (c) to determine the amount of N_2 fixed by a field crop or pasture and (d) to determine the importance of N_2 fixation to the functioning of an ecosystem. Think about how you will use the resulting data once you have obtained them. Do you really need quantitative (kg N/ha) data to answer your hypothesis?

If the objective is simply to assess if a particular legume is fixing N_2, then determining the presence of root nodules (including number and size) and their colour (pink, white, green or pale yellow) will provide practical insight. In some situations even the colour (dark green) of newly expanded leaves of the plant can provide some indication. The capability of plants to fix N_2 in association with particular bacteria can be tested, in principle, by simply growing them in a pot in N-free media; however, this does not tell you if, and how much, the plant might fix in the relevant field setting or ecosystem.

Furthermore, to simply determine if the N_2 fixation system under study is effective, comparisons of plant (crop) growth in the presence and absence of fertiliser N under conditions of very low soil mineral N will give a good indication (but no certainty) of the contribution of N_2 fixation. If the main objective is to compare relative differences between genotypes, species or treatments, rather than to estimate absolute amounts of N_2 fixed, techniques that enable ranking of different crop varieties or treatments are available.

2.1.1 What kind of accuracy or precision do you need?

If you want to quantify N_2 fixation, you need also to determine the accuracy required. If other measured parameters are determined at a very low accuracy, it may not be justifiable to invest lots of resources to obtain highly accurate N_2 fixation estimates. For example, in tree or grassland systems, accurate estimation of total biomass production is often the limiting factor. Continuous leaf fall, sampling and subsampling difficulties with large trees, the presence of grazing animals, and high spatial variability create large uncertainties in total N determinations. Additionally, many studies have focused only on determining N_2 fixed in shoots of plants, ignoring the considerable amounts of fixed N stored in roots and ceded to the soil as root exudates and decayed root matter. Recent studies suggest that below-ground N of legumes has been substantially underestimated (e.g. McNeill et al. 1997; Khan et al. 2002; McNeill and Fillery 2008). Parameters such as these often represent the major source of error in quantifying areal N_2 fixation and so the focus should not be solely on the N_2 fixation process itself.

Assessment of legume nodulation provides additional information to support N_2-fixation data. Plants are usually dug up at random from a field or treatment plot and examined for, variously, nodule mass, number, colour and distribution.

At this stage, it may be useful to define accuracy and precision. **Accuracy** is the closeness of a measured or estimated value to the actual or true value. **Precision** is the degree to which repeated measurements provide the same or similar value, i.e. the reproducibility of an estimate.

It is possible to be precise but not accurate, or accurate but not precise (see Figure 3). In an ideal world we would like our measures of N_2 fixation to be both accurate and precise. As it is easier to be precise than accurate, and because precision is usually much easier to determine than accuracy, there is a tendency for people to focus on precision rather than accuracy. For a given investigation you will need to know both how accurate and how precise you need to be.

The only times we can know the true value of N_2 fixation is under defined conditions when the organism in question either fixes no N_2 or is totally dependent upon N_2 fixation for growth. Thus, plants grown with high mineral N supply and without an association with N_2-fixing bacteria must take up all N from the soil and have nil (0%) contribution from N_2 fixation. On the other hand, plants growing with their competent N_2-fixing bacteria in the absence of any other plant-available sources of N will derive all N (100%) from N_2 fixation, except for a very small amount in the sown seed.

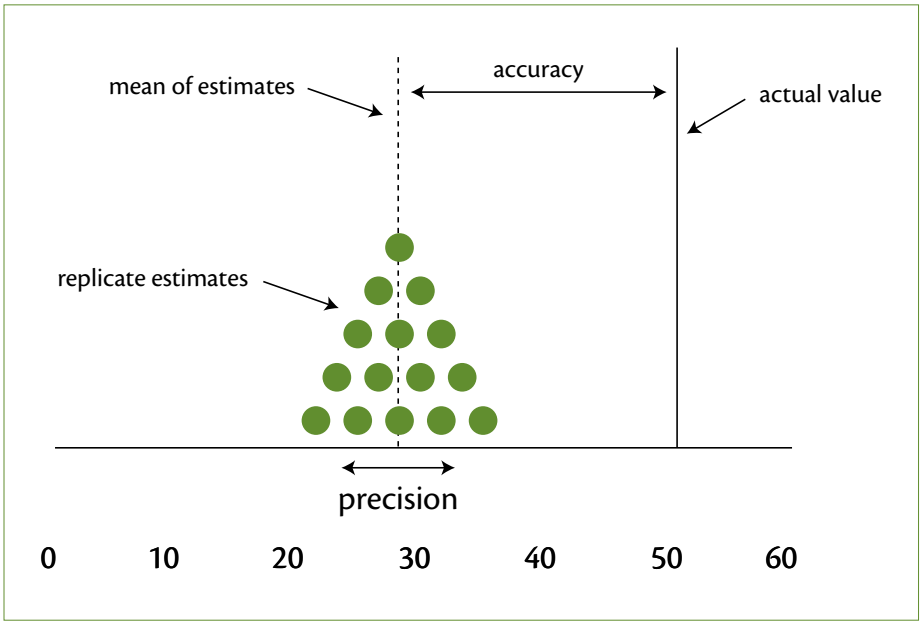

Figure 3. The difference between precision and accuracy

2.1.2 What is the time frame of study?

Methodologies for quantifying N_2 fixation may be instantaneous or time-integrated. Time-integrated techniques are preferred for assessing the total amount of N_2 fixed for a specified period (e.g. months, crop growth cycle). Other techniques provide a measure of activity only at the time of sampling, and while these may have a higher sensitivity for assessing diurnal (daily) variation, they may require more frequent measurements and assumptions or calibrations to achieve a long-term assessment of N_2 fixation.

Special care has to be taken when planning time sequences and long-term field measurements. You need to make sure, in particular, that there is minimum plot disturbance, and that sufficient experimental area is available for adequate replication and for more than one harvest of sufficient sample size to provide a representative determination of plant biomass and N content. Several methods utilise non N_2-fixing 'reference plants' for comparison with the N_2-fixing species, and both species must be grown under identical soil conditions.

2.1.3 Is it only N_2 fixation, or is it the benefit of fixed N in the system that you want to assess?

Nitrogen fixation should not be viewed in isolation. It is important to recognise the value of fixed N to a whole ecosystem. For example, to assess N_2 fixation and the residual benefit of fixed N to subsequent non N_2-fixing rotation crops, it may be necessary to employ techniques that enable the uptake of N derived from the N_2-fixing species by following crops to be assessed (e.g. Harris and Hesterman 1990).

2.1.4 A matter of scale: from laboratory to landscape

The choice of scale depends on the research question. Laboratory and glasshouse trials have value in generating scientific knowledge about specific mechanisms or factors regulating the N_2 fixation process through treatments imposed under uniform and controllable conditions that are not achievable in the field. However, they cannot provide estimates of amounts of N_2 fixed that can be extrapolated to the field. Thus, any quantification of agronomically relevant N_2 fixation must be undertaken in the field. Additionally, any practical testing of a management treatment should be done in the field. Nevertheless, glasshouse experiments are highly valuable for developing an understanding of the sensitivity of N_2-fixing systems to a range of environmental and nutritional variables, and for becoming familiar with N_2 fixation measurement methodologies.

Spatial variability within the landscape, district, country or continent may be considerable, so that scaling up localised, highly precise estimates of N_2 fixation from one field may not be accurate at a larger scale. It might therefore be more useful to try to account for this variability rather than investing the majority of the resources to obtain a single, accurate value for N_2 fixation. In some situations, highly uniform soils, cropping and management systems may occur, making scaling up less problematic.

2.1.5 What resources do you have available?

Apart from knowledge, the availability of time, space and financial resources can also affect the choice of technique for measuring N_2 fixation. The various ^{15}N techniques, well suited to measure N_2 fixation of field- and glasshouse-grown plants (described in detail in Chapter 7), are relatively expensive. Even though the costs of isotope-N determination have been reduced through the development of automated preparation and analysis systems, this approach may remain beyond the financial resources of many researchers.

It is not always necessary to have the particular analytical equipment in the researcher's laboratory. Mass spectrometers for ^{15}N isotope analysis, for example, are costly and have very high skills and maintenance requirements. For such cases, it would be wiser to establish collaborative activities with specialised groups that have access to suitable equipment and expertise. The International Atomic Energy Agency (IAEA, Vienna, Austria) offers services and training facilities for many developing countries (including specific country contracts) and also has special programs in agriculture (see <http://www.iaea.org/OurWork/ST/index.html>). Alternatively, less expensive methodologies may provide entirely satisfactory N_2 fixation data that are completely suitable for your objectives.

2.2 Introduction to techniques for measuring N_2 fixation

The different approaches that have been used to quantify N_2 fixation by crop, pasture and woody legumes and non-nodulating plants have been extensively reviewed (e.g. Chalk 1985; Shearer and Kohl 1986; Boddey 1987; Chalk and Ladha 1999; Boddey et al. 2000; Unkovich and Pate 2000; Giller 2001; Peoples et al. 2002). We do not intend in this chapter to provide an exhaustive critique of all the various applications of those methodologies as per those reviews, but rather to summarise the principles, assumptions, advantages and disadvantages for each of them. It will become clear that the methodologies we

describe fall into three broad approaches. The first estimates N_2 fixation as the net increase in total N of a plant–soil system (N balance method). The second aims to separate plant N into the fraction taken up from the soil and the fraction derived from the N_2 fixation (N difference, ^{15}N natural abundance, ^{15}N isotope dilution and ureide methods). The third measures the activity of nitrogenase, the enzyme responsible for N_2 fixation (acetylene reduction and hydrogen evolution methods).

2.2.1 Nitrogen balance

Principles behind the method

- If all possible external inputs, except N_2 fixation, and outflows of N can be accounted for and incremental changes in soil N quantified, a net positive N balance in the system under study may be attributed to N_2 fixation.

Assumptions

- Potential inputs of N that are difficult to measure, such as wet and dry deposition associated with rainfall and dust, extraction of N from deep soil horizons or from the watertable, and NH_3 absorption by leaves, are small and insignificant compared with inputs via N_2 fixation.

Advantages

- In theory the method is simple. Any inputs of N in fertilisers or organic sources are relatively easily quantified, as are amounts of N removed from the site in plant and animal products.

Potential limitations

- Losses of N through NH_3 volatilisation, denitrification, leaching, run-off and erosion can be substantial, and difficult to measure. Failure to include estimates of losses through these processes will result in an underestimation of N_2 fixation inputs.
- In pasture systems, spatial variability of both N inputs and N losses associated with excreta from grazing animals can be difficult to quantify.
- Since the amount of N in soil organic matter in a plant's rooting zone can be as much as 12,000 kg N/ha in rich, clay soils, even small errors arising from soil sampling or analysis, or in measures of bulk density, result in large discrepancies when estimating total soil N. Consequently, many soil cores are needed to accurately determine changes in total soil N (Chalk 1998).

Conclusions

- The method relies on many independent and unrelated measurements, each made with a differing degree of accuracy, so the confidence in the final estimate of N_2 fixation can be low.
- Because of the very large size of the soil-N pool, substantial inputs of fixed N are necessary to detect increased soil N. Consequently, use of N balance tends to be restricted to long-term experiments with land use dominated by the N_2-fixing association of interest. Thus, it may be more suited to perennial species in agroforestry and/or pasture systems than to annual crops.

2.2.2 Nitrogen difference

Principles behind the method

- N difference compares total N of the N_2-fixing species with that of a neighbouring non N_2-fixing species, with the difference between the two measures assumed to be due to N_2 fixation.

Assumptions

- The N accumulated by the non N_2-fixing control is derived only from soil N, and its N content represents the amount of soil mineral N available for plant growth.
- The N_2-fixing plants use the same amount of soil mineral N as the non N_2-fixing control.

Advantages

- It is a simple, low-cost method that can be applied when facilities for only dry matter determinations and total N analyses are available.

Potential limitations

- The method requires a non N_2-fixing control to be included in the experimental design.
- Differences between N_2-fixing and non N_2-fixing plants in root morphology and rooting depth can result in different capacities to use soil N (Chalk 1998).

- There may be errors in accurately quantifying total N accumulated by the N_2-fixing plants and control plants.

Conclusions

- The technique is likely to be most reliable under conditions of low plant-available N and where there are large differences in N yield between the N_2-fixing plants and non N_2-fixing control.

2.2.3 Ureides

Principles behind the method

- In many tropical and subtropical legumes, the N-solute composition in xylem sap and stem segments changes from one dominated by the ureides allantoin and allantoic acid in N_2-fixing plants, to one dominated by nitrate and amino acids in plants utilising soil N.

Farmers and scientists inspecting nodulation of field-grown groundnut in Vietnam

- The substantial differences in the principal forms of N transported in the xylem between symbiotic and non-symbiotic plants allow incoming fixed N and soil N to be distinguished.

Assumptions

- The N-solute composition of xylem sap and stem segments reflects current N assimilation by the legume.
- The abundance of ureides relative to other N solutes can be used as an indirect measure of the percentage of legume N derived from the atmosphere, i.e. %Ndfa.

Advantages

- The procedures used to sample xylem sap and stem segments of field-grown legumes are not technically demanding.
- Ureides, nitrate and amino acids in xylem sap and stem segments can be easily and rapidly analysed using simple colorimetric assays in a test tube. There is no need for expensive or sophisticated equipment.
- No special experimental design is required, so the method can be used for on-farm measurement of N_2 fixation.
- Many samples can be collected and analysed in a single day.
- Plants can be sampled non-destructively from the top of the stem, or from lateral branches, with the base left intact to continue to grow (Herridge et al. 1988).
- The method provides an estimate of %Ndfa, which can be used to assess the N_2 fixation response of the legume to experimental treatments, without the need to measure legume total N yield.
- %Ndfa, when combined with a measure of legume total N, can be used to calculate amounts of N fixed during a period of growth (e.g. Herridge et al. 1990).

Potential limitations

- Its use is restricted to ureide-exporting legume species (e.g. *Glycine, Vigna, Phaseolus, Macroptilium*).
- The method provides an indirect measure of %Ndfa, necessitating calibration against another method, e.g. ^{15}N isotope dilution (Herridge and Peoples 1990; Alves et al. 2000a).

- Different calibrations may be needed during vegetative and reproductive stages of development.
- The technique provides only a 'point-in-time' estimate of the legume's symbiotic dependence at, or shortly before, the time of sap sampling, so repeated sampling of xylem composition and plant N may be required during a growing season.
- Volumes of xylem sap and solute composition vary diurnally, and can be affected by delays in sap collection (Herridge et al. 1988).

Conclusions

- It is a versatile and useful technique that can be applied in glasshouse and field experiments, or used in farmers' fields, to assess N_2 fixation by ureide-exporting tropical and subtropical legumes.
- It can provide estimates of N_2 fixation (%Ndfa and total N_2 fixed) for field-grown legumes similar to those from more sophisticated techniques.

2.2.4 ^{15}N isotope techniques

There are two main stable isotopes of N—^{14}N and ^{15}N—with ^{14}N naturally more abundant than ^{15}N. In absolute terms, the isotopic abundance of ^{15}N is usually expressed as a percentage of the total N (atom% ^{15}N). The isotope ^{15}N occurs in atmospheric N_2 at a constant abundance of 0.3663 atom% (Mariotti 1983).

Principles behind all ^{15}N-based methodologies

- If the ^{15}N concentration in atmospheric N_2 differs significantly from that of plant-available soil N, and these values are known, it is possible to calculate N_2 fixation on the basis of ^{15}N analyses of the putative N_2-fixing plant and a non-N_2 fixing plant.

Assumptions common to all ^{15}N-based methodologies

- Either there is no discrimination or identical discrimination between ^{14}N and ^{15}N during the uptake and metabolism of plant-available soil N and N_2, or the discrimination can be accounted for.
- Any variability in the ^{15}N composition of the air and soil is small compared to the difference between them.

Potential limitations of all ^{15}N-based methodologies

- These include the high cost of a mass spectrometer, the technical skills required to accurately determine the isotopic composition of plant (and sometimes soil) samples and the expense of analyses.

2.2.5 Using $^{15}N_2$

Principles behind the method

- The roots of intact or detached plants are placed in a chamber with an atmosphere enriched in $^{15}N_2$. The amount of ^{15}N in the plant at the end of the incubation period is a direct measure of the rate of N_2 fixation.

Assumptions

- Exposure to $^{15}N_2$ is sufficiently long to allow adequate equilibration and gaseous exchange with nodules, and for measurable amounts of ^{15}N to be fixed.
- In the case of legumes the assayed nodules are representative of all nodules on the legume root system.
- Rates of N_2 fixation under assay conditions are related to rates of N_2 fixation achieved in situ.

Advantages

- It is a direct measure of N_2 fixation.
- The uptake of $^{15}N_2$ by an organism is the only technique (apart from growing plants in N-free medium) to unequivocally prove active N_2 fixation.

Potential limitations

- These include the high cost of $^{15}N_2$ and the instruments used to quantify ^{15}N (mass spectrometer or emission spectrometer).
- There may be difficulties in keeping incubation systems completely sealed while maintaining adequate environmental conditions inside the chamber, e.g. temperature, oxygen level.

Conclusions

- The $^{15}N_2$ method is generally used for short-term laboratory incubations and is much less suitable for field use.

2.2.6 Where the ^{15}N composition of soil N is greater than atmospheric N_2

With appropriate analytical procedures and a suitably precise mass spectrometer, it is possible to measure the slight elevation in the ^{15}N abundance of plant-available soil N above atmospheric N_2 (commonly 0.001–0.007 atom% ^{15}N) that naturally occurs in many soils (Hogberg 1997). This small difference can be utilised to distinguish between legume N originating from the soil and from the air (the ^{15}N natural abundance method of Shearer and Kohl (1986)). It is also possible to obtain or generate different sources of N, e.g. urea, ammonium sulfate, plant residues, that are artificially enriched in ^{15}N (5–99 atom% ^{15}N) and can be added to the soil to expand the difference in the ^{15}N compositions of soil N and atmospheric N_2 (the ^{15}N isotope dilution method of Chalk (1985)).

Principles behind the methods

- Where a N_2-fixing plant–bacteria association is growing in a medium free of mineral N and the plant is completely reliant upon N_2 fixation for growth, the isotopic composition of the plant will be similar to that of atmospheric N_2.
- Conversely, the ^{15}N enrichment of a non N_2-fixing plant growing in soil should closely resemble the ^{15}N enrichment of the assimilated soil mineral N.
- In the case of a plant assimilating both atmospheric N_2 and soil mineral N, the ^{15}N of the plant will gradually decline as atmospheric N_2 of lower ^{15}N abundance is progressively fixed. The term 'isotope dilution' is commonly used to describe this process since the lower ^{15}N abundance of the atmospheric N_2 'dilutes' the higher ^{15}N concentration of soil-derived N.
- The ^{15}N content of the plant will be somewhere between the ^{15}N signature of the plant-available soil N (%Ndfa of zero) and a value close to 0.3663 atom% ^{15}N (%Ndfa of 100%).

Assumptions

- Non N_2-fixing reference plants growing at the same location as the putative fixing species can be used to provide a measure of the ^{15}N signature of plant-available soil N.
- Reference plants are totally reliant upon soil mineral N for growth and, during the growing season, utilise the same soil pools of N of identical ^{15}N concentrations as the N_2-fixing species.

- The application of N (as ^{15}N) to enrich soil N pools does not alter the plant's ability to fix N_2 (Chalk 1985).

Advantages

- It provides a time-integrated estimate of %Ndfa for a period of growth.
- %Ndfa values can provide useful information about a plant's N_2-fixing performance and the N_2 fixation response to experimental treatments without the need to measure plant N yield.
- Amounts of N_2 fixed can be estimated from a single analysis of the ^{15}N contents of the non N_2-fixing reference and N_2-fixing species, and a measure of biomass and N content of the N_2-fixing species at the time of peak biomass (Peoples et al. 2002). Amounts of N_2 fixed can be calculated for shorter periods (days and weeks), limited only by the ability to accurately determine changes in plant N accumulation.

Well-nodulated soybean plants sampled from a field trial in Australia to compare different inoculant formulations. These plants were inoculated with granular inoculants.

- The ^{15}N natural abundance variation of the method allows N_2 fixation to be monitored in almost any location where both N_2-fixing and non N_2-fixing plants are present, as nothing needs to be added before measurements can be undertaken. Thus, the potential exists to measure N_2 fixation in farmers' fields and in experiments not originally designed with N_2 fixation in mind.

Potential limitations

- The efficacy of the non N_2-fixing reference plant to provide an accurate measure of the isotopic composition of plant-available soil N is compromised by differences in the rooting depths and patterns of N uptake of the reference and N_2-fixing plants.
- The ^{15}N composition of plant-available soil N can change with soil depth and time during the growing season, particularly where ^{15}N-enriched materials have been applied to soil (Chalk 1985).
- ^{15}N-enriched materials (when used) have a high cost.
- The ^{15}N abundance of plant-available soil N can be either too low and/or too variable for the methodology to be applied.
- Isotopic discrimination during N_2 fixation is generally assumed to be zero in ^{15}N enrichment studies, but needs to be considered when using ^{15}N natural abundance.

Conclusions

- Nitrogen isotope techniques are arguably the benchmarks for quantifying plant-associated N_2 fixation against which other techniques are compared and calibrated.
- Use of centralised laboratories for analysing N isotopes through payment for analysis or collaborative networks provides a means for resource-poor researchers to use the techniques.

2.2.7 Acetylene reduction

Principles behind the method

- The enzyme nitrogenase, which reduces N_2 to NH_3, is also capable of reducing acetylene (C_2H_2) to ethylene (C_2H_4). Thus, C_2H_2 can be used as an alternative substrate to N_2 (Hardy et al. 1968, 1973).

- Root systems are placed in an airtight vessel, or contained within a cuvette that can be connected to a flowing gas-stream, and exposed to a C_2H_2-enriched atmosphere (usually 10% C_2H_2 in air). The rate of C_2H_4 accumulation in gas samples collected over a set interval is measured using a gas chromatograph.

Assumptions

- Substituting C_2H_2 for N_2 does not affect nitrogenase activity.
- In the case of legumes, the assayed nodules are representative of all nodules on the root system.
- Measures of nitrogenase activity obtained under assay conditions are related to rates of N_2 fixation in situ.

Advantages

- The C_2H_2 reduction assay is a very sensitive diagnostic tool for detecting nitrogenase activity.
- It is simple, rapid and relatively inexpensive, and many measurements can be undertaken daily (Hardy et al. 1973).

Potential limitations

- Measurements reflect nitrogenase activity for only the duration of the assay.
- There are marked diurnal and seasonal changes in enzyme activity, and many individual measurements are required to provide an estimate of N_2 fixation for an entire growing season.
- While there is theoretical and empirical support for a conversion ratio of C_2H_2 reduced to N_2 fixed of 3–4 to 1 (Hardy et al. 1973), C_2H_2 reduction assays should ideally be calibrated using $^{15}N_2$. Relationships between C_2H_2 reduction and N_2 fixation may change during plant growth, and can differ across legume–rhizobial strain combinations (Witty and Minchin 1988).
- It is virtually impossible to quantitatively recover whole root systems from field-grown legumes (and non-legumes), resulting sometimes in gross underestimations of total nitrogenase activity (Vikman and Vessey 1992).
- C_2H_2 can induce a decline in nitrogenase activity in some legume species, and plant handling, nodule detachment and excision of nodulated roots have all been demonstrated to interfere with nodule gas exchange and to lower nitrogenase activity (Minchin et al. 1983a, 1986).

- Although measurements on undisturbed plants are likely to be more reliable than the traditional closed-incubation system, the flow-through C_2H_2 reduction chambers that have been developed for use in the field are cumbersome. This and other issues have prevented their widespread use (Giller 2001).
- C_2H_2 is explosive and poses a possible hazard to the experimenter.

Conclusions

- While the C_2H_2 reduction assay may be quantitative for pot studies under some conditions, and can provide a useful tool for detecting N_2-fixing activity in both leguminous and non-leguminous plants, the calculated rates of N_2 fixation cannot be extrapolated beyond the incubation vessel. As a consequence, the method is unsuitable for measuring N_2 fixation at field scales.

2.2.8 Hydrogen evolution

Principles behind the method

- Hydrogen gas (H_2) is an obligate by-product of N_2 fixation in legume nodules, and its production may account for about 35% of the energy consumed in nitrogenase activity (Hunt and Layzell 1993).
- An indirect measure of nitrogenase activity can thus be obtained by placing a nodulated root system in a cuvette and quantifying the increase in H_2 concentration in a gas stream using a flow-through H_2 sensor or gas chromatograph.

Assumptions

- The assayed nodules are representative of all nodules on the legume root system.
- Measures of nitrogenase activity obtained under assay conditions can be related to rates of N_2 fixation achieved by the legume in situ.

Advantages

- Measuring H_2 evolution is a simple approach that has been used since the 1960s as an assay of nitrogenase activity (Hardy et al. 1973).
- Flow-through H_2 analysers are extremely sensitive and cheap, and the procedure is less labour intensive than assaying C_2H_2 reduction.

- Measurements of H_2 evolution in air do not inhibit nitrogenase activity, so repeated measurements can be performed on the same plant material (Hunt and Layzell 1993).
- There does not appear to be the same marked diurnal change in H_2 evolution as is observed with C_2H_2 reduction assays.

Potential limitations

- H_2 evolution in air provides a measure of apparent nitrogenase activity because H_2 represents only a portion of the total electron flux through nitrogenase. To measure total nitrogenase activity via H_2 evolution, it is necessary to incubate the nodulated roots in an atmosphere lacking N_2, such as argon:oxygen (Hunt and Layzell 1993).
- While brief exposure of nodulated roots to an argon:oxygen atmosphere does not inhibit N_2 fixation, extended exposure causes a decline in nitrogenase activity.
- Measurements reflect nitrogenase activity for only the duration of the assay. While many agronomically important legumes evolve H_2 from their nodules, some legume symbioses possess a hydrogenase enzyme in the nodules that is able to take up and recycle some or all of the H_2 produced by nitrogenase. The technique cannot be used in these circumstances.
- Logistical problems for field-based experiments include quantitative recovery of nodules when assaying detached nodulated roots, and difficulties in establishing open-topped or flow-through gas exchange systems when attempting to monitor H_2 evolution from the roots of intact plants.

Conclusions

- While the technique has application in the laboratory and in controlled-environment experiments, it is not suitable for quantifying N_2 fixation in field-grown plants.

2.2.9 Which technique to use?

Table 3 gives our view of the potential suitability of the various techniques for quantifying N_2 fixation in agricultural systems. It is not intended as an absolute guide, but as a pathfinder in the challenging task of deciding which method to adopt. Further to this, Table 4 lists some of the characteristics, strengths and possible applications of the techniques. Remember it is important that you bear in mind the resources you have available (skills, equipment, money for analysis, time, technical assistance, land area) and the level of certainty you need in terms of accuracy and precision.

Table 3. Suitability of methods for quantifying biological N_2 fixation in agricultural systems. The more '√', the more suitable is the methodology.

Species	System	Non-isotopic methods				N isotope methods	
		N balance	N difference	Ureide[d]	C_2H_2 reduction[f]	^{15}N natural abundance	^{15}N isotope dilution
Crop legumes	Monocrop	–[a]	√√	√√√	–	√√√	√√
	Intercrop	–[a]	–[c]	√√√	–	√√√	√√
Pasture legumes	Monocrop	√[b]	√	√√√	–	√√√	√√
	Intercrop	√[b]	√	√√√	–	√√√	√√
Tree legumes	Monocrop	–	√	√√√	–	√√	√
	Intercrop	–	–	√√√	–	√√	√
	Native forest	–	–	[e]	–	√	√
Grasses/cereals	Mono/intercrop	√[b]	√	–	–	√	√√[g]
Azolla		√[b]	–	–	√	√√√	√√

a Not suitable for short-term experiments
b Possible if long-term experiments
c Not suitable if legume proportion in mixture is small
d For ureide-producing species only
e Not known
f Useful for assessing activity but not quantification
g Only with relatively stable labelled soil

Table 4. Characteristics of methods to quantify biological N_2 fixation (the more '$', the greater the cost)

Method	Non-isotopic methods				Isotopic methods				
Characteristics	N balance	N difference	Ureide	C_2H_2 reduction	^{15}N natural abundance	^{15}N isotope dilution	^{15}N enriched A-value	Gas ($^{15}N_2$)	^{13}N
Direct								√	√
Indirect	√	√	√	√	√	√	√		
Time integrated	√	√			√	√	√	√	
Reference plant needed		√			√	√[d]	√		
Non-destructive			√[b]		√[b]				
%Ndfa measured		√[a]	√		√	√	√	√	
Quantify kg N/ha fixed	√	√	√		√	√	√		
Laboratory			√	√		√		√	√
Glasshouse	√	√	√	√	√	√	√	√	
Field	√	√	√		√	√	√		
Possibility to assess fate of fixed N in system						√			
Short-term			√	√	√	√	√	√	√
Long-term	√	√	√		√	√	√		
Precision	low	low	good	low	low–good[c]	medium	low	low	low
Costs	$$	$	$	$	$	$$	$$	$$$	$$

[a] Can be calculated indirectly

[b] If only %Ndfa is required

[c] Depending on natural enrichment of soil

[d] Not when cultivated in N-free media

Additional measurements

None of the methods for assessing N_2 fixation is perfect, and it is thus desirable to obtain additional information to support the N_2 fixation data. The following suggested measures require relatively little technical skill.

2.3.1 Assessment of nodulation

Nodulation is generally assessed by examining the roots of a number of plants from each treatment, dug up at random. Measures include earliness of nodulation, root nodule number, mass and colour, distribution and longevity of the nodule population, and visual nodulation scores.

It is recommended that nodulation be assessed during mid–late vegetative growth, when it is still relatively easy to excavate the majority of the root system. Nodule mass per plant is the most informative measure but can be very time consuming for species such as groundnut that have high nodule numbers. A practical alternative is to assess nodule number or, if nodule numbers are large, to use a scoring method (see Figure 4). This particular system scores nodulation from 0 to 5, taking into account nodule number, size, pigmentation and distribution. The system was devised for soybean and was adapted from that used by Corbin et al. (1977) for field-grown chickpea. The nodule score is determined by the number of effective nodules in the crown-root zone (regarded as the region up to 5 cm below the first lateral roots) and elsewhere on the root system (Figure 4).

To test nodules for active N_2 fixation, check their internal colour. They should be pink–red due to the presence of the oxygen carrier leghaemoglobin, which is essential for legume N_2 fixation. White, greenish or dark colours are indicative of ineffective nodulation and might correlate with low N_2 fixation rates.

The scoring in Figure 4 and described in Corbin et al. (1977) should be regarded as a guide only and may need to be modified for other species in different environments. Visual ratings should ideally be made by the same person throughout an experiment, but if more than one person is involved, each should score all samples. Thus, division of labour should be on the basis of replicates and not treatments. The procedure involves carefully digging up an appropriate number of plants at random across a crop or from a replicate plot (ensuring the root system and nodules are recovered) and scoring each

plant using criteria similar to that of Figure 4. The scores from all plants are added and then divided by the number of plants to obtain a mean nodule score. A mean nodule score of:

4–5 represents excellent nodulation; excellent potential for N_2 fixation

3–4 indicates good nodulation; good potential for N_2 fixation

2–3 represents fair nodulation; N_2 fixation may not be sufficient to supply the N demand of the crop

0–2 indicates poor nodulation and probably little or no N_2 fixation.

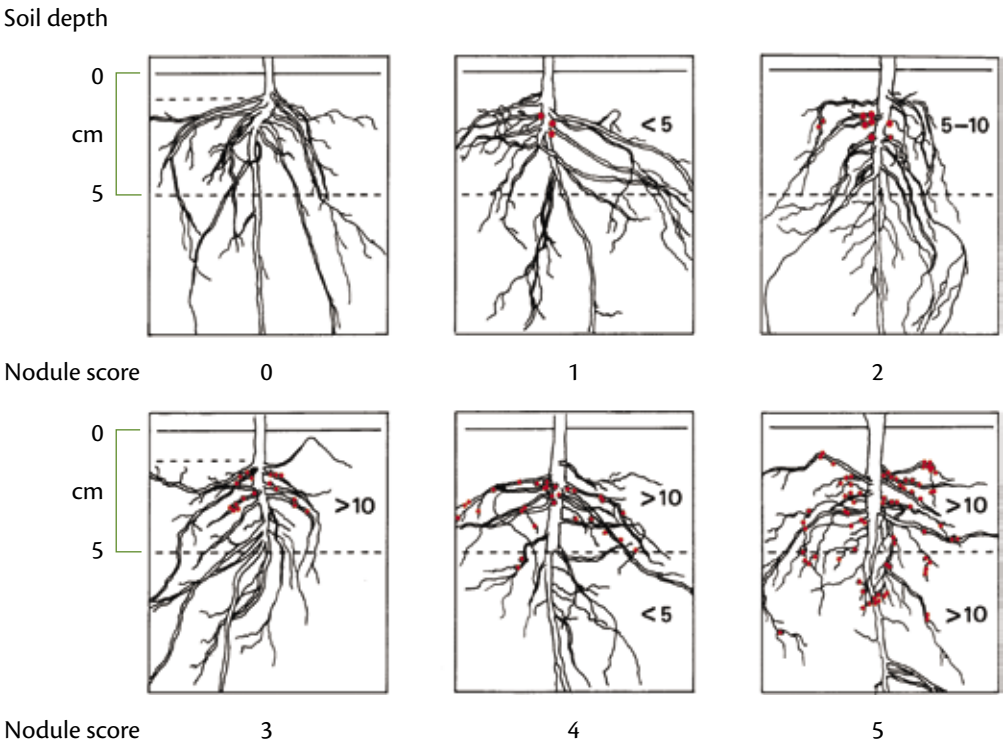

Figure 4. System for scoring nodulation of legumes, using a 0–5 ranking. Numbers within boxes are the numbers of nodules observed, and their associated ranking (score) is given underneath each box. Scores between whole numbers (e.g. 2.5, 3.5) can also be incorporated. The system is suitable for most annual species but may need to be modified for perennial plants.

Nodule recovery can be a problem, particularly in hard-setting, heavy-clay soils. The picture shows scientists digging up pasture legumes from a field site in the south-west of Australia. In this instance, nodule recovery from the coarse-textured, sandy soils was straightforward.

Nodule assessment of perennial legumes is more difficult, as root systems are likely to be extensive and nodules can be distributed throughout the soil profile. In some ecosystems, nodules will occur only at depths of several metres (Shearer et al. 1983; Pate and Unkovich 1999).

2.3.2 Growth analysis

Knowledge of the temporal pattern of N uptake of both N_2-fixing and companion non N_2-fixing plants often assists with interpretation of results. Measurements of total plant N several times during the growing season can be obtained via a variety of direct or indirect methods (see Rowe and Cadisch 2002).

2.3.3 Rooting patterns of N_2-fixing and companion non N_2-fixing plants

Exact determination of root length densities is very time consuming and often not necessary. However, a good knowledge of the potential maximum rooting depth and relative distribution pattern can be valuable. Qualitative assessment of this, using a profile wall or soil coring, is usually sufficient (see Anderson and Ingram 1993; doRosario et al. 2000).

2.3.4 Determination of mineral N in soil

High concentrations of soil mineral N will suppress N_2 fixation but low concentrations can promote N_2 fixation (and nodulation in the case of legumes). It is thus useful to know something about soil mineral N at the study site and in the various treatments. A minimum dataset would include sampling at sowing of the crop and at the end of the study period. If you are conducting a N balance (see Chapter 4), it is necessary to sample to the crop rooting depth. How to measure soil mineral N is covered in section 3.5.2.

2.3.5 Soil analysis

Results from a standard soil analysis (e.g. Anderson and Ingram 1993) may assist when interpreting experimental results, particularly where they reveal possible constraints to plant growth. Key analyses include pH, organic C, total N, available phosphorus (P), a suite of macro- and micronutrients and soil salinity, sodicity and cation-exchange capacity. As such analyses are expensive, it is often useful to do an analysis on a small number of bulked soil samples. Then, if anything is indicated that may impact negatively on plant growth (e.g. extremely acidic or alkaline pH, nutrient deficiencies or toxicities), further analysis of replicate soil samples may be warranted. This will help to characterise the general soil fertility and identify potential limitations to growth. Particular emphasis should also be given to assessment of any major physical constraints to root growth (e.g. hard pans).