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Measuring plant-associated nitrogen fixation in agricultural systems

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Murray Unkovich, David Herridge, Mark Peoples,
Georg Cadisch, Bob Boddey, Ken Giller,
Bruno Alves and Phillip Chalk

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Telephone: 61 2 6217 0500
aciar@aciar.gov.au

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Foreword

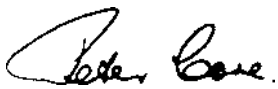
Nitrogen (N) is one of the key drivers of global agricultural production. Between 150 and 200 million tonnes of mineral N are required each year by plants in agricultural systems to produce the world's food, animal feed and industrial products. To meet those requirements, close to 100 million tonnes 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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Authors

Murray Unkovich
Soil and Land Systems Group
School of Earth and
Environmental Science
Waite Campus
The University of Adelaide
SA 5005 DP 636
Australia

David Herridge
NSW Department of Primary
Industries
Tamworth Agricultural Institute
4 Marsden Park Road
Calala NSW 2340
Australia

Mark Peoples
CSIRO Plant Industry
GPO Box 1600
Canberra ACT 2601
Australia

Georg Cadisch
Centre for Agriculture in the
Tropics and Subtropics
University of Hoenheim
Garbenstraße 13
70593 Stuttgart
Germany

Bob Boddey
Embrapa Agrobiologia
Km 7, Rodovia BR 465
Seropédica
23890-000 RJ
Brazil

Ken Giller
Plant Production Systems
Department of Plant Sciences
Wageningen University
PO Box 430
6700 AK Wageningen
The Netherlands

Bruno Alves
Embrapa Agrobiologia
Km 7, Rodovia BR 465
Seropédica
23890-000 RJ
Brazil

Phillip Chalk
Rua Paula Freitas 21/802
Copacabana RJ
Brazil

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).

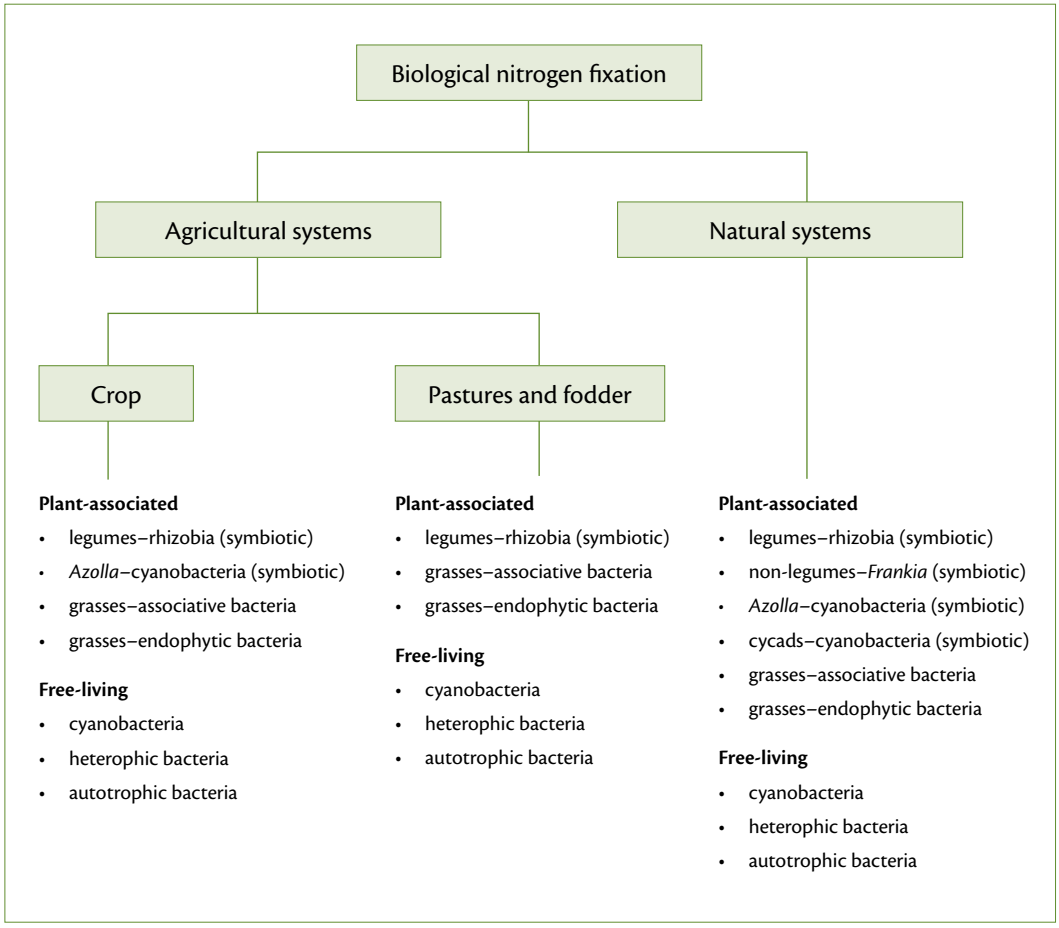


Figure 1. The biological N_2-fixing agents in agricultural and natural systems

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).

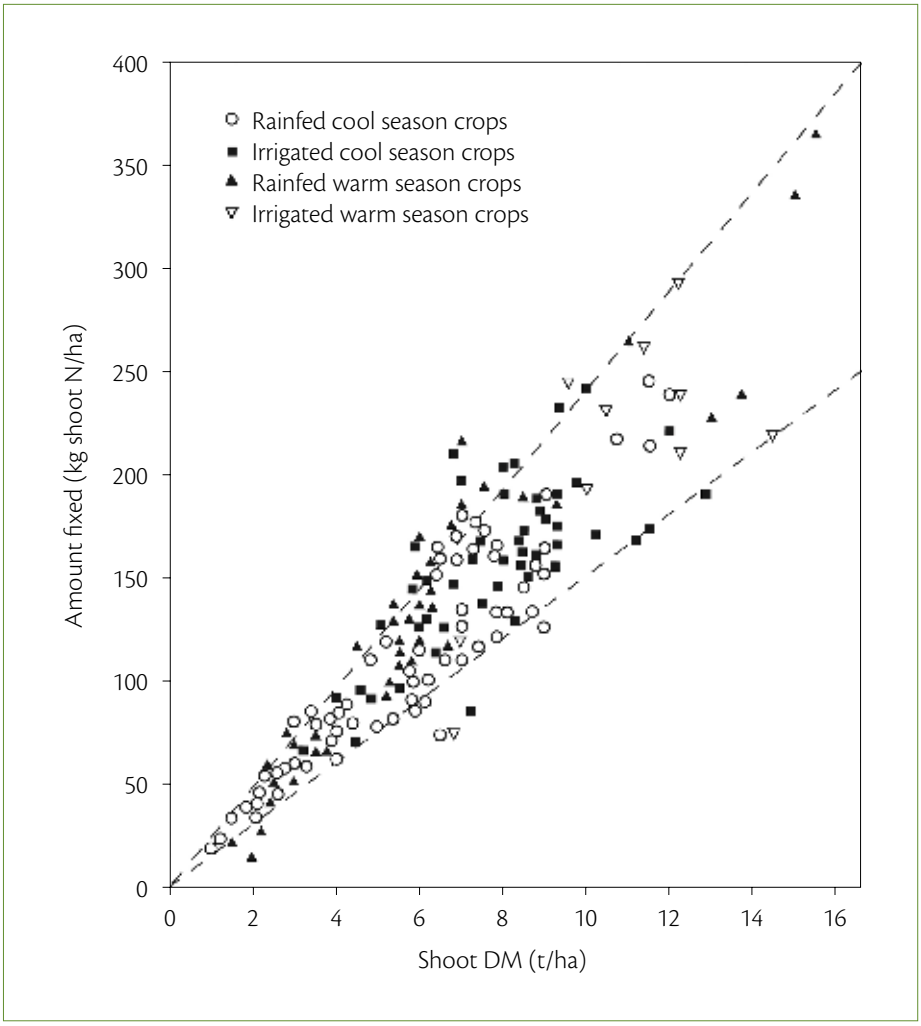


Figure 2. Relationship between amounts of shoot N fixed (kg N/ha) and shoot dry matter (DM, tonne/ha) for rainfed and irrigated warm season (black gram, green gram, cowpea, soybean) and cool season legumes (chickpea, faba bean, pea, lentil, grasspea, lupin) growing in different geographic regions. The 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.

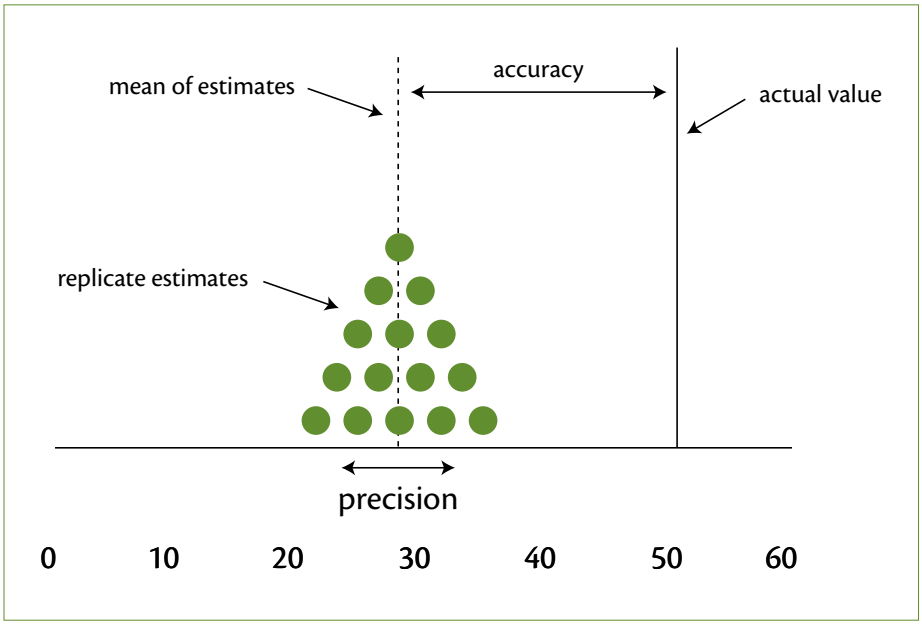


Figure 3. The difference between precision and accuracy

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.

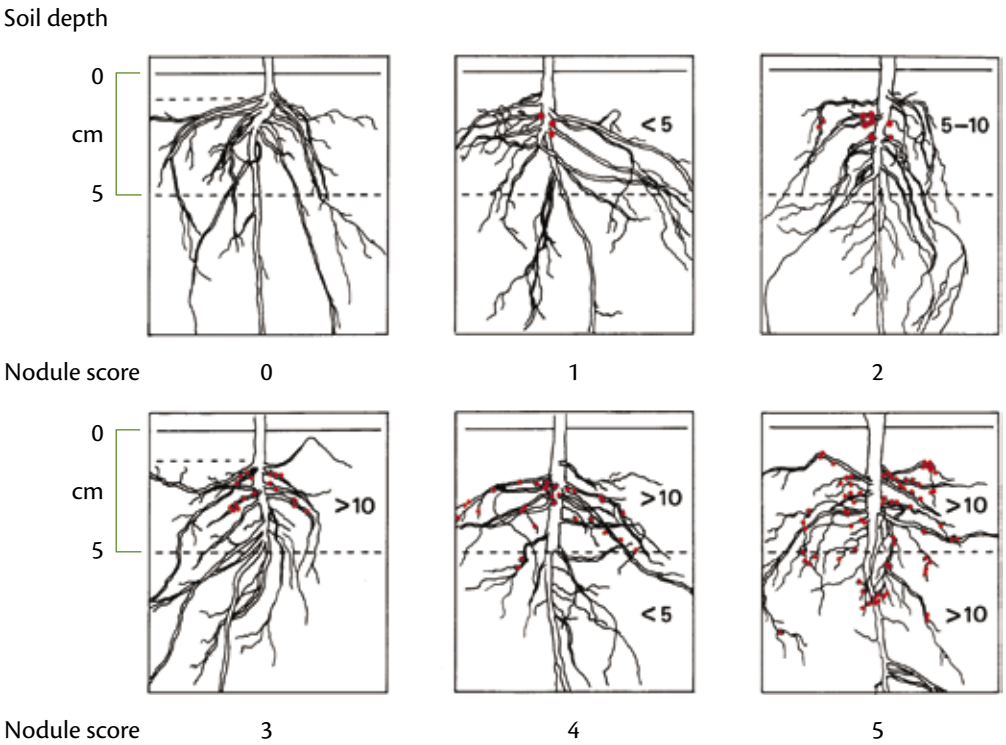


Figure 4. System for scoring nodulation of legumes, using a 0–5 ranking. Numbers within boxes are the numbers of nodules observed, and their associated ranking (score) is given underneath each box. Scores between whole numbers (e.g. 2.5, 3.5) can also be incorporated. The system is suitable for most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

3

Analysis of nitrogen

The amount of N_2 fixed by plants (kg/ha) is calculated by multiplying total plant N (kg/ha) by the percentage derived from N_2 fixation (%Ndfa). Regardless of the method used to measure %Ndfa, if N_2 fixed is to be calculated in terms of kg N/ha or g N/plant, it is necessary to determine total plant or crop N from total dry matter and dry matter %N. Interpreting N_2 fixation data will be greatly enhanced by measuring total N in non N_2-fixing reference crops and plant-available N in soils (nitrate (NO_3^-) + ammonium (NH_4^+)). Methods for analysing total plant N (and total soil N) and plant-available mineral N in soils vary from relatively simple, wet-chemistry techniques that can be undertaken in a basic laboratory through to more sophisticated, automated methods. The latter can be quite cost-effective and provide a throughput of hundreds of samples per day but typically requires a large capital investment and advanced technical support.

3.1 Total plant nitrogen

There are three fundamental approaches to measuring total N. The first, based on the original Dumas technique, involves oxidation of the sample in the presence of copper oxide to produce N_2 gas, the volume of which is measured. Incomplete combustion can be a problem with older systems, with only 70–80% of the sample converted to N_2 without mixing during combustion, 90% with mixing, and up to 99% conversion with addition of potassium perchlorate (Fiedler 1984). There are also problems with this method when the combustion products (N_2) are used for determination of ^{15}N at natural abundance. However, the more recent Dumas-type combustion systems have been specifically designed for coupled total N and precise ^{15}N analysis (Barrie et al. 1995). In the second approach, the 'wet' Kjeldahl digestion method, organic and mineral N are reduced to NH_4^+ in hot, concentrated sulfuric acid in the presence of a catalyst. The NH_4^+ is recovered by distillation or diffusion and estimated by titration or colorimetrically (Bergersen 1980a). The third approach is based on near infrared spectroscopy and is relatively rapid and inexpensive, but requires careful calibration.

3.1.1 Plant sampling, drying and grinding

Plant sampling for biomass

Plant samples should be collected from the field using a predetermined pattern that should be followed for all plots or sampling areas. Generally, samples are taken from treatment replicates as fixed lengths of rows (e.g. 1 m)

or quadrat areas (e.g. 0.5 × 0.5 m). Quadrat sizes should be chosen bearing in mind the size of the plots or field and the extent of variation within the plots or field. Sometimes it will be necessary to use individual plants in field studies, but this usually gives less accuracy and, if small numbers are involved, can bias the estimate (see Hunt et al. 1987; Swan et al. 2003).

Accounting for below-ground N

Plant biomass and N are usually determined from measures of shoot biomass. In the past it was generally assumed that N in the roots represented only a small fraction (5–15%) of the total plant N, and that shoot N provided a reasonable surrogate measure of whole plant N. However, evidence is now emerging that below-ground N associated with, or derived from, (nodulated) roots can represent 30–50% of the total plant N of both legumes and cereals (e.g. Russell and Fillery 1996; McNeill et al. 1997; Unkovich and Pate 2000; Khan et al. 2002; Herridge et al. 2008; McNeill and Fillery 2008). Therefore, total inputs of fixed N could be 50–100% greater than those determined from shoot-only measurements. Failing to take into account this below-ground pool of plant N will have major implications for any conclusions drawn about the contributions of fixed N to agricultural systems. This is particularly the case for N_2-fixing grain legumes where large amounts of N tend to be removed in seed at harvest (Rochester et al. 1998), but will also be true for pasture systems (Peoples et al. 2001).

There is no single value for below-ground N, with variations in published estimates reflecting the influence of species, soil and climate, and other factors affecting the partitioning of dry matter and N between the shoot and the root (Khan et al. 2003; Gregory 2006). To account for below-ground N when calculating total plant or crop N and N_2 fixation, we suggest a multiplication factor of 2.0 for the pasture/fodder legumes and chickpea (assumes 50% of plant N is below ground), 1.5 for soybean (assumes 33% below-ground N) and 1.4 for the remainder of the pulse and oilseed legumes and cereals (assumes 30% below-ground N). Although these are approximations, we believe that the errors associated with their use are far less than those incurred by ignoring below-ground N or perhaps using values for physically recovered roots. We recommend that you try to improve these approximations wherever possible.

Sampling woody perennials

In some cases it may be necessary to sequentially harvest trees or shrubs to calculate above-ground net primary productivity (and total N accumulation) from incremental changes in biomass, while in other situations the cumulative

regrowth of coppiced or hedgerowed trees might be used (e.g. Peoples et al. 1996). Cutting down and sampling mature trees inevitably generates very large amounts of material, placing logistical limitations on transport and processing of the samples. One approach is to cut down the tree, or a representative branch from it, take the fresh weight of this immediately, then take a subsample of this back to the laboratory to determine the fresh:dry weight ratio (e.g. Unkovich et al. 2000).

A non-destructive method for estimating biomass and N content derived from basal stem diameter or height of shrubs has been described by Rowe and Cadisch (2002). A similar approach may also be possible in mature tree plantations by applying allometric regression equations based on trunk diameter (Boddey et al. 2000). The other problematic component of net primary productivity is quantifying the amount of dry matter and N added to the system annually in litter fall. This requires both intensive and repeated sampling.

Drying plant samples

Ensure that sample bags are clearly labelled with the date (including year), plot or treatment number or name, the plant species and the part of the plant harvested. Plant samples are best kept in paper bags and allowed to breathe prior to drying. Try to avoid tight packing of samples as this can result in 'stewing' of material and subsequent changes in plant N forms. Plastic bags are not recommended. Where plants have been labelled with isotopes, it is important to keep labelled and unlabelled material in separate boxes to avoid contamination. The same applies for oven drying—keep labelled and unlabelled material separate, especially when dry and brittle and material is easily broken off. Many laboratories maintain separate systems (rooms, ovens, balances, storages) for ^{15}N-labelled and unlabelled samples.

Samples should be transported to ovens for drying as soon as possible after collection. If this cannot be arranged within a few hours, plant samples should be kept in a coolroom or cool place overnight. In circumstances where there is a large amount of sample material and insufficient oven space, aim to treat all samples the same. Preliminary air drying in a glasshouse may be possible in dry environments. Under conditions of high humidity, samples should be stored in a coolroom until oven space is available. The objective of drying is to remove the water from the plant material without loss of organic compounds, and without the growth of contaminating micro-organisms (bacteria and fungi). If plant samples have attached soil (or dust), it should be washed off while the plants are fresh as it cannot be separated from dried material.

Plant material must be dried to constant weight at 65–80 °C (usually for at least 48 hours) in an air-circulating oven. Very bulky samples can be spread on metal trays and returned to the sample bags when dry. Do not pack sample bags tightly into an oven as this can readily cause a fire. Ensure airspace between sample bags for safe and efficient drying. Drying plant material at 100 °C is not recommended as this can cause rapid proteolysis (protein breakdown), thermal decomposition and loss of N. High temperatures also increase the risk of an oven fire. Where suitable facilities are available, freeze-drying is a good alternative to oven drying, especially for material with a high sugar or starch content.

The dry weight of the total sample should be recorded immediately after drying, before any grinding and subsampling. Samples can conveniently be weighed in their paper bags, with the bag weight subtracted from the sample weight. The weight subtracted should be the average weight of similarly oven-dried bags. Once dried and weighed, the samples can be stored indefinitely provided that they are kept in dry, airtight conditions. It is preferable, however, to store samples in a ground-up state as this requires less space and the samples can be kept in containers to prevent damage from insects and mice etc. Samples should be stored in paper bags and cardboard boxes for only short periods (days–weeks) to avoid possible sample damage or loss.

Take care with your samples

Short cuts and carelessness in sample handling and storage can result in major catastrophies. The cost of setting up experiments and sampling is much greater than the cost of taking care with appropriate sample labelling, handling and storage.

3.1.2 Grinding and subsampling

Weighed, oven-dry samples should be chopped or coarsely ground prior to subsampling. For very bulky or woody samples, garden shredders may be used but care must be taken to avoid any sample loss at this point. A range of cutting-type grinders are suitable for grinding to pass a 10-mesh (2 mm) screen prior to subsampling. This is done by mixing the whole, thoroughly ground sample on a sheet of paper or plastic, and taking from this a subsample of 5–10 g which is then stored in an airtight, screw-cap container. The entire subsample can then be ground later, using a finer screen or hammer mill fitted with an 80-mesh (approx. 0.2 mm) screen. Where sample size for analysis is <1 g, a ring, roller or ball mill will be essential to produce very finely ground samples. Mills and grinders should be cleaned between

samples using an air jet or by careful brushing out. This is particularly important when isotope analysis is to follow. It is often most efficient to grind like samples or treatments in batches together, reducing the extent and effect of cross-contamination between samples and treatments.

There can also be problems in preparing material for ^{15}N analysis. While it is relatively easy to grind leaves, the preparation of woody trunks, branches and roots for analysis can be much more difficult. One approach is to subsample the woody components using an electric saw and collecting the sawdust to be ground for N and ^{15}N analysis (Peoples et al. 1996). Once the ^{15}N abundance has been determined for each part, a 'weighted' ^{15}N composition based on the ^{15}N abundance and proportional amounts of N in each organ of a whole plant can be used to calculate total above-ground ^{15}N and N contents (see Chapter 7).

Ensure that samples are evenly mixed before subsamples are taken. Vibration during storage leads to non-uniformity within containers, even when the contents were originally well mixed. The fine and coarse material that separate during storage usually differ in both N concentration and isotope abundance.

Where isotope analysis is required and if separate grinding equipment for labelled and unlabelled materials is not available, grind unlabelled samples first, then the ^{15}N-labelled samples. It may be useful to grind 'blank' material between batches of samples or between treatments to reduce the possibility of cross-contamination. By blank material we mean spare plant material that is not artificially enriched in ^{15}N, and has a low total N content (<2%) and a low oil content. Acid-washed sand can be a very effective cleaner of the grinder between batches.

If samples are to be sent by mail or courier to another country for weighing and analysis, it is essential to enclose them in heat-sealed or 'ziplock' plastic bags. Although small paper envelopes may appear to seal well, the shaking that samples experience during transport means that they tend to leak, with a danger of cross-contamination.

A note on grinding and grinders

Sample grinding is a key element of plant (and soil) analysis, but can be time consuming. Shortcuts can lead to poor sample preparation and large variations in analyses due to subsampling and heterogeneity errors. At least two stages of grinding are generally required, an initial grind to produce a sample that can be readily subsampled, then a fine grinding of that sample to be used for analysis. Only for very small samples such as single plants, or parts of individual plants, can the first step be avoided. In some cases three stages of grinding will be required, each using a different grinder. No single laboratory

Studies of N_2 fixation are likely to be enhanced by analysis of soil N, both plant-available mineral N (NO_3^- and NH_4^+) and soil total N. Plant-available N is an important determinant of both nodulation and N_2 fixation of legumes. An understanding of the availability of mineral N relative to plant growth is thus of great value when interpreting N_2-fixation data. In this picture, scientists are coring a field soil in Myanmar.

grinder can handle the wide range of sample sizes, materials and grinding requirements for different analyses. As a consequence, laboratories will usually need two to three grinders of different types.

Initial grinding can be achieved with high-volume rotor, cross-beater or cutter-type mills. Two of these types of grinders (large and small) in combination are often very effective. Some laboratories use small, inexpensive coffee-type grinders, but the authors have found these to be insufficiently robust for ongoing laboratory use.

Where very small (<1 g) samples are to be analysed, grinding with a roller, ball or puck mill is usually required. An inexpensive but very effective roller mill can be built using an electric motor, steel rods, sprockets and chain (see Figure 5 and Arnold and Schepers (2004)). Grinders such as these have a low labour input and can be built from readily available items. More sophisticated, pulverising grinders that are quite effective include the very useful two-sample MM200 ball mill from Retsch Germany and a larger, single-sample mill produced by Roklabs Pty Ltd, Auckland, NZ. Versions of all of the above grinder types are manufactured by companies such as Retsch and Fritsch in Germany; Glen Creston in the UK; and Thomas-Wiley, Spex Certiprep and GlenMills, all of New Jersey, USA.

3.2 Total nitrogen analysis by Kjeldahl digestion

The standard Kjeldahl method for determining total N in plants and soils involves the digestion of the plant or soil sample with a catalyst in hot sulfuric acid, converting the organic N to NH_4^+, then determining the concentration of NH_4^+. The method can be done with limited resources and is useful for analysis of total N and ^{15}N from a single sample distillation. Full details of the methodology are given in Peoples et al. (1989b). Unkovich et al. (1993) provide details of modifications required for high-precision ^{15}N analysis. Clifton and Clifton (1991) describe a system for doing a Kjeldahl digestion in the field, but their method would not be suitable if ^{15}N analysis was also required. Protocols for assessment of soil total N are detailed in Bremner and Mulvaney (1982).

3.3 Total nitrogen analysis by dry combustion

Several commercial instruments are available for the automated determination of total C, N and S in plants and soils. These instruments provide rapid analysis of large numbers of samples but are expensive and require skilled operators. Readers should refer to the technical manuals that accompany each instrument, or to the laboratory technician who is analysing their samples, for specific advice on sample preparation and analysis. We give only a brief outline of the methodology here. In recent years these combustion systems have also been coupled to isotope-ratio mass spectrometers so that both total N and ^{15}N can be determined on a single sample. Commercial services for such analyses are also available (see Appendix 9).

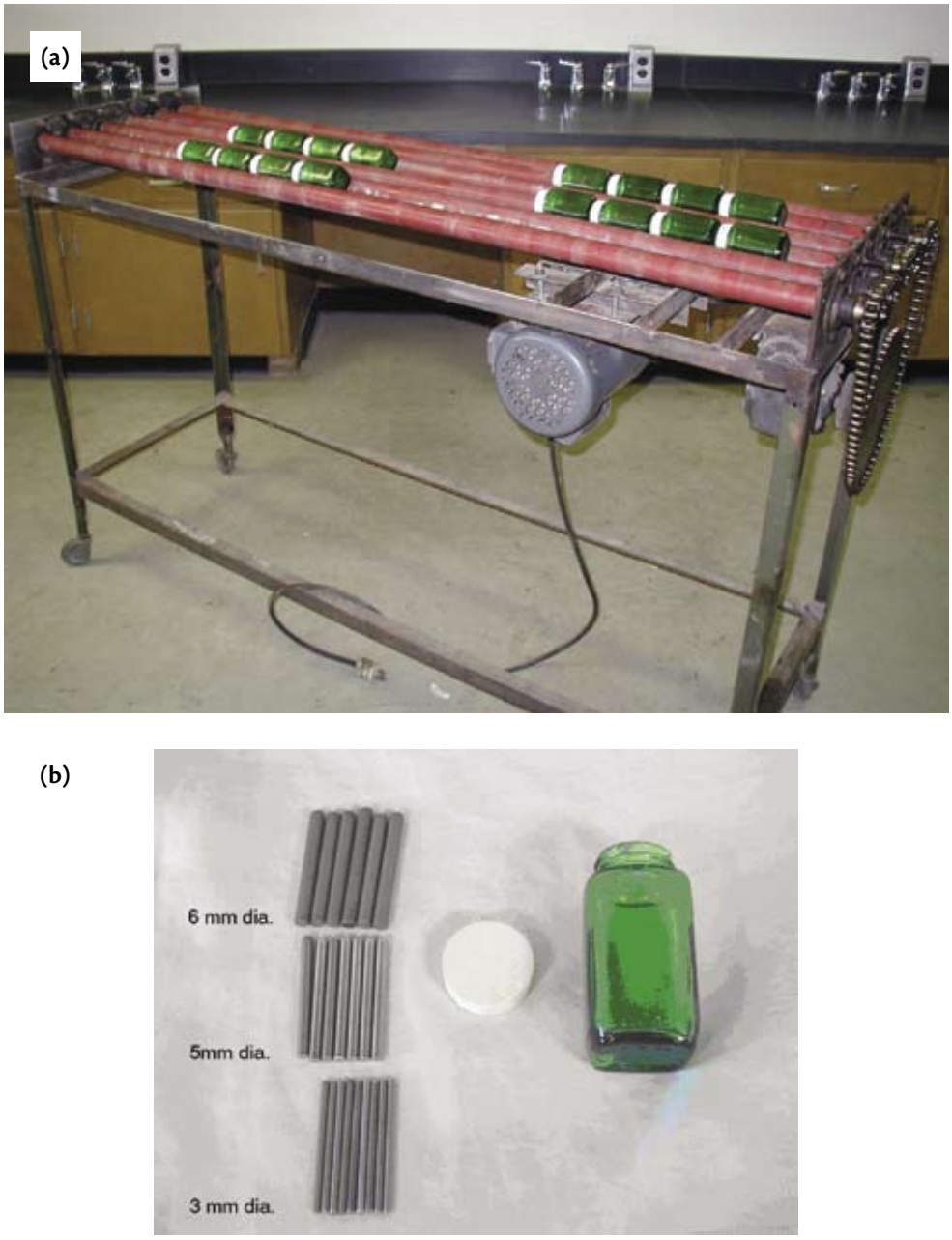


Figure 5. A simple (a) roller grinder that can be made from readily available materials, including (b) steel rods and square glass bottles (see Arnold and Schepers 2004)

Figure 6 shows an example of a dry combustion system, illustrating the principal features. The system is purged with helium carrier gas (1). Samples are weighed into tin capsules (3) and loaded into a carousel (4) that can hold many (40–120) samples. Sample cups are dropped into a heated (>1,000 °C) combustion chamber (5), which is flushed with oxygen (2). Flash combustion occurs in the chamber in the presence of catalysts. The resulting gases are swept through to the reduction chamber (6) by helium carrier gas where N oxides are reduced to N_2 and C oxides to CO_2. Water, which is produced in both the combustion and reduction processes, is removed via a perchlorate trap (7). The remaining gases can be analysed in a thermal conductivity detector (10) after separation in a gas chromatography capillary column (9), or the CO_2 be trapped out (8) and only the N_2 analysed. If the combustion analyser is attached to an isotope-ratio mass spectrometer, the mass spectrometer can also be used to determine total N, total C, ^{15}N and ^{13}C.

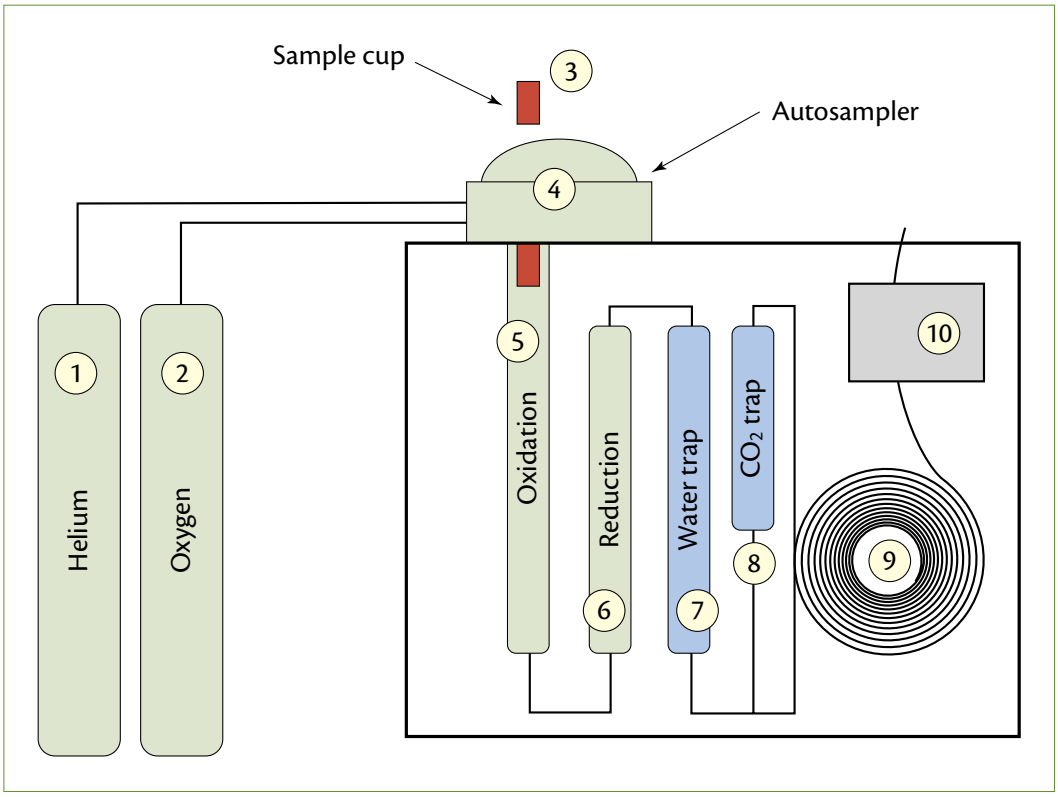


Figure 6. Schematic diagram of an automated combustion system for analysis of total carbon, nitrogen or sulfur in plants or soils. A description of the components and operation is given in the text.

Limitations

Sample size varies from gram to milligram (mg) quantities between different systems, so check sample requirements with the analytical laboratory. For isotope analysis systems, the sample weights are usually in the mg range. The smaller the sample size, the greater is the need for fine grinding and high sample homogeneity. Samples must be dry before analysis and weights must be recorded very accurately. For mg quantities a very sensitive balance is required (±0.00001 g), and scrupulous hygiene is necessary to prevent contamination. In some cases the small sample size is of benefit but, in most cases, it necessitates additional grinding and very careful sample handling, particularly subsampling. Instruments must be well maintained with careful attention paid to the saturation of the water trap, reduction tube (Cu) and oxidation catalysts.

Total plant nitrogen analysis by near infra-red spectroscopy

3.4

3.4.1 How it works

Electromagnetic energy with wavelengths in the range 780–2,500 nm is termed near infra-red (NIR) energy. When NIR energy interacts with matter, such as fresh or dry plant material, some energy is absorbed and this raises the energy of chemical bonds in the material to a higher state. Bonds between small atoms and large atoms, especially O–H, N–H and C–H in biological materials, absorb this energy most strongly, thereby providing the basis for analysis of water, proteins and carbohydrates in plant tissues. Osborne et al. (1993) describe the basic principles of NIR spectroscopy. Typical plant spectra of fresh and dry, and low and high N, rice-shoot samples are shown in Figure 7.

There are two steps to NIR spectroscopic analysis. The first is calibration of the NIR spectrometer, and the second sample analysis.

3.4.2 Calibration of the NIR spectrometer

Because NIR energy is strongly absorbed by N–H bonds of proteins, it is relatively easy to develop reliable calibrations to measure total N. The steps to calibrate an NIR spectrometer are as follows:

1. Select a set of samples of the species with similar physiological age (do not mix green shoots and dry straw samples) and the widest possible range in N values. Preserve and prepare the samples using the drying and grinding techniques as described in section 3.1.

2. Scan the samples on the NIR spectrometer. Then, keeping in mind errors that may be caused by changes in the moisture content of the sample, analyse the samples for total N using either a Dumas combustion technique or by Kjeldahl wet chemical analysis (see sections 3.2 and 3.3). As a guide, at least 100–200 samples should be scanned and analysed. One approach is to analyse all samples using NIR (perhaps hundreds or thousands) and then select 100–200 samples for crosschecking on the basis of spectral diversity. The set may be selected using commercial software such as WINISI (InfraSoft International LLC, 362 South Atherton Street, State College, PA 16801, USA). Another approach (Williams et al. 1983) is to scan the samples using a preliminary calibration and select samples with the widest possible range in %N to form a 'boxed' population (uniform number of samples in each %N increment across the total range).

3. Develop a calibration (Figure 8). This involves extracting information from the NIR spectra of the calibration samples that explains the variation in %N. If the samples are uniform and the laboratory data are reliable, a multiple regression approach will produce a robust calibration. A filter instrument with as few as four to six specifically selected bands

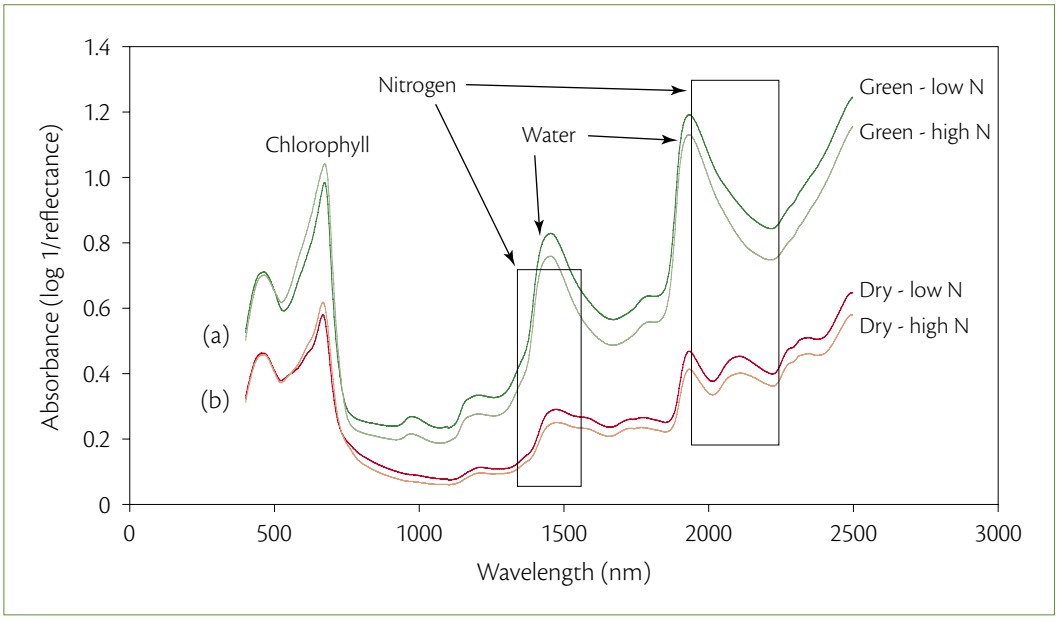


Figure 7. Typical spectra of (a) fresh and (b) dry, ground rice tissue, showing the location of N, water and chlorophyll absorption bands

in the NIR region will be acceptable. Modern software packages available with NIR spectrometers, and from third-party companies, now offer more sophisticated mathematical manipulations and achieve slightly more reliable calibrations. NIR calibrations that explain 99% of the variation in N in samples analysed by traditional laboratory methods and a standard error of ± 0.08%N are now routine. By comparison, standard errors of laboratory measurement of total %N using Dumas may be ± 0.04%N. The small loss in performance is more than offset by the advantages of NIR analysis (see below).

4. Maintain the calibration. For reasons not yet understood, like samples collected in different seasons vary subtly and may cause bias in NIR analyses. Recent studies suggest that as few as 15–20 samples from a new season should be added into the calibration set to correct for this bias (Guthrie et al. 2005, 2006). Check samples are essential for quality control and are used to monitor for errors due to variations in temperature, moisture, sample presentation and instrument malfunction (Blakeney et al. 1995, 2008).

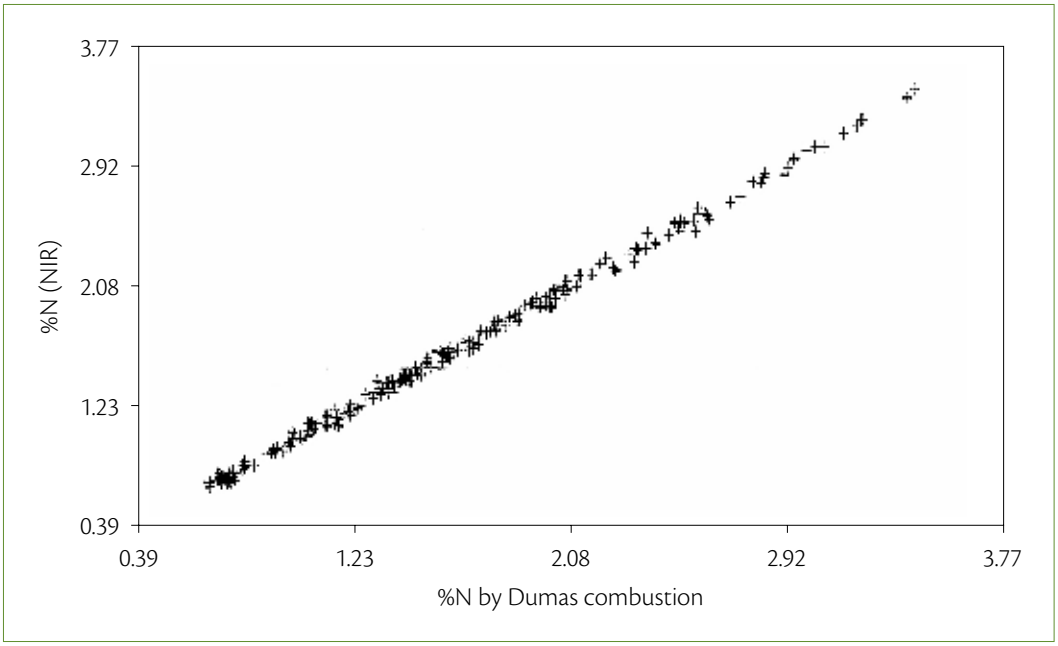


Figure 8. Example of correlation between %N of rice shoots determined by Dumas combustion and near infra-red reflectance (NIR). Such NIR calibrations need to be done for each instrument and plant material type and species.

3.4.3 Routine analysis of samples

1. Collect a representative sample. If it is to be analysed 'as is' go to step '4'.
2. Dry the sample. Microwave drying (Batten et al. 1991) is rapid and can be done at the site of collection, including on-farm (in kitchen). Plant samples may be dried using the full power setting in a 600–1000 watt microwave oven. A fresh weight of ~100 g would require about 1 minute to heat through and make the water boil. Continued microwave heating removes water at a linear rate. Approximately 6 minutes is required to achieve constant weight. If samples char (look inside the stems), chop them into to approximately 1 cm^2 pieces.
3. Mill the sample using a grinder that achieves a consistent range in particle size.
4. Present the sample to the NIR spectrometer using a consistent technique to ensure repeatability.
5. Calculate the concentration of the constituent(s) in the sample using the calibration(s) developed previously. Results may be reported as % total N on a fresh or 'as is' basis.

Limitations

A sample size as small as 0.3 g may be analysed (Ciavarella et al. 1995), or even less if an optic fibre probe is used. A large sample area is recommended, however, to reduce sampling errors even with finely ground material. Fresh samples are more difficult to analyse with accuracy because absorption by the water in the sample causes loss of information in the areas adjacent to the water bands in the spectra (Figure 7) and increases heterogeneity in the sample.

Benefits compared to other methods

NIR-based analysis is rapid and non-destructive, has a low cost per sample and is more repeatable in routine use than traditional analytical methods used to calibrate it (Williams et al. 1983).

Sample preparation and presentation

Plant leaf, shoot or stem material is usually scanned in the reflectance mode. The (ground) sample may be presented in a covered cell, an open cell, a glass or other vial, a plastic bag or to the tip of a fibre optic probe.

Sampling a field-grown faba bean crop for biomass and N determinations. Generally, samples are taken from treatment replicates as fixed lengths of rows (e.g. 1 m) or quadrat areas (e.g. 0.5 × 0.5 m).

Dubious results

The software programs used to calculate %N from NIR spectra usually flag samples that have atypical spectra and may have a large residual relative to the true value. Unless these samples are found to be atypical for a known reason, they should be considered as potentially valuable and included in the calibration set.

3.4.4 Final word

Analysis of %N of plant tissues using NIR spectroscopy is now common because it is rapid, reliable and inexpensive. Analysts consistently find that laboratory errors for traditional analyses are detected and eliminated more rapidly when there is an NIR spectrometer in the laboratory. However, the reliability of the NIR data must be constantly validated by cross-referencing against traditional analyses. Simultaneous determinations of plant moisture, carbohydrates, macro- and some micro-elements and other constituents are possible. This ability to make multiple determinations further enhances the value of the NIR technique.

3.5 Analysis of soil nitrogen

Studies of N_2 fixation are likely to be enhanced by analysis of soil N, both plant-available mineral N (NO_3^- and NH_4^+) and soil total N. Plant-available N is an important determinant of both nodulation and N_2 fixation of legumes, and is generally used by the plant in preference to N_2 fixation. An understanding of the availability of mineral N relative to plant growth is thus of great value when interpreting N_2 fixation data. Soil total N can also be a useful indicator of soil fertility status, especially in relation to cropping systems over long periods of time. Short-term changes in total soil N are very difficult to determine because of the large background pool of total N in most ecosystems.

Careful attention needs to be paid to sampling procedures as soils are generally variable in chemical and physical properties over short distances and depths. Soil total N is concentrated near the soil surface (0–20 cm), but mineral N can be distributed more evenly through the soil profile. Fertiliser N may be concentrated in bands, and thus there may be substantial differences between the crop row and the inter-row. As a general rule, soil total N is usually determined on 0–10 cm or 0–15 cm samples, and soil mineral N on from between 0–15 cm and 0–150 cm depth. A detailed practical guide to soil sampling can be found in Dagliesh and Foale (2000) (also freely accessible online at <http://www.apsru.gov.au/apsru/Products/APSoil/SoilMatters/Default.htm>). Soil N is calculated as follows (equation (1)):

$$\text{N in soil (kg N/ha)} = \text{soil N (mg/kg)} \times \text{bulk density} \times \frac{\text{depth of soil (cm)}}{10} \qquad (1)$$

This can be used for either soil total N or soil mineral N.

Subsampling and soil drying

Soil samples are difficult to subsample when wet, so subsampling is best done in the laboratory after the samples have been dried. Samples can be transported in calico (cloth) or plastic bags. Calico bags are better in hot climates where plastic bags tend to heat up the samples, resulting in significant biological and chemical changes. Heavy paper bags are suitable for drier soils but plastic bags will be essential for very wet soils. Wherever possible keep samples out of direct sunlight prior to drying. As with plant samples, it is possible (though not ideal) to store fresh soil samples for short periods (days) in a cool room (4 °C) prior to processing. Soil samples that are too wet for sieving and subsampling are usually slurried with water and a subsample of known proportion by volume used for the analysis. They can also be partially air dried under cool conditions (< 30 °C). Soil samples are best air dried at 40 °C prior to storage or analysis. A forced-draught oven can be used for this but do not exceed 40 °C. Soil samples can be digested wet. However, you will need to know the moisture content of each sample to be able to accurately calculate soil total N (mg/kg dry soil).

To convert N concentration (mg/kg) to kg N/ha, soil bulk density is required. To estimate soil bulk density, samples are usually taken with a cylindrical coring device of known diameter (75–100 mm) to a defined depth. This volume of soil is weighed accurately to give a bulk density in g/cm^3. Soil bulk density is surprisingly difficult to determine and is often a significant source of error in estimates of total or available soil N. Details on how to measure soil bulk density can be found in Dagliesh and Foale (2000).

3.5.1 Total soil nitrogen

Kjeldahl digestion and Dumas combustion are the two principal methods for analysing soil total N. The Kjeldahl technique is similar to that for plants. Dumas-type analysers can provide rapid analysis of soil total N but need to be calibrated against wet chemical methods (Kowalenko 2001). For combustion systems, larger (≥1 g) sample sizes are preferable due to lower subsampling errors, but for smaller samples, fine grinding using a ball or puck mill is required to avoid subsampling errors. The N concentration of the soil is calculated on weighed oven-dried samples; however, to relate this to the field where the soil came from, the soil bulk density and initial moisture content need to be recorded so that the calculated mg N/kg soil can be converted to kg N/ha. Detailed protocols for Kjeldahl digestion of soils can be found in Bremner and Mulvaney (1982) and for combustion analysis in Kowalenko (2001).

3.5.2 Extractable mineral nitrogen

Of greater importance in N_2 fixation studies is the concentration of plant-available soil mineral N. In moist and wet soils, mineral N is subject to many biological and chemical reactions during transport and storage that may affect both the amount of available N (Westfall et al. 1978) and its N isotope composition. The following procedures have been found to minimise the effects of storage and handling on mineral N and N isotope composition.

Sample handling for extraction of NH_4^+ and NO_3^- from soils

Unless soils are dry when sampled, soil sampling and handling always results in changes in soil mineral N, as soil N is biologically active. To reduce the effects of this, soil samples can be immediately chilled on ice, then stored at –10 °C as soon as possible and kept frozen until thawed for immediate analysis (extraction). In the absence of ice or freezers, samples can be carefully air dried at no more than 40 °C, recognising that this may result in changes in the amount of mineral N in the soil, particularly the ratio of NH_4^+:NO_3^- and the natural abundance of ^{15}N in the N fractions. Such problems are more likely for soils with a high moisture content (>50% field capacity) and soils with high N or mineral N contents. An alternative way of preventing such biological and chemical transformations of N is to commence extraction immediately after sampling (e.g. see Herridge et al. 1984). The salts in the KCl extractant effectively prevent biological modification of the mineral N. In this case, parallel cores need to be taken for estimations of fresh weight, dry weight and bulk density.

If soil samples are too wet for sieving and mixing when taken from the coring device, a 300 g bulk sample can be mixed with 100 mL of distilled H_2O to form a slurry. Two aliquots (140 g each) of the slurry are weighed and 60 mL of 3.5M (molar) KCl added to bring the aqueous suspension to 2M concentration. Record the total volume of liquid added.

3.6 Final word

Nitrogen analysis of plants and/or soils will inevitably be required in N_2 fixation studies. Suitable protocols can be found in standard textbooks, keeping in mind the precautions mentioned above. Where N isotope analysis is to be conducted, it is important to consult your analytical laboratory to ensure that sample preparation protocols do not compromise subsequent analysis by mass or emission spectrometry (see Craswell and Eskew 1991; Mulvaney 1993; Preston 1993; Unkovich et al. 1993; Barrie et al. 1995).

4

Nitrogen balance method

The N balance and N difference methods are the simplest tools for estimating N_2 fixation. Nitrogen balance compares the total N of a plant–soil system on two separate occasions, with any increase attributed to N_2 fixation after other possible inputs and outputs of N have been accounted for. In contrast, N difference compares total N accumulated by the N_2-fixing plants with that of neighbouring non N_2-fixing plants, with the difference assumed to be due to N_2 fixation. Both methods can be used in either the glasshouse or field. The methods can be confused in some situations, but the N balance method clearly makes a comparison *within a system between times*, and the N difference method compares two *different systems at one point in time*.

4.1 How it works

The N balance method is illustrated in its simplest form in Figure 9, where plants are grown in N-free media (e.g. acid-washed sand) and the only source of N for growth, apart from seed- and inoculum-N, is N_2 fixation. This can be calculated directly as (equation (2)):

$$\text{Amount of N fixed} = \text{N yield}_{\text{N fixing plant}} - \text{N}_{\text{seed+inoculum}} \quad (2)$$

Time zero (T_0) in this case is when the seed is planted, and total N at this time is equal to seed N plus that added in any rhizobial (or other) inoculant. Plants can be harvested at any point in time, or on several occasions thereafter and the amount of N_2 fixed calculated between each harvest. In this form, the method is often used to compare N_2 fixation of plants inoculated with different strains of rhizobia. The basic assumption is that N_2 fixation, N in sown seed and N in the inoculant are the only sources of N. Thus, we assume there is no N in the potting mix, no N added in watering of the pot and no N deposited from the atmosphere in the form of dust or ammonia, and that any N remaining in the pot after plant harvest is negligible.

Principal assumption

The principal assumption of the method is that all N inputs (fertiliser, deposition) and all N outputs (leaching, denitrification, run-off, volatilisation, harvest) of the system under study are accounted for. If there is a positive net total N balance in the system between two points in time, then the N gain is assumed to be from N_2 fixation (Giller et al. 1997).

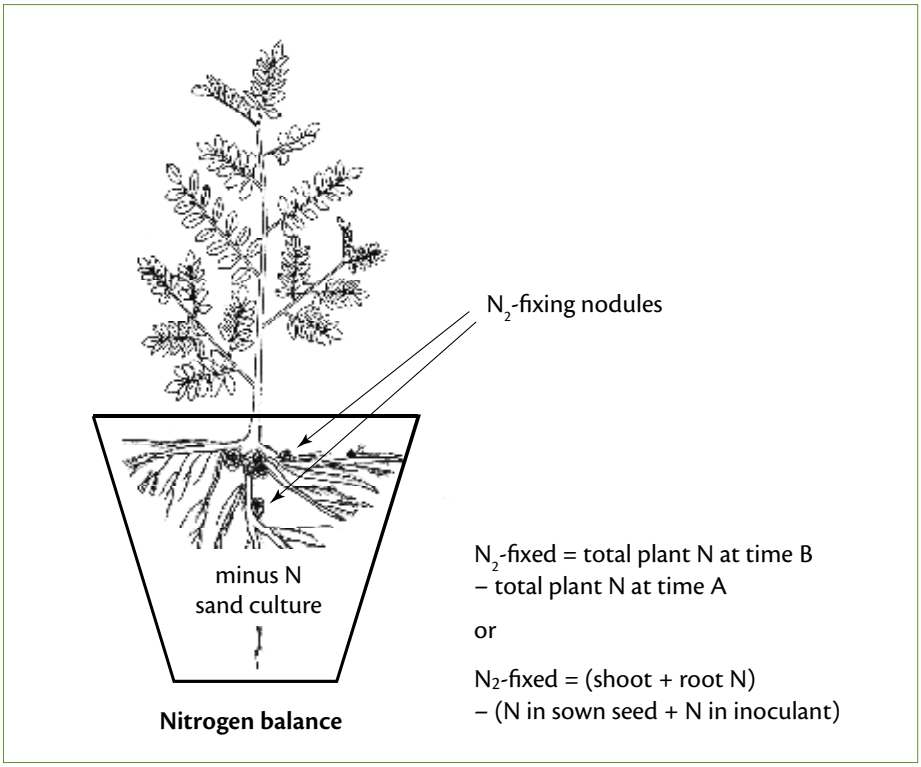


Figure 9. Estimating N_2 fixation by N balance. For a N_2-fixing plant grown in minus N culture, total plant N minus sown seed N equates to N_2 fixation. In this case it is assumed that any N remaining in the pot soil after plant harvest is negligible compared with total plant N.

While this is relatively easy to do under glasshouse conditions, it is very difficult to do in the field, where changes in soil total N need to be measured in addition to many potential sources of N input and loss from the system. The various inputs and outputs of N that might need to be accounted for are given in Table 5.

While inputs of N to the plant–soil system under study via fertilisers and organic sources may be relatively easy to quantify, difficulties quantifying the remaining inputs and outputs can create large uncertainties. Many of these flows of N are highly dynamic, with rates that vary substantially with changing environmental conditions, diurnally, and from day to day. Integration of such dynamic flows of N from point measurements is therefore prone to error, but failure to estimate them and include them in the budgets will definitely result in errors in the calculation of N_2 fixed.

Table 5. Potential gains and losses of N that need to be accounted for when constructing N balances for agroecosystems. Items in the bottom half of the table are more difficult to measure.

Inputs	Outputs
Manures and other organic sources of N	Crop and animal removal in products or other harvested material
Fertiliser N	
N in irrigation water	
Wet deposition (NH_4^+, NO_3^- in rainfall)	
Dry deposition (NH_3, NO_2^-, organic N in dust)	Gaseous N losses (NH_3 via volatilisation and NO_x and N_2 via denitrification)
Run-on	
Uptake from lateral subsoil flow	Soil erosion and run-off
N_2 fixation (symbiotic, associative, free-living)	Leaching of NO_3^-

As the total amount of N in the soil is generally large compared with the amount of N in plants and the amounts added via N_2 fixation, any error in the estimation of soil total N will result in a large discrepancy in the estimate of N_2 fixation. A very large number of field samples is usually required to accurately determine soil total N (see section 3.5).

4.2 N balances in pot experiments

In pot experiments it should be possible to control all inputs and outputs of N, so the N balance method can provide a useful, integrated estimate of N_2 fixation. It is usually advisable to choose a growth medium that contains little or no N (see Figure 9). This will increase the sensitivity of the estimate of N_2 fixation. There are no hidden assumptions in measuring an N balance. If the experiment aims to detect only small amounts of N, care must be taken that the air does not contain significant levels of ammonia (NH_3). Plants can absorb NH_3 through their leaves, particularly when N deficient, and this would be incorrectly attributed to N_2 fixation. Acid traps, such as those described in Schjoerring (1998), can be used as NH_3 sinks and to check for

NH_3 contamination. Ammonia-based cleaning products should not be used in glasshouses. If pots containing mineralisable organic matter are used for plant culture, then the N difference method is preferable.

In the case of N-free plant-growth systems, the medium should be inert and the nutrient solution contain no traces of mineral N. Care has to be taken that the medium used is indeed free of N. Because amounts of mineral N can be released from vermiculite when incubated under warm, moist conditions (Giller et al. 1986), this medium should be avoided. The degree of error through such contamination depends on its relation to the amount of N_2 fixed. In such cases the additional use of a non N_2-fixing control plant would be necessary, although it is preferable to use completely N-free media.

An application of an N-free plant-growth system is the use of Leonard jars for comparing the effectiveness of strains of inoculant rhizobia for legumes (for details see Gibson (1980)). In this method plants are grown in sterilised, acid-washed sand and fed with N-free nutrient solution (see Appendix 1). An uninoculated control should be included. The method is easy to implement and allows the screening of large numbers of rhizobia or legumes for N_2 fixation efficiency. However, the results of N-free screening in the glasshouse indicate only the relative potentials of rhizobia under non-limiting conditions. They are not directly transferable to soil and field situations where large populations of other micro-organisms occur along with a combination of soil and environmental stress factors that can influence either the activity or persistence of the microbes. It may thus be advisable to screen soil cores for promising rhizobia where strains compete with native microbes and are subjected to similar soil chemical and physical conditions as might be experienced in the field (Sylvester-Bradley et al. 1983). Under no circumstances should results from N-free media be used to extrapolate amounts of N_2 fixed by a legume (or other N_2-fixing plants) under field conditions.

N balances in field experiments

4.3

In field studies, N balances of systems are normally measured over several years and can provide an integrated measurement of net input of N that approximates N_2 fixation. Due to the large amounts of N in soil organic matter, varying from around 800 kg N/ha in degraded sandy soils to more than 12,000 kg N/ha in rich clay soils, accurate estimation of this pool is essential. Errors in estimating bulk density, or problems with standardising methods for measuring soil N from year to year, often introduce errors into

long-term measurements of N balances. Nitrogen balances measured over a single season are unlikely to provide reliable estimates of N_2 fixation because it is difficult to measure small changes in total N against the large bank of soil N. An example N balance from a field experiment is shown in Figure 10. Note that differences in soil total N could not be detected after 3 years.

Details of sampling procedures and calculations required to quantify soil total N are given in section 3.5 and in Anderson and Ingram (1993) and Dagliesh and Foale (2000). Soil sampling should be conducted to the full depth of rooting of the deepest crop grown in the system (as much as 2–3 m if perennials are involved). Analysis methods are also important in terms of sample size, with larger sample sizes giving more reliable results. Quality control of sample analysis over time (years) is particularly important. If at all possible, initial and annual soil samples should be archived for re-analysis, or samples

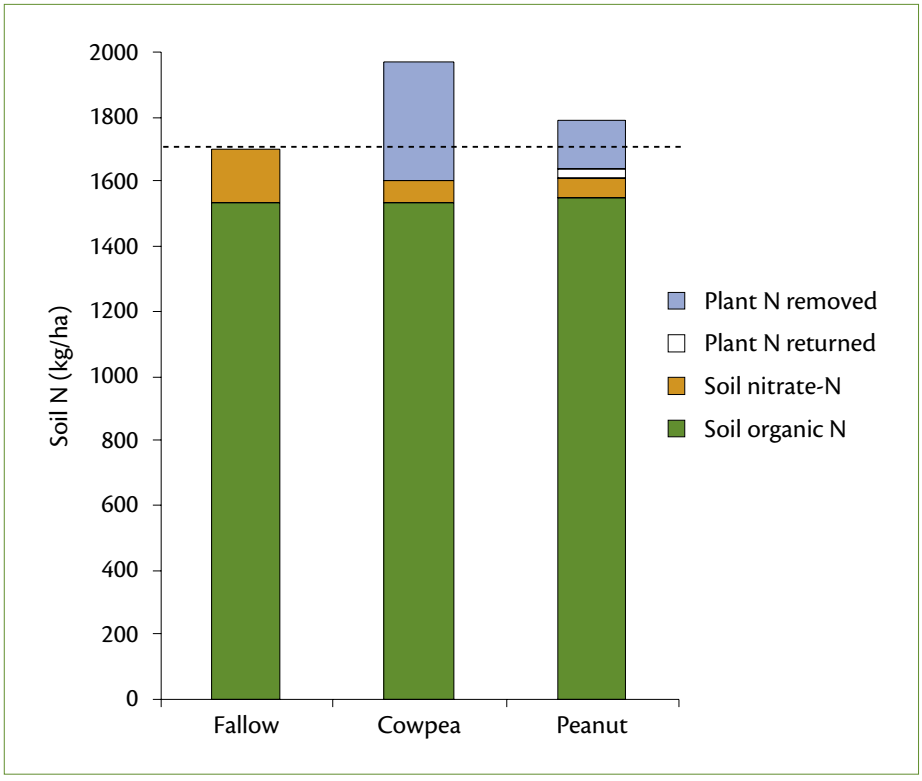


Figure 10. Field-based N balances for three cropping systems. Crops were grown for three consecutive years, with cowpea cut for forage and peanut grown as a grain crop (from Wetselaar et al. 1973). Compared with the fallow, both systems with legumes returned a positive N balance.

for all years analysed at a single time. Since soil is notoriously heterogeneous, sampling and bulk density estimations are likely to be the largest sources of error in field N balance studies, and many hundreds of soil samples and analyses are likely to be required if differences in soil total N between two points in time or treatments are to be validated (see e.g.Vallis 1973; Chalk 1998). For field studies it is important to define clearly the boundaries of the system, e.g. soil depth, as all subsequent input/output measures need to be taken accordingly.

Subsoil exploitation or uptake from deep water sources

This is also difficult to assess, and at least some root investigations are necessary to ascertain whether plants are able to access sources of N deep in the soil profile. The majority of soil N is present in organic forms in the surface horizons. However, water rich in nitrate (NO_3^-) can be present in the soil at depth, and the uptake of this N may be seen as 'apparent N_2 fixation' if not accounted for. Extraction of subsoil N is most likely in long-lived perennial plants on lighter textured soils where water and N tend to move more freely.

4.3.1 N inputs

Fertiliser and manure N inputs

Any N supplied to the system under study as manure or fertiliser must be included in the N balance calculations. The N contents of fertilisers vary to some extent with manufacturer. Urea fertiliser is typically 46% N and di-ammonium phosphate (DAP) 18% N. Manures are much more variable in N concentration. Farmyard and cattle manure averages about 0.6% N by weight, goat manure 2.8% N, chicken and sheep manure about 1.5% N and pig slurry about 0.2–4% N (Farnworth 1997). Manures must be analysed for their N content and the total amount of N added to the system in fertilisers, manures and other organic sources (e.g. leaf litter and compost) needs to be calculated for each year, and then all years summed for the period of study.

Atmospheric deposition

Inputs of N from the atmosphere occur both as wet (N in precipitation) and dry deposition (particulate material or direct adsorption of NH_3 onto leaf surfaces and soil). Wet N deposition is usually measured by continuous sampling of rainfall and determination of the concentration of N in the collected precipitation. Dry N deposition can be measured using simple acid traps that give integrated measurements over time (Schjoerring 1998).

Atmospheric inputs from wet and dry deposition should be ignored in N balance calculations only when they are likely to be insignificant compared with the expected inputs of N via N_2 fixation. Nitrogen inputs via rainfall are likely to be in the order of 3–5 kg N/ha per year in drier environments (McNeill and Unkovich 2007) and up to 50 kg N/ha per year in wetter, polluted environments (Giller and Merckx 2003).

Sedimentation, run-on and irrigation water

Inputs from run-on and sediments are difficult to assess. If plots are irrigated, the volumes of water used by the crops can often be large, so that even a small concentration of mineral N in the irrigation water can amount to a substantial input, particularly if the experiment is conducted over a number of years. The N concentration of irrigation waters should be measured and N inputs calculated.

4.3.2 N outputs (outflows)

Leaching

Nitrogen leaching is the process whereby NO_3-N moves down the soil profile with drainage water. It is more common in sandy soils, in high rainfall environments and under irrigation. Estimation of N leaching requires an accurate water balance to assess drainage of water through the soil, together with measurements of the concentration of NO_3-N in the leachate (e.g. Anderson et al. 1998). As it is difficult to measure and sample drainage water continuously, simulation models can be used to integrate point measurements to amounts of N leached over a period of time (Addiscott et al. 1991). A simpler, time-integrated measurement is provided by resin cores installed at the boundary soil depth (e.g. McNeill and Fillery 2008). Leaching is most likely to occur under fallows or annual crops when high-intensity rainfall occurs and the soil profile is wetted to field capacity.

Erosion and run-off

Detailed measurements of nutrient losses through erosion and run-off require special plots and instrumentation (e.g. Bonilla et al. 2006). Erosion losses can be estimated using the universal soil loss equation (for further details see Morgan 1986).

Long-term field experiments in which N_2-fixing plants are grown side-by-side with non N_2-fixing plants are useful for estimating N_2 fixation using the N balance and N difference methods. The N balance method makes a comparison *within a system between times*, and the N difference method compares two *different systems at one point in time*.

Gaseous losses

Nitrogen can be lost to the atmosphere through denitrification (as gaseous oxides of N or N_2) or through volatilisation of NH_3. Both processes are highly dynamic, depending on the N concentrations of the substrate (NO_3^- or NH_4^+) and environmental conditions. Denitrification is a microbial process that takes place mostly under anaerobic (waterlogged) conditions, and is highly sensitive to temperature and the redox potential. Ammonia volatilisation is a chemical process that occurs only at high pH and is sensitive to wind speed, largely through effects on the NH_3 concentration gradient. Neither is easy to measure in the field. The principal sources of NH_3 in agriculture are urea fertilisers and animal urine. As much as 30–50% of urea N may be lost as NH_3 when the fertiliser is applied to the surface of alkaline soils. Similarly, 20–30% of urinary N may be lost as NH_3 during warm conditions (i.e. >20 °C, see Bolan et al. 2004).

Grazing systems

It is more difficult to construct reliable N balances with livestock in the system. If a cut-and-carry system is used to feed animals, then a budget can be constructed relatively easily, but it is not so easy if animals are grazed on plots. More than 70% of the N ingested by animals is excreted as urine and dung and, if not returned to the plots under study, would need to be accounted for as an N output.

Crop and animal removal in harvested products

With crops, substantial amounts of N are transferred out of the system in the harvested grain, ranging from as little as 20 kg N/ha to >200 kg N/ha. In grazing systems, the harvested products are meat and wool, typically accounting for 5–50 kg N/ha per year. These amounts can be relatively easily and accurately recorded.

4.3.3 Final word

Due to the difficulty in measuring many inflows and outflows of N, the N balance method can provide only an indication of potential N_2 fixation by legumes in field studies. It is not a direct measure of N_2 fixation. The method can grossly underestimate N_2 fixation in many situations (e.g. where there are large unmeasured losses of N), and overestimate it in others (e.g. where all external inputs of N have not been quantified, or where changes in soil bulk density over time as a result of compaction have not been accounted for). The method is probably more useful for examining system fertility management over long periods, rather than quantifying N_2 fixation. Where substantial N_2 fixation is indicated, other, more reliable methods should be applied for confirmation. Under glasshouse conditions the technique can be quite robust.

5

Nitrogen difference method

5.1 How it works

Nitrogen difference can be used to compare total N accumulation by N_2-fixing plants and non N_2-fixing plants grown in pots in a glasshouse or in the field. The simplest form of the methodology is depicted in Figure 11. Ideally, the non N_2-fixing (e.g. uninoculated plants or non-nodulating mutant) and N_2-fixing plants will be of the same species. In practice it is difficult to prevent contamination with rhizobia (in the case of legumes) and infection of plants, especially in soils, and so species incapable of N_2 fixation are more commonly used.

Principal assumptions

The principal assumption of the methodology is that the N_2-fixing plants and non N_2-fixing (reference) plants use exactly the same amount of soil N, and that the total N of the N_2-fixing plants is never less than that of the non N_2-fixing plants. To mimimise differences in soil N uptake by N_2-fixing and reference plants, the reference species needs to be as close as possible in phenology (development rate) and stature (particularly rooting intensity and depth) to that of the N_2-fixing plant. The technique is most effective at low soil N fertility where differences in soil N uptake will be small and dependence on N_2 fixation is likely to be large.

5.2 Calculating N difference

A non N_2-fixing (control or reference) plant is used to approximate the amount of available soil N taken up by the N_2-fixing plant. The amount of N_2 fixed is calculated as the difference in uptake of N of the N_2-fixing and reference plants (equation (3)).

$$N_2 \text{ fixed} = \text{N yield } N_2\text{-fixing plant} - \text{N yield reference plant} \quad (3)$$

The non N_2-fixing reference plant can be a non-legume, a non-nodulating legume of the same species as the N_2-fixing plant (e.g. a mutant not able to nodulate) or an uninoculated legume in a system without a background population of compatible rhizobia.

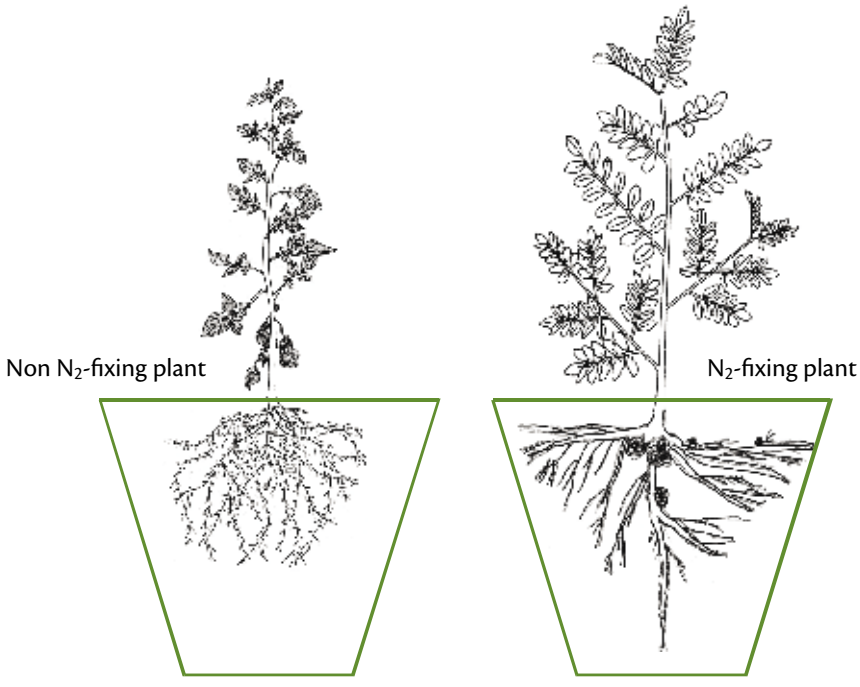


Figure 11. The N difference method for estimating N_2 fixation in a pot study. The difference in total N accumulation between the N_2-fixing and non N_2-fixing plants grown in the same soil is attributed to N_2 fixation. The assumption here is that the non N_2-fixing and N_2-fixing plants extract the same amount of N from the soil.

A modification of the procedure has been suggested to improve the accuracy of the methodology for legumes when the legume and reference plant are not well matched in terms of soil N uptake (Evans and Taylor 1987). In this method the difference in postharvest soil mineral N is also determined in the N_2-fixing and reference plots, and added to the difference in total N yield of the two crops. Thus, equation (3) becomes (equation (4)):

$$N_2 \text{ fixed} = (\text{N yield } N_2\text{-fixing plant} - \text{N yield reference plant}) + (\text{soil mineral N under } N_2\text{-fixing plant} - \text{soil mineral N under reference plant}) \quad (4)$$

The use of this modified equation assumes that mineralisation, leaching and denitrification are identical under each crop or species. These assumptions may not always be valid under field conditions.

An example of the use of the N difference method is presented in Table 6. Here, the choice of non N_2-fixing species (wheat or linseed) as the reference for lupin greatly affected the estimate of N_2 fixation. The inclusion of postharvest soil mineral N to 20 cm depth as in equation (4) did not greatly reduce the disparity between the two reference crops. The accuracy may have been improved by measuring soil mineral N to 100 cm depth (Evans and Taylor 1987). However, from a comparison of the amounts of N accumulated by the two control species (Table 6), it could be suggested that linseed growth was limited by factors other than N. Thus, wheat might have been a more appropriate reference. In the original publication (Evans et al. 1987), it was noted that the linseed ceased growing before either the lupin or wheat. This adds weight to the fact that wheat was probably the more suitable reference plant. The data highlight the significant impact the choice of reference plant has on N difference estimates of N_2 fixation. This is the major limitation of the methodology.

Table 6. Calculating N_2 fixation by lupin using N difference with two different non N_2-fixing reference species, with (equation (4)) and without (equation (3)) accounting for residual soil mineral N. Data are from Evans et al. (1987).

	N_2-fixing plant	Reference crop	
	Lupin	Wheat	Linseed
Total crop N	192	119	67
Postharvest mineral N (kg N/ha)	30	18	24
Lupin kg N fixed/ha excl. mineral N	Equation (3)	73	125
Lupin kg N fixed/ha incl. mineral N	Equation (4)	85	131
%Ndfa by lupin excl. mineral N	Equation (3)	38	65
%Ndfa by lupin incl. mineral N	Equation (4)	44	68

Application of the N difference method for estimating N_2 fixation

Isolines (cultivars) of some legumes are available that are genetically unable to nodulate, and these can be useful reference plants. However, the use of a non-nodulating isoline does not necessarily mean that it will have a similar N uptake pattern and rooting depth as the N_2-fixing isoline (Herridge 1982a). In cases of environmental stress (e.g. severe N or P deficiencies, pests), the growth of the two isolines might deviate significantly. Unfortunately, widespread application of such isolines is limited to a relatively small number of species for which non-nodulating isolines are available. In recent years, more non-nodulating legume lines have become available (Table 7).

Table 7. Legume species for which non-nodulating genotypes are available and which may make suitable reference plants for quantifying legume N_2 fixation (see Giller 2001)

Arachis hypogaea	Peanut
Cajanus cajan	Pigeonpea
Cicer arietinum	Chickpea
Glycine max	Soybean
Medicago sativa	Lucerne (alfalfa)
Phaseolus vulgaris	Common bean
Pisum sativum	Field pea
Stylosanthes guianensis	Stylo
Trifolium incarnatum	Crimson clover
Trifolium pratense	Red clover
Trifolium subterraneum	Subterraneum clover
Vicia faba	Faba bean
Vigna unguiculata	Cowpea

Uninoculated legumes can also be used as non N_2-fixing reference plants, but only where soils are devoid of native populations of rhizobia that are effective on the legume species of interest. This is sometimes possible where a legume species is newly introduced to a particular area, but care must be taken to

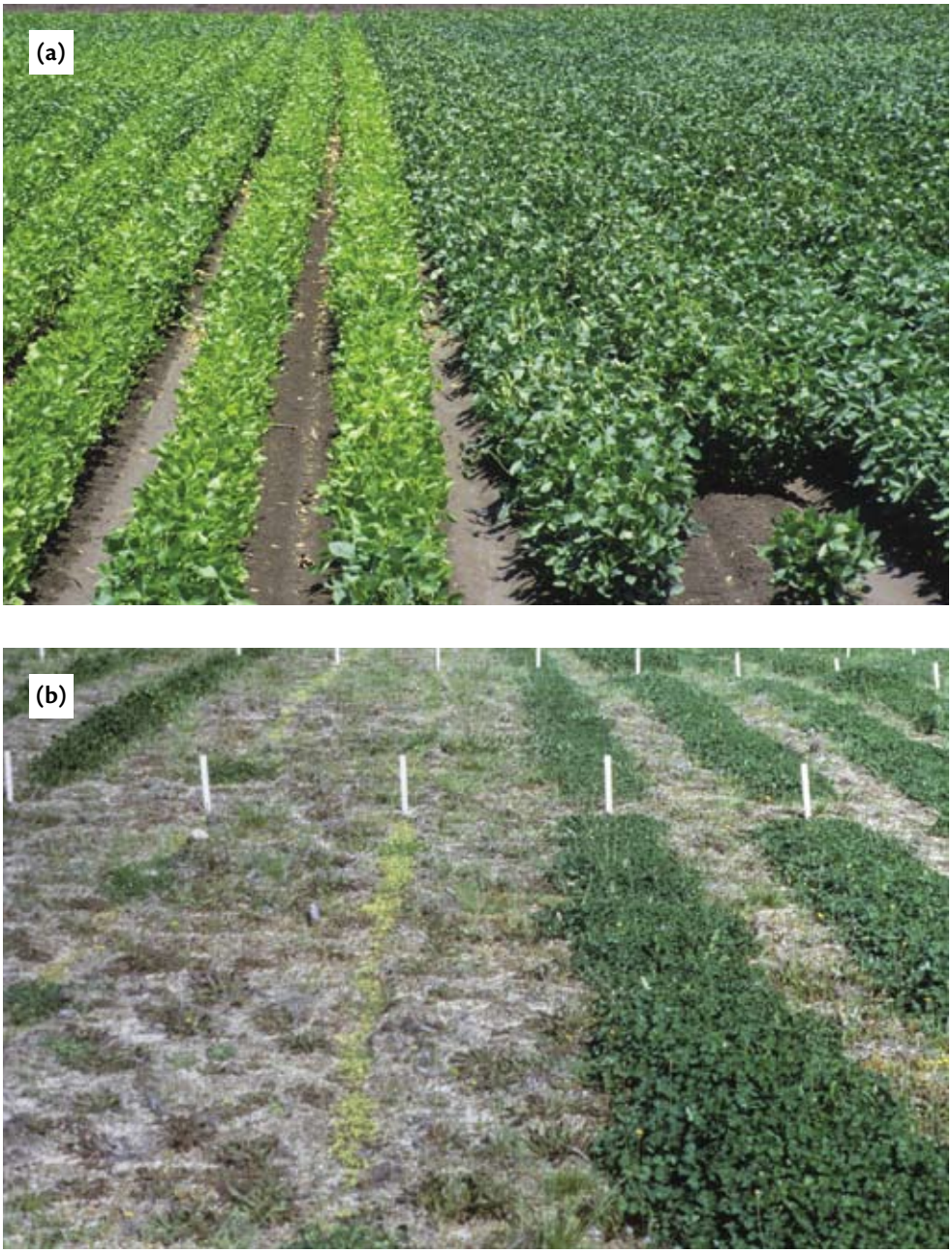


Figure 12. Using N difference to estimate N_2 fixation by field-grown (a) soybean and (b) subterranean clover. In both cases, the difference in total N accumulation between the inoculated and uninoculated plants was attributed to N_2 fixation.

Chickpea growing in free-draining plastic pots in a glasshouse. In this experiment, N_2 fixation by the chickpea was estimated as the difference in plant N between nodulated and unnodulated plants growing in separate pots.

ensure that no cross-contamination with the inoculated treatment occurs. The final option is using non-legume species such as highlighted in Table 6. Nodulation status of reference plants should always be assessed whenever non-nodulating or uninoculated legumes are used, to ensure that no nodulation and N_2 fixation has occurred (see Figure 4). This must be verified, not assumed. A field example of the use of uninoculated soybean for N difference is illustrated in Figure 12.

5.3.1 Glasshouse experiments

The N difference method can readily be used in glasshouse studies where plants are grown in pots containing soils collected from the field. The N_2-fixing plants and non N_2-fixing reference plants are grown in separate pots of equal

size with exactly the same amount of identical soil. Both treatments should receive equivalent watering and nutrients if required. A common problem with such pot systems is the increased N mineralisation that occurs on rewetting disturbed soils, which have possibly been sieved and air dried prior to use. The resulting accumulation of mineral N suppresses N_2 fixation and, in the case of the N difference method, obscures it. The use of intact cores that minimise disturbance and mineral N effects is one way of reducing this problem (Sylvester-Bradley et al. 1983).

5.3.2 Field experiments

In field studies the crops must be grown under identical soil conditions. This means ensuring that N_2-fixing and reference plants are sown in adjacent plots, in soil that has had exactly the same cropping history. While replicates of N_2-fixing and reference plant treatments are typically laid out in a randomised block design, having a reference plant plot adjacent to each N_2-fixing plant plot can reduce errors associated with spatial variation in plant-available soil N. The reference plant should not be fertilised with N if the N_2-fixing plants are not fertilised with N, and both should receive identical applications of P and other nutrients. The use of more than one non N_2-fixing reference species is recommended.

As the N difference method is yield dependent, it is particularly important that plots are sufficiently large to facilitate normal growth and to capture spatial variability in soil N fertility. Assessment of plant total N requires sampling for plant dry matter, and this is often the largest source of error with the N-difference technique. It may also be necessary to collect fallen leaves. Small micro plots as described later for isotope studies are not adequate and should be avoided. Sufficiently wide borders or guard rows should also be used to avoid 'edge effects'. Additional measurements such as those listed in section 2.3 are advisable.

When using uninoculated legumes as control plants, it is advisable to undertake all field preparations and sow the uninoculated treatment first. The inoculum (or inoculated seeds of the test plant) should be applied last and be covered with soil to avoid wind dispersion. The inclusion of drainage channels between plots to avoid cross-contamination via run-on is also recommended, especially in tropical areas where rainfall intensity can be high.

Spatial variability in soil fertility, and differences in growth potential and rooting depths between test and reference plant, can limit the successful application of this method. Nevertheless, as N yields are commonly measured in most experiments, the N difference method can often be used as a secondary assessment method with little extra effort.

Data interpretation

5.4

Two examples of N difference for estimating N_2 fixation are given in Table 8. The first example (Table 8a) is from a pot study involving common bean where three different grass species and a non-nodulating line of common bean were used as reference plants. Plant total N was highest in the nodulated legume, as would be expected, but there were large differences in the total N of the various reference plants. Wheat accumulated 40–70% more total N than the other three reference plants, which were similar. Based on these data, one would estimate N_2 fixation of the nodulated bean as the average of the estimates based on the three similar non N_2-fixing plants (non-nodulating bean, sorghum and guinea grass) and assume that the wheat was unusually aggressive in soil N uptake. This dataset highlights the value of (a) using multiple reference plants and (b) including a non-nodulating line of the legume under study as an additional reference plant.

Our second example (Table 8b) shows the application of the N difference method to estimate N_2 fixation by seven, field-grown, tropical forage legumes. Total N accumulation by the legumes ranged from 85 to 131 kg N/ha, and for the three grass species used as reference plants was in the range 33–56 kg N/ha. All the legumes accumulated more total N in shoots than the non-legumes and thus the principal assumption of the methodology was not compromised. Two of the grass reference species gave very similar estimates of N_2 fixation, while the grass with the highest total N accumulation (Tanner grass) yielded values mostly around 20 kg/ha lower. In this case it is not clear which reference plant provided the most accurate estimate (highlighting the need to collect additional data and use complementary approaches). The data do, however, allow a comparative analysis of N_2 fixation of the various forage legumes.

Advantages

5.5

The main advantages of the N-difference method are that it is cheap and easily applied in most situations, and it provides a time-integrated measure. While increased plant dry-matter accumulation and/or tissue N concentration indicates improved plant N nutrition, these measures alone are not sufficient evidence of N_2 fixation. Comparative N yields of N_2-fixing and non N_2-fixing plants can provide reliable evidence.

Table 8. Examples of N difference technique for estimating N_2 fixation in (a) a pot study and (b) a field study. Nitrogen fixed was calculated by subtracting total N of the non N_2-fixing reference plant from legume total N.

(a) Pot study of Viera-Vargas et al. (1995b)

	Plant	Total N (mg/pot)	N_2 fixed by common bean using each reference plant (mg/pot)
Legumes	Common bean	294	
	Non-nodulating common bean	182	112
Grasses	Wheat	275	19
	Sorghum	193	101
	Guinea grass	161	133

(b) Field study of Viera-Vargas et al. (1995a)

	Plant	Total shoot N (g/m^2)	Legume N_2 fixation estimated using the three different reference plants (kg N/ha)		
			Beard grass	Guinea grass	Tanner grass
Legumes	Centrosema (hybrid)	10.7	69	74	52
	Galactia	11.7	79	84	62
	Desmodium	10.2	64	69	47
	Pueraria	8.5	47	52	30
	Centrosema	11.2	74	78	56
	Stylo	13.1	93	98	76
	Soya perenne	8.8	50	54	32
Grasses	Beard grass	3.8			
	Guinea grass	3.3			
	Tanner grass	5.6			

Limitations

The major assumption behind this technique is that the N_2-fixing and non N_2-fixing reference plants remove identical amounts of N from the soil. This assumption is often compromised because the growth rates, rooting depth and soil-N demand differ between the two species. Matching growth rates, rooting depth and total uptake of soil N remains the major challenge of this methodology. Rooting profiles of legumes and grasses are commonly very different. Even if non-nodulating legumes are used as the reference, the N_2-fixing crop will often explore a greater volume of soil due to its higher plant vigour and associated larger root system, to acquire not only N but also other nutrients and water (Ruschel et al. 1979). In such a case it is likely that the N_2-fixing plant will take up more mineral N than the reference plant and an overestimation of N_2 fixation will occur. The opposite occurs (i.e. underestimation of N_2 fixation) with nitrate-tolerant N_2-fixing plants that continue to fix N_2 in the presence of high soil mineral N. For example, Turpin et al. (2002) showed that faba bean was significantly more tolerant of increasing soil nitrate than chickpea (Figure 13). However, adding the nitrate balance to the N difference method (equation (4)) should largely account for this.

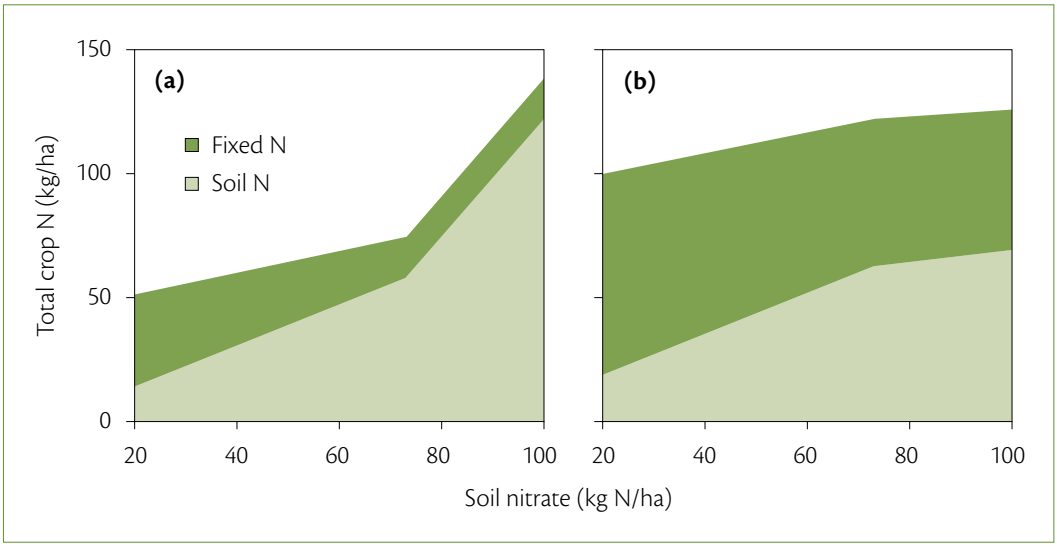


Figure 13. N_2 fixation and uptake of mineral N by field grown (a) chickpea and (b) faba bean at different levels of soil nitrate (from Turpin et al. 2002). Note that at the same levels of soil nitrate, substantially more N was fixed by faba bean than by chickpea.

Further limitations arise from the fact that the method relies on quantitative differences between treatments and thus has a low accuracy if only small differences in N yield occur, e.g. in short-term experiments (Vose et al. 1982). Where soil N or fertiliser N supply are relatively high, N_2 fixation may be obscured by sampling, analytical and other experimental errors (Giller 2001). The N difference method cannot be used if fertiliser applications vary between the N_2-fixing crop and non N_2-fixing control.

Intercropping systems

The N difference method can also be applied in intercropping or mixtures of fixing and non-fixing plants. The amount of N_2 fixed is then the difference in total N yield of the mixture (legume and non-fixing control) minus the N yield of the non-fixing monocrop, plus the difference in mineral N between the two systems. While the method may work reasonably with an added design element (maintaining the same spacing) or in temperate mixed pasture systems, it may work less satisfactorily in other systems. In tropical pastures, for example, the performance of legumes is often poorer than that of C4 grasses, leading to negative fixation rates.

5.7 Final word

The principal assumption that N_2-fixing and non N_2-fixing plants acquire the same amount of N from the soils is not robust and, in most cases (apart from N-free media), the N difference method can thus only approximate the amount of N_2 fixed. In some instances it has been shown to give similar estimates to more reliable methodologies but, in the absence of such complementary approaches, it cannot be relied upon. It is most robust when used in low-N soils and when differences in growth between the N_2-fixing and reference plants are large. Over-interpretation of results should be avoided and it is strongly recommended that the method be used in combination with others (e.g. ^{15}N, Chapter 7).

6

Ureide (N solute) method

Legumes export the majority of fixed N from root nodules, either as the amides asparagine and glutamine, or as the ureides allantoin and allantoic acid (Figure 14). Most legumes are amide exporters, with ureide exporters being restricted to species in the tribes Phaseoleae and Desmodieae within the Papilionoideae subfamily (Sprent 2001) (see Table 9). Thus, the ureide (N solute) method is restricted to the ureide-producing legumes and cannot be used for quantifying N_2 fixation across the broad range of other N_2-fixing plants (other legumes, and cereals and grasses).

Nitrogen is taken up from the soil by plant roots, principally as nitrate and/or ammonium ions. Once inside the plant, the N solutes derived from soil mineral N are transported in the xylem as free nitrate or, after nitrate reduction and/or ammonium metabolism in the root, as organic products (principally the amides asparagine and glutamine) (Figure 14). In most arable, agricultural soils where nitrification takes place rapidly, nitrate is the predominant N source for plants. Ammonium nutrition, however, could become important when legumes are grown in soils in which nitrification is inhibited, e.g. in anaerobic rice paddies, acidic soils. Early in the development of the methodology, the assumption was made that nitrate was the principal form of mineral N assimilated by nodulated legumes in the field, and

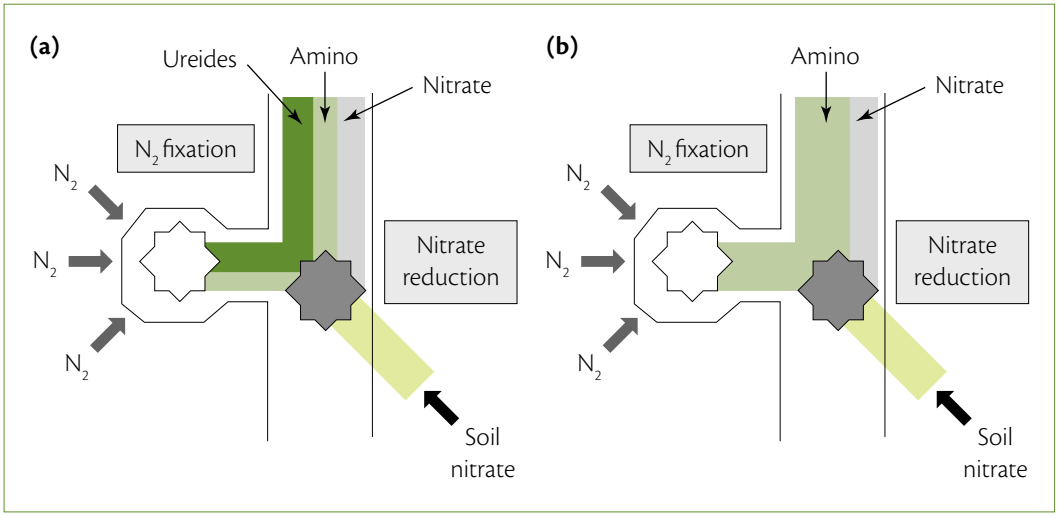


Figure 14. Assimilation and export of N from the nodulated roots of (a) ureide- and (b) amide-producing legumes. Note that the ureide species have three major groups of N solutes in the xylem stream—ureides, amino compounds and nitrate—while the amide legumes have only two—amino compounds and nitrate.

calibrations of the ureide technique were done accordingly (see section 6.4). Effects of ammonium N uptake on ureide export were, however, examined in some instances (e.g. Hansen et al. 1993; Alves et al. 2000c).

How it works

With the ureide method, the relative concentrations of the N solutes—allantoin, allantoic acid, amino compounds and nitrate—in readily sampled plant tissues and xylem sap reflect the sources of N currently (within 30 minutes) being assimilated by the legume. When calibrated, they can be used as a measure of the dependence of the plant on N_2 fixation (%Ndfa) (Herridge et al. 1978).

Sampling of stems, petioles, leaves and xylem sap, and subsequent analysis of their contents, are long-standing procedures for assessing the nutritional status of plants. Stems, petioles and leaves are readily collected and either dried for later analysis or immediately subjected to solute extraction. Xylem sap can be collected either as sap bleeding spontaneously under pressure from the stump of the intact root following decapitation of the shoot (root-bleeding sap), or under mild vacuum applied to freshly harvested shoots (vacuum-extracted sap).

Ureide plants

The presence of the ureides allantoin and allantoic acid in plants was established 80 years ago, although another 30 years passed before their role in transport of N in the xylem stream was clearly defined (Pate 1980). Research in Japan in the mid 1970s showed a strong relationship between root nodulation of soybean and ureide contents of plant tissues (Tajima and Yamamoto 1975). Subsequent $^{15}N_2$-labelling experiments with soybean and cowpea (Matsumoto et al. 1977; Herridge et al. 1978) confirmed that the ureides were synthesised principally in the nodules of legumes and were the major transport compounds involved in the export of fixed N from the nodules to shoot tissues (Figure 14). In the following decade, much attention was focused on the mechanisms of allantoin and allantoic acid synthesis and utilisation in legumes.

At the same time, research was conducted at a number of laboratories to quantify the link between N_2 fixation activity of the legume and the relative abundance of ureides as a potential assay of N_2 fixation (McClure et al. 1980; Pate et al. 1980; Herridge 1982b; Patterson and LaRue 1983). Different laboratories established that the composition of N solutes in xylem sap and aqueous stem extracts changed from one dominated by ureides in N_2-dependent plants to

Table 9. Legumes that transport either ureides or amides in xylem sap as the dominant products of N_2 fixation

Ureides (allantoin, allantoic acid)	Amides (asparagine, glutamine)
Grain legumes	
Soybean (*Glycine max*)	Chickpea (*Cicer arietinum*)
Pigeon pea (*Cajanus cajan*)	Lentil (*Lens culinaris*)
Mung bean (*Vigna radiata*)	Pea (*Pisum sativum*)
Black gram (*Vigna mungo*)	Faba bean (*Vicia faba*)
Adzuki bean (*Vigna angularis*)	Lupin (*Lupinus angustifolius*)
Cowpea (*Vigna unguiculata*)	Groundnut (*Arachis hypogaea*)
Moth bean (*Vigna aconitifolia*)	Vetch (*Vicia sativa*)
Rice bean (*Vigna umbellata*)	Grasspea (*Lathyrus sativus*)
Bambara groundnut (*Vigna subterranea*)	
Common bean (*Phaseolus vulgaris*)	
Lima bean (*Phaseolus lunatus*)	
Runner bean (*Phaseolus coccineus*)	
Tepary bean (*Phaseolus acutifolius*)	
Winged bean (*Psophocarpus tetragonolobus*)	
Guar (*Cyamopsis tetragonoloba*)	
Hyancinth bean (*Lablab purpureus*)	
Kersting's groundnut (*Macrotyloma geocarpum*)	
Forage and tree legumes	
Codariocalyx gyroides	Medics (*Medicago* spp.)
Desmodium spp.	Clovers (*Trifolium* spp.)
Desmodium rensonii	*Sesbania* spp.
Siratro (*Macroptilium atropurpureum*)	*Gliricidia sepium*
Calopogonium caeruleum	*Prosopis juliflora*
Centrosema pubescens	*Leucaena* spp.
Kudzu (*Pueraria phaseoloides*)	*Calliandra calothyrsus*
Hardenbergia spp.	*Acacia* spp.
	Crotalaria spp.
	Flemingia macrophylla
	Stylosanthes spp.

Sources: Hansen and Pate (1987); Peoples et al. (1989b); Giller (2001)

one dominated by nitrate and amino-N in plants utilising soil N (Figure 15). In calibration experiments, the relationship between the percentage of N of xylem sap or stem extracts as ureides (termed relative ureide-N) and %Ndfa was shown to be close to unity (e.g. McClure et al. 1980; Herridge and Peoples 1990).

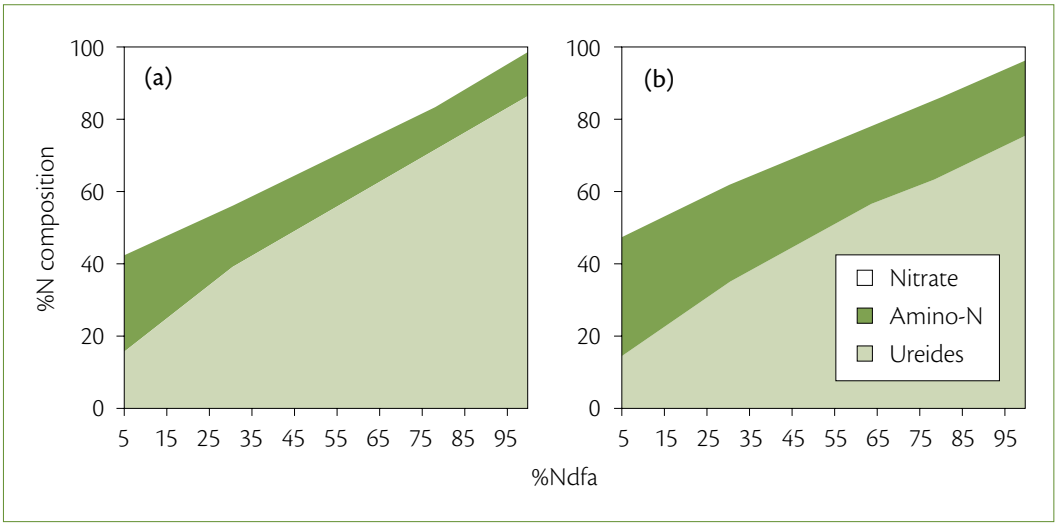


Figure 15. Changes in the composition of N solutes in (a) root-bleeding and (b) vacuum-extracted xylem sap of nodulated soybean, supplied with different concentrations of ^{15}N-labelled nitrate to generate different values of %Ndfa. Data are from Herridge and Peoples (1990).

Amide plants

Most of the legumes, however, are not ureide exporters, but rather export fixed N from the nodule and transport it in the xylem stream as the amides asparagine and glutamine. The response of amide-exporting legumes to changes in N source is less well defined than for ureide producers, as the products of N_2 fixation and nitrate uptake are essentially the same (Figure 14). Some amide exporters have a high capacity for nitrate reduction in their roots, and the spectrum of N compounds transported in the xylem stream is not greatly altered if the plant's source of N changes from atmospheric N_2 to soil mineral N (see Hansen and Pate 1987; Peoples et al. 1987). In other species, however, the proportion of the nitrate reduced in the roots is much lower and there is a progressive increase in xylem nitrate and decrease in amide and amino acid N as plants increasingly rely upon soil nitrate (Peoples et al. 1986). Under these conditions, the relative amount of nitrate in xylem sap reflects the contribution of soil N to plant growth.

Details of calibration experiments for the amide plants—groundnut, chickpea, lentil, pea, faba bean and lupin—can be found in Peoples et al. (1987, 1996) and Herridge (1988). Except for the report of Herridge and Doyle (1988) that showed good agreement between solute-based estimates of N_2 fixation of lupin and other methods, field calibrations for amide exporters have not been explored.

6.2 Calibrations of relative ureide-N and %Ndfa

Calibration experiments typically involve growing the particular legume in large pots containing an N-free medium such as sand, supplied with a range of ^{15}N-labelled nitrate nutrient solutions (e.g. 0–8 mM nitrate (Herridge and Peoples 1990)) to induce a range of %Ndfa levels. The plants are sampled at intervals throughout growth for measurement of plant N, %Ndfa using ^{15}N isotope dilution (see Chapter 7) and ureides, amino-N, and nitrate in xylem sap and plant tissues. The relative abundance of ureide-N in xylem sap is calculated as (equation (5)):

$$\text{relative ureide-N (\%)} = 400a/(4a + b + c) \quad (5)$$

where a is the molar concentration of ureides (ureides contain four N atoms per molecule), b the molar concentration of nitrate-N and c the molar concentration of α amino-N (Herridge 1984). Takahashi et al. (1992) proposed using $2c$ rather than c in the equation to account for the two N atoms of the amides. Ohtake et al. (1995) showed subsequently that the average number of N atoms in the amino fraction of soybean xylem sap was 1.7. However, there appears to be no reduction in accuracy or precision in assuming a single N atom for α amino-N (D.F. Herridge, unpublished data). Thus, all the calibrations listed in Table 2 assume one N atom per α amino molecule, i.e. they use c, rather than $2c$ or $1.7c$.

The relative abundance of ureide-N in extracts of whole stems or stem segments is calculated as (equation (6)):

$$\text{relative ureide-N (\%)} = 400a/(4a + b) \quad (6)$$

where a and b are, respectively, the molar concentrations of ureides and nitrate. Note here that α amino-N is not included in the equation, principally because the relationship between relative ureide-N and %Ndfa was sufficiently robust without it (Herridge 1982a,b; Herridge and Peoples 1990).

Farmers inspecting a legume crop in Zimbabwe. Many of the tropical legumes are ureide exporters, which means that the ureide method can be readily used to quantify N_2 fixation.

Examples of relationships between relative ureide-N in xylem sap, collected as both root-bleeding sap and vacuum-extracted sap, and %Ndfa are shown in Figure 16. Species are cowpea and mung bean, and data were collected from vegetative through to late pod-fill stages of growth. Clearly, ureide-N in xylem sap and %Ndfa are highly correlated, but are quite different for the two species and for the different forms of xylem sap. Because low levels of ureides are detected in non N_2-fixing ureide producers (typically about 10%), the calibration lines do not pass through the origin. Calibrations have now been published for a number of species of crop, forage and shrub legumes (Table 10).

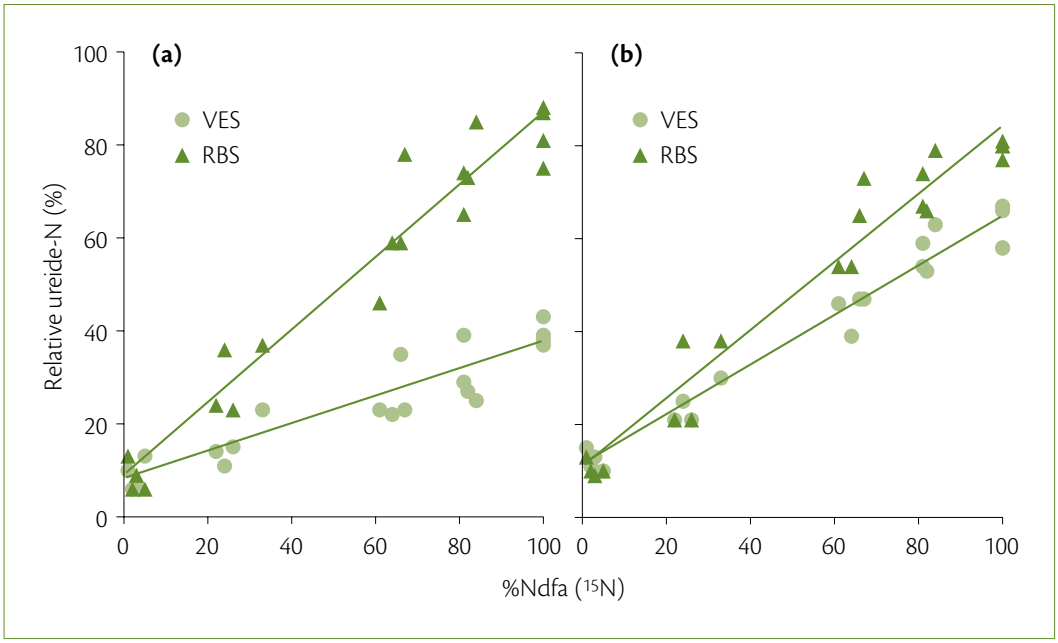


Figure 16. Relationships between %Ndfa and relative abundance of ureide-N in vacuum-extracted sap (VES) and root-bleeding sap (RBS) of (a) cowpea and (b) mung bean (green gram). Data are from Herridge and Peoples (2002a).

Ureide-based estimates of N_2 fixation can be crosschecked with ^{15}N-based estimates as follows:

Perennial plants

One potential limitation of the ureide method is that each measurement reflects N_2 fixation at, or shortly before, the time of assay. In the cases of tree and perennial fodder legumes this is unlikely to be important because the periods of study are often the intervals between coppicing, cutting or grazing. For example, Peoples et al. (1996) reported very similar ureide- and ^{15}N-derived %Ndfa values for the shrub legume codariocalyx (*Codariocalyx gyroides*) for a series of growth periods between coppicing (Figure 17A). This was despite the fact that the point-of-time ureide assay provided a %Ndfa value at the specific time of sampling, in contrast to the ^{15}N-derived value that was an average for the interval between the current and previous samplings.

Similarly, Alves et al. (2000c) reported, for the perennial forage legume *Desmodium ovalifolium*, almost identical estimates of %Ndfa from the ureide assay based on aqueous stem extracts and ^{15}N isotope dilution. Values for

Table 10. Species for which the ureide method has been calibrated. In each case, %Ndfa was calculated using ^{15}N isotope dilution.

Species	Calibration equation (y = RU-N; x = %Ndfa)	Sap/ tissue[a]	Reference
Grain legumes			
Soybean	$y = 0.64x + 7.7$ (veg., flower.) $y = 0.64x + 15.9$ (pod-fill) $y = 0.83x + 4.8$ (veg., flower.) $y = 0.67x + 21.3$ (pod-fill) $y = 0.0057x^2 + 0.31x + 1.4$ (veg., flower.) $y = 0.0034x^2 + 0.50x + 10.7$ (pod-fill) $y = 1.08x - 9.0$ (well watered) $y = 0.74x + 26.0$ (pod-fill)	VES VES RBS RBS Stem Stem Petiole Petiole	Herridge and Peoples (1990) Purcell et al. (2004)
Pigeon pea	$y = 0.51x + 5.8$ (veg., flower.) $y = 0.51x + 13.5$ (pod-fill)	VES VES	Peoples et al. (1989a)
Rice bean	$y = 0.0066x^2 - 0.06x + 7.6$ $y = 0.0086x^2 - 0.12x + 13.9$	VES RBS	Rerkasem et al. (1988)
Cowpea Mung bean, black gram Cowpea, mung bean, black gram	$y = 0.31x + 7.2$ $y = 0.49x + 11.7$ $y = 0.75x + 8.6$	VES VES RBS	Herridge and Peoples (2002a)
Common bean	$y = 0.94x - 25.2$ (veg.) $y = 0.83x - 5.3$ (flower, pod-fill)	RBS RBS	Hansen et al. (1993)
Common bean	$y = 0.54x + 3.3$ $y = 0.72x - 0.10$	VES RBS	Herridge and Peoples (unpublished data)
Tepary bean	Equation not provided	RBS	Crews et al. (2005)
Forage and tree legumes			
Desmodium ovalifolium *Centrosema* sp.	$y = 0.89x - 2.1$ (sand culture) $y = 1.04x + 7.43$ (soil culture) $y = 0.85x - 2.6$ (sand culture)	Stem Stem Stem	Alves et al. (2000a,b)
Desmodium rensonii *Codariocalyx gyroides*	$y = 0.45x + 18.3$ $y = 0.28x + 8.5$	VES VES	Herridge et al. (1996)

[a] VES, vacuum-extracted sap; RBS, root-bleeding sap; Stem, extract of dried stem

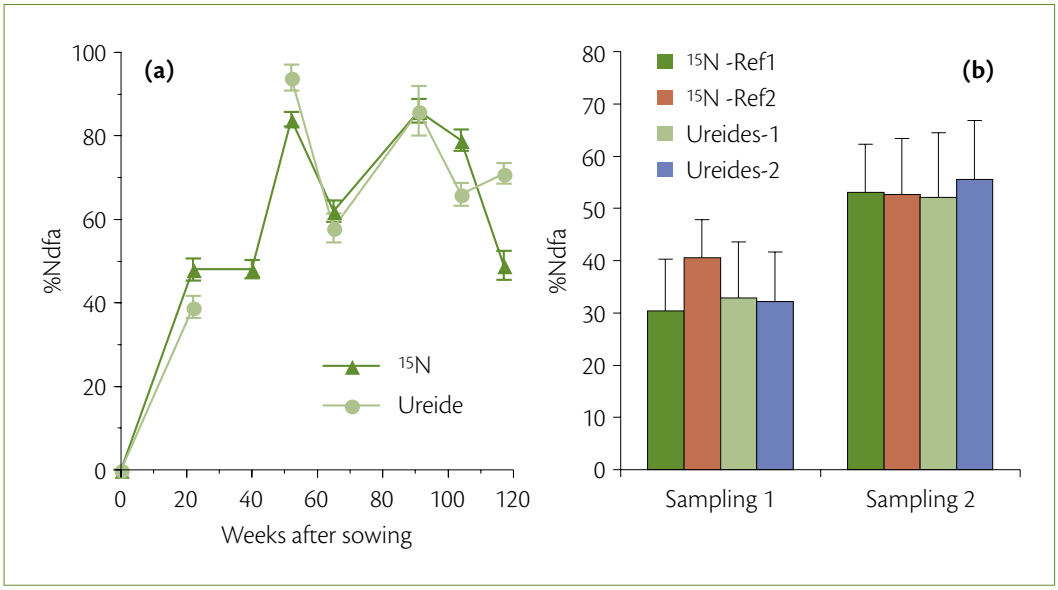


Figure 17. Crosschecking ureide and ^{15}N methods for determining %Ndfa for (a) *Codariocalyx gyroides* grown in the field in northern Australia (Peoples et al. 1996) and (b) *Desmodium ovalifolium* grown in the field in Brazil (^{15}N-Ref 1 *Brachiaria humidicola* used as the non N_2-fixing reference; ^{15}N-Ref 2 *Panicum maximum* used as the non N_2-fixing reference; Ureides -1 and Ureides-2 refer to calibrations in inoculated and uninoculated soils, respectively) (Alves et al. 2000c)

%Ndfa for sampling time 2, for instance, were 53% for both ^{15}N estimations (^{15}N-Ref1 and ^{15}N-Ref2) and 52% and 56% for the two ureide-based estimates (Ureides-1 and Ureides-2) (see Alves et al. (2000c) for methodological details). From these examples it appears that the ureide method and ^{15}N-based methods can provide very similar estimates of %Ndfa for perennial legumes when the objective is to assess N_2 fixation during specific growth periods.

Annual plants—estimating N_2 fixation inputs

With annual crop legumes, a common objective is to determine inputs of fixed N during the legume phase of the cropping system. Thus, the total amount of N fixed by the legume needs to be quantified. Recommended protocols to obtain this information involve multiple (as many as 6–8) samplings of plants for xylem sap or stem segments at regular intervals from mid vegetative to late reproductive stages. Seasonal profiles of %Ndfa are then calculated from the seasonal profiles of relative ureide-N of xylem sap or stem segments using newly established calibrations or the calibrations in

Table 10. Next, the %Ndfa values are used to partition accumulated crop N, estimated from repeated biomass samplings, between fixed N and N taken up from the soil (see example dataset in section 6.9). Thus, Herridge et al. (1990) reported reasonably good agreement between ureide- and ^{15}N-determined %Ndfa values for six soybean genotypes grown at five field sites in Australia using this approach (Figure 18). Estimates of %Ndfa were in the ranges 6–56% (ureide) and 0–68% (^{15}N).

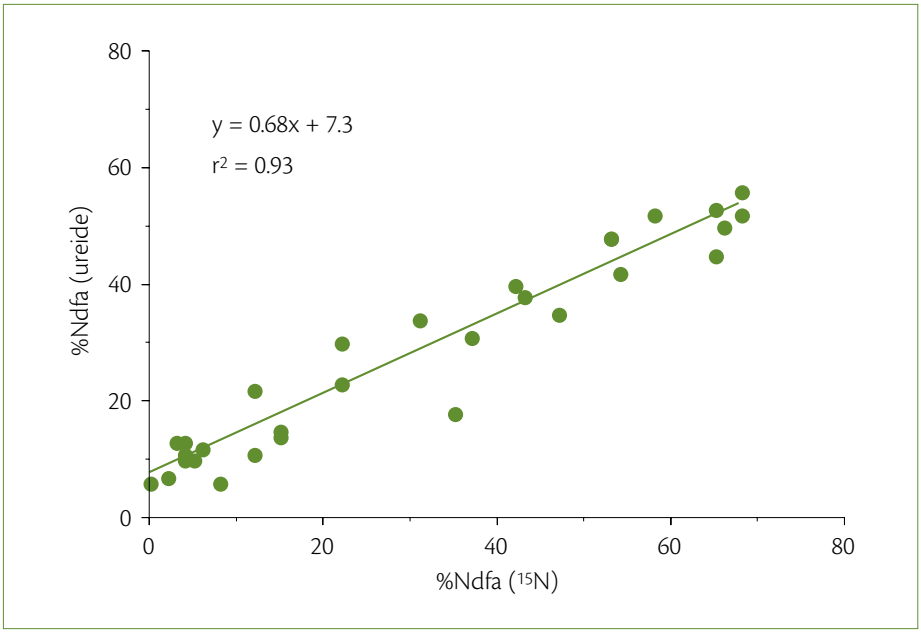


Figure 18. Correlation between %Ndfa of soybean genotypes, estimated using stem and xylem ureide (multiple samplings) and natural ^{15}N abundance (single sampling just prior to physiological maturity) techniques (see section 8.1). Source: Herridge et al. (1990)

The ureide method would have more appeal if the number of samplings could be reduced to only one or two. The ^{15}N methods integrate all N_2 fixation activity from the onset of growth until the time of sampling, so total N fixed can be estimated by sampling the plants only once at the time of maximum biomass N (late pod-fill, just before physiological maturity for most annual plants). A single sampling of xylem sap or stem segments for N-solute analysis would not necessarily coincide with the sampling for maximum biomass N and ^{15}N, but would be done when the point-of-time, ureide-determined %Ndfa had a similar value to the integrative, ^{15}N-determined %Ndfa.

To advance this proposition, Herridge and Peoples (2002b) reported two studies in Australia involving soybean, cowpea, mung bean and black gram that showed %Ndfa could be estimated with reasonable accuracy from a single sampling of xylem ureides during early pod-fill (R3.5) (see Fehr et al. (1971) for descriptions of the various stages of development for soybean). Estimates from the R3.5 sampling were compared with ^{15}N-determined %Ndfa, assessed at the time of maximum biomass N (R6–7) (see Figure 19).

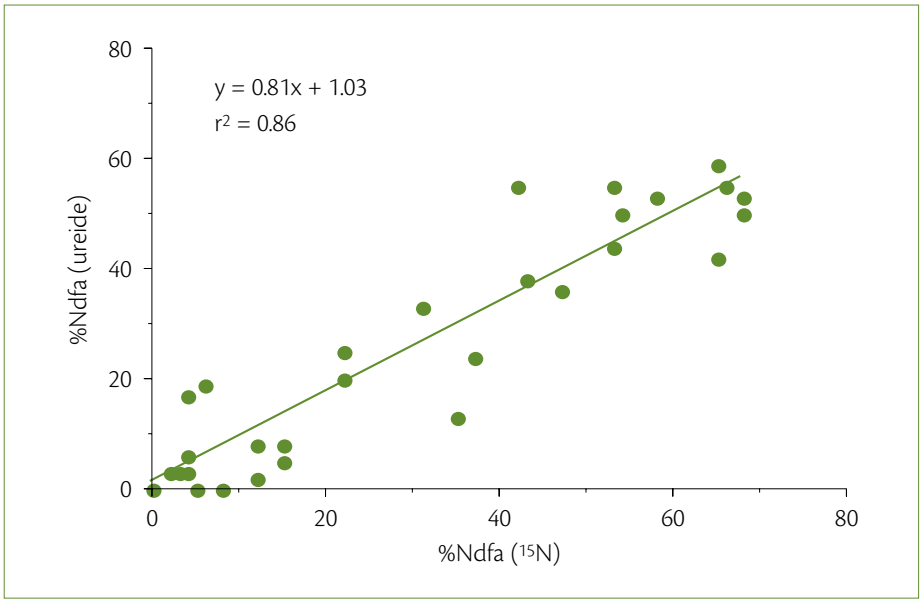


Figure 19. Correlation between %Ndfa of soybean genotypes, estimated using xylem ureide (single sampling at R3.5) and natural ^{15}N abundance techniques (single sampling at R6.5) (see section 8.1). Source: Herridge et al. (1990)

Clearly, there was good agreement between the two methods, suggesting that a single sampling for N solutes during early pod-fill can provide a reasonable estimate of %Ndfa. It is interesting that LaRue and colleagues (Patterson and LaRue 1983; Glenister and LaRue 1986) concluded almost 20 years ago that the relative ureide contents of petioles and stems at R3 were highly correlated with seasonal N_2 fixation.

Although the profiles of relative ureide-N of xylem sap and stem segments and %Ndfa (ureide) of all four species in the two Herridge and Peoples (2002b) field studies were relatively uniform, a period of stress, coinciding with the critical R3.5 sampling, could create unacceptable errors (e.g. drought

A soybean crop in Australia. Most of the published applications of the ureide method for assessing N_2 fixation involve soybean. However, the technique can be equally applied to any of the ureide exporters, which are listed in Table 9.

stress, see Purcell et al. 2004). Furthermore, rapid changes in %Ndfa (ureide) of the short-duration cowpea, mung bean and black gram during pod-fill highlighted the need for accurate assessment of phenology and timely sampling. Substantial error could result from sampling just a week too early or too late. For these reasons, multiple samplings of plants for sap and stem segments are still recommended, as shown in the example in section 6.9, although in situations where sampling is restricted (see below) it should be done during early pod-fill.

Annual plants—comparing treatments for N_2 fixation activity

When comparing treatments for N_2 fixation, as, for instance, would be the case when evaluating species or genotypes of legumes or strains of rhizobia for symbiotic competence, a single sampling for xylem sap or stem segment N solutes can be effective. Examples of this scenario can be found in the

published experiments of Herridge and Rose (2000), involving 13 soybean genotypes at three sites, and Song et al. (1995), involving nine genotypes of soybean at one site. In both cases, relative ureide-N values from early pod-fill samplings (physiological stage R2–R4) were highly correlated with $\delta^{15}N$ values of shoots and grain (data from Herridge and Rose (2000) shown in Figure 20).

6.3 Applications of the ureide technique

The studies described above provide examples where the ureide technique was cross-referenced with ^{15}N techniques to quantify %Ndfa and, in some instances, amounts of N fixed. In other studies, the ureide technique was used as the sole means of quantifying seasonal N_2 fixation (e.g. Hughes and Herridge 1989; Ying et al. 1992; Wang et al. 1993).

The technique has also been used as a relative measure of N_2 fixation activity, i.e. to compare treatment effects on N_2 fixation. For example, Hungria et al. (2006) reported that inoculating soybean in established soybean-growing regions of Brazil increased N_2 fixation (relative ureide-N of stem segments) by about 6% and grain yield by about 5%. Shutsrirung et al. (2002) measured

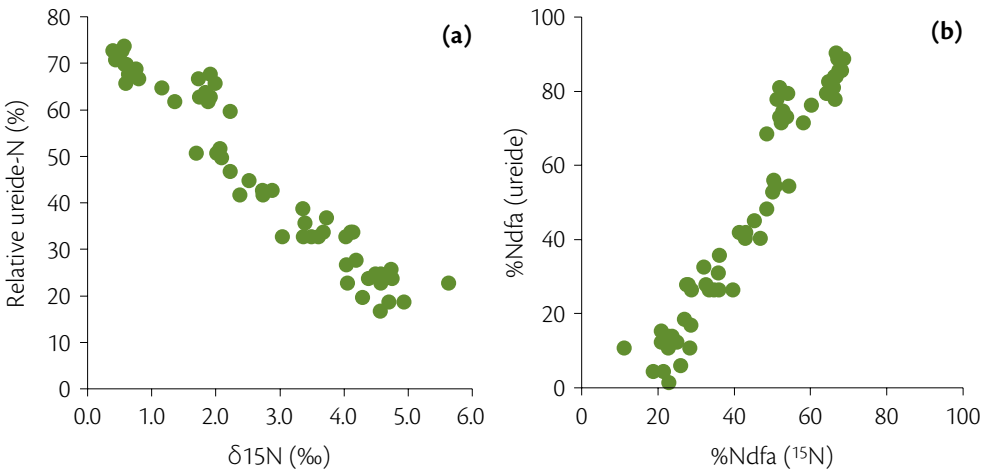


Figure 20. Correlations between (a) natural ^{15}N abundance values of harvested grain of field-grown soybean and relative ureide-N values of xylem sap collected during early pod-fill (physiological stage R3) and (b) %Ndfa estimates from grain ^{15}N and xylem sap ureides (see section 8.1). Source: Herridge and Rose (2000)

nodulation, plant yield and xylem-relative ureide-N to evaluate symbiotic compatibilities of 17 soybean cultivars and naturalised soil rhizobia in northern Thailand. Similarly, Nohara et al. (2005) showed that local cultivars of soybean had higher nodulation and N_2 fixation (relative ureide-N in xylem sap) than American cultivars in N-fertile fields in a 2-year study in Japan.

Effects of fertiliser N on N_2 fixation and yield of soybean have also been assessed using the ureide technique. Tewari et al. (2004) reported increased N_2 fixation when soybean plants were fertilised with deep-placed, slow-release formulations of N. The amounts of N fixed were about 50% higher for the slow-release, calcium cyanamide and coated urea formulations compared with the non-fertilised control. Osborne and Riedell (2006), working with soybean in the cool environment of the northern Great Plains of the USA, found that low fertiliser N at sowing (starter N) had a negative effect on N_2 fixation, assessed using the relative ureide-N values for whole shoots during early flowering but not during late pod-fill. Hungria et al. (2006) reported negative effects of fertiliser N on N_2 fixation (relative ureide-N of stem segments), with reductions of as much as 50% with 200 kg N/ha applied at sowing.

Advantages and potential sources of error of the ureide technique

6.4

Technically, the sampling of xylem sap or stem segments from field-grown plants is simple, and the analysis of the N solutes—ureides, amino N and nitrate—can be done by colorimetric assays in test tubes (see section 6.7). As a consequence there is no need for expensive or sophisticated equipment to collect and analyse samples. With a team of three or four people, it is possible to sample 100–150 plots in a day, and to complete the N-solute analyses in another couple of days. It is not necessary to dig out legume roots and recover nodules to obtain measures of N_2 fixation. Neither is it necessarily a destructive technique as stem segments, rather than complete stems, can be sampled for either immediate vacuum extraction of sap (Herridge et al. 1988) or hot water extraction of the dried, ground material. Since sampling is confined to the accessible aerial parts of the plant, the solute method particularly lends itself to measuring N_2 fixation by twining groundcover or forage legumes (Norhayati et al. 1988) and woody perennial legumes (Herridge et al. 1996; Peoples et al. 1996).

Apart from species differences in the relationships between relative ureide-N in xylem sap and stem extracts and %Ndfa (see Table 10), effects of sampling procedures and other physiological and environmental variables should be considered before glasshouse-derived relationships can be legitimately applied

to field-grown crops (Table 11). One potential source of error is delayed vacuum-extraction of xylem sap following harvest of the shoot (Herridge et al. 1988; Peoples et al. 1989a). The time-related changes in relative ureide-N can be avoided by vacuum-extracting sap within 5 minutes of shoot harvest.

Table 11. Factors to consider when using the ureide technique for assessing legume N_2 fixation

Variable	Comments
Plant species	Relationships between relative ureide-N and %Ndfa (N_2 fixation) are similar among the group of ureide exporters (Table 9). However, it is recommended that use of the calibrations to determine %Ndfa not be extended beyond the species for which it was done (Table 10).
Cultivar/ genotype	Relationships between relative ureide-N and %Ndfa appear to be unaffected by cultivar/genotype within a species (e.g. McClure et al. (1980); Peoples et al. (1989a), Herridge and Peoples (1990)).
Strain of rhizobium	There are conflicting reports on effects of rhizobial strain on relationships between relative ureide-N and %Ndfa—a nil strain effect reported for pigeon pea (Peoples et al. 1989a) and soybean (Herridge and Peoples 1990; Yamakawa and Ishizuka 2002), but large effect reported for soybean in Brazil (Neves et al. 1985) (see additional comments below).
Plant age	With some species, the relationships calibrated between relative ureide-N and %Ndfa differ according to the age of the plant (see Table 10).
N stress and senescence	Relationships between relative ureide-N and %Ndfa appear to be invalid when plants are under severe N stress or are in senescence, since ureides may also be synthesised from degradation products of nucleic acids (e.g. Purcell et al. 2004). This is indicated by xylem N-solute concentrations of <1–2 mM (see additional comments below).
Effects of drought	Purcell et al. (2004) found that, for soybean, the relationship between relative ureide-N and %Ndfa was affected by drought.
Ureides not associated with N_2 fixation	High levels of ureides in vacuum-extracted xylem sap of *Gliricidia sepium* were not associated with N_2 fixation. The %Ndfa–relative ureide-N link needs to be firmly established.
Source of soil N	Relationships between relative ureide-N and %Ndfa may be affected if most of the soil N is taken up as ammonium (Hansen et al. 1993), although the form of soil N had no effect on the calibration of relative ureide-N and %Ndfa for pigeon pea (Peoples et al. 1989a). This is unlikely to be an issue in most agricultural soils (see additional comments below).

Table 11. (continued)

Variable	Comments
Sampling of vacuum-extracted sap	Sampling should be conducted between 0900h and 1600h, because of diurnal variation (see Herridge et al. 1988). Relative ureide-N is unaffected by source or strength of vacuum. A time delay of >5 minutes between plant sampling and extraction of xylem sap progressively increases relative ureide-N levels. For non-destructive sampling of single plants for xylem sap, best results in terms of volume of sap collected, separation of treatments etc. are obtained at late flowering, using the shoot detached at internode 5.
Sampling of stems and petioles	Purcell et al. (2004) sampled petioles from the top of soybean canopies for analysis, and showed that the relative ureide-N and %Ndfa relationship for the petiole extracts was different to the full stem + petiole extracts. Data published by Herridge and Peoples (1990).
Storage of xylem sap	Xylem sap is stable at 25 °C for at least 14 days when diluted 1:1 in ethanol; undiluted sap is stable at 25 °C for just 1 day; at 4 °C for 7 days.
Errors in analysis of xylem sap, such as colour interference	False readings when analysing xylem sap for ureides may occur with certain species, e.g. *Sesbania grandiflora* (Herridge et al. 1996)

The influence of the source of soil mineral N—nitrate or ammonium—on the relationship between %Ndfa and relative ureide-N was referred to at the beginning of this chapter and remains somewhat contentious. Peoples et al. (1989a) reported no effect of mineral N source on the relationship for pigeon pea. The issue was revisited by Hansen et al. (1993) and by Alves et al. (2000c), with data from both studies inferring higher relative ureide-N values for common bean and soybean plants supplied with ammonium than for the nitrate-supplied plants, particularly at low levels of nodulation and %Ndfa. Thus, Alves et al. (2000c) cautioned that use of a calibration derived with ^{15}N-labelled nitrate on field-grown plants that were predominately supplied with ammonium would result in an overestimation of %Ndfa. As stated at the outset, the majority of the ureide species in agricultural settings would assimilate soil mineral N predominately as nitrate. Peoples et al. (1989a) reported that nitrate had been detected in all of >5,000 samples of xylem exudate collected during 10 years of field experiments and that concentrations in those samples generally reflected soil N fertility. Thus, it is logical that ^{15}N-labelled nitrate was used in calibration

experiments. The calibrations in Table 10 should be applied to field-grown plants to estimate %Ndfa until additional calibrations, such as those done by Alves et al. (2000c), show significant errors.

A second contentious issue is the effect of rhizobial strain on the relationship between %Ndfa and relative ureide-N. A series of studies in Brazil involving the legumes soybean and common bean, and rhizobial strains with varying levels of relative efficiency (expressed as the amount of H_2 evolved relative to C_2H_2 reduction), showed that plants nodulated with the more efficient strains produced higher relative ureide-N in xylem sap than plants nodulated with the less efficient strains (Neves et al. 1985; Hungria and Neves 1987). Differences were expressed only when the plants were grown in full sunlight (Santos et al. 1997). Unfortunately, %Ndfa using ^{15}N was not measured in any of the three studies. There may be a need for new calibrations for Brazilian rhizobial strains, particularly of the various species of *Bradyrhizobium* for soybean (e.g. Hungria et al. 1998).

A third contentious issue relates to the large amount of effort that has gone into unravelling the role of ureides in drought-stressed plants (see Sinclair et al. 2006), and how this might affect the efficacy of the ureide technique (Purcell et al. 2004). There is substantial, but somewhat conflicting, evidence that the accumulation of ureides in leaves and nodules of drought-stressed soybean is associated with a decline in N_2 fixation (Vadez and Sinclair 2001; King and Purcell 2005; Ramos et al. 2005). Furthermore, the ureides act in a feedback mechanism to restrict further N_2 fixation (see King and Purcell 2005). As a consequence, the relationship between relative ureide-N in the plant tissues and N_2 fixation changes when plants are drought stressed. Thus, Purcell et al. (2004) reported that drought-stressed soybean had higher relative ureide-N than well-watered plants for the same levels of symbiotic dependence (%Ndfa). This represents a source of error in the technique and would result in an overestimation of %Ndfa for drought-stressed plants if ignored. Ideally, the calibrations should be done under similar conditions of growth as the plants to be assayed.

6.5 Setting up experiments and on-farm surveys

A clear advantage of the ureide technique for quantifying legume N_2 fixation is that there is no need for special preparation or treatment of plants prior to sampling. The only decisions that have to be made are about experimental design (layout of treatments and replication) and the size of sampling units (number of plants).

6.5.1 Glasshouse experiments

Experiments described by Herridge and Peoples (2002a) to calibrate the ureide technique for cowpea and mung bean provide an example of the design and execution of glasshouse experiments. In this instance, plants were grown in a 3:1 (v:v) mixture of sand and vermiculite in 14-L free-draining pots in a naturally lit, temperature-controlled (day 27–32 °C, night 17–20 °C) glasshouse. For each species, 10–12 seeds were sown into each pot. Effective rhizobia (*Bradyrhizobium* sp. strain CB1015) were then added as peat inoculant in a water suspension. Seedlings were subsequently thinned to 3 per pot, resulting in overall densities of 12 plants/m^2 of bench. There were 4 replicate pots of each treatment for each sampling time, arranged in a completely randomised design. Thus, 12 plants were sampled for each treatment, i.e. 4 replicates and 3 plants/replicate, at each time of sampling. Plants were fed a range of NO_3^-–N concentrations of 0–10 mM.

6.5.2 Field experiments

Typical designs for field experiments are randomised complete blocks with 4–6 replicates and individual plots 5–20 m long depending on available land. Plots should be wide enough to accommodate buffer rows. Commonly, only the middle rows are sampled for xylem sap, stems, whole shoots, grain etc. Sampling for xylem sap might involve 8–15 plants/replicate plot, with the sap bulked for later analysis (e.g. Herridge and Peoples 2002b). Note that sampling for dry matter and total plant N requires larger quadrat-type sampling than for ureide analysis, as dry matter and total plant N are likely to be more variable than ureide N (see section 3.1.1).

6.5.3 On-farm surveys

The ureide technique lends itself to 'on-farm' assessment of N_2 fixation. Ideally, 5–8 replicate areas should be sampled from a single field, with the number depending to some extent on the size of the field and possible variations in crop growth and soil N fertility within the field. The samples for each replicate area would consist of xylem sap from 5–10 plants.

6.6 Sampling of N solutes

It is possible to collect xylem sap as it bleeds spontaneously from intact root stumps of crop legumes following decapitation of the shoot from both glasshouse-grown plants and from plants growing in the field in the humid tropics (e.g. Norhayati et al. 1988). It may be difficult, however, to collect root-bleeding sap from field-grown plants in drier environments. Pre-watering field plots may assist plants to bleed but this is not always convenient or successful. As a result, an alternative method for sampling xylem sap was developed in which the xylem contents of the harvested stems or stem segments are extracted in the field using a mild vacuum (Herridge et al. 1984, 1988). The sap displaced from the stem xylem strands is then available for immediate analysis. Vacuum-extraction of sap has worked well with many species of crop, fodder and shrub legumes growing in quite diverse field environments. The third method for sampling N solutes involves harvesting of stems or stem segments followed by drying, grinding and aqueous extraction of the solutes (Herridge 1982b). Procedures for the three methods are described below. Irrespective of the method used, it is advisable to sample the plants between 0900 and 1600 hours to avoid error due to diurnal variations in relative ureide-N (Herridge et al. 1988, 1996; Peoples et al. 1989a; Alves et al. 2000a).

6.6.1 Root-bleeding sap

1. Cut the shoot below the first node close to ground level with a pair of secateurs or a very sharp blade.

2. Place a sleeve of silicon- or latex-rubber tubing, 2–4 cm long with an internal diameter slightly smaller than the stem, over the exposed root stump (Figure 21).

3. The sap exuding under root pressure can easily be collected from within the tubing sleeve using a Pasteur pipette or syringe. We recommend that root stumps be allowed to exude for no more than 20–30 minutes since changes in N-solute composition that might occur over longer periods of bleeding could introduce errors in the subsequent analyses and interpretation of xylem data. The accumulated sap should be collected about every 10 minutes and placed in a sealable tube or vial (e.g. Vacutainer®, Becton Dickinson, Rutherford, New Jersey, USA) kept on ice to minimise potential decomposition or metabolism of xylem N-components.

4. Sap samples should be kept chilled on ice until frozen at –15 °C for long-term storage, or stabilised immediately after collection by mixing with an equal volume of ethanol in the collection tube if ice is unavailable.

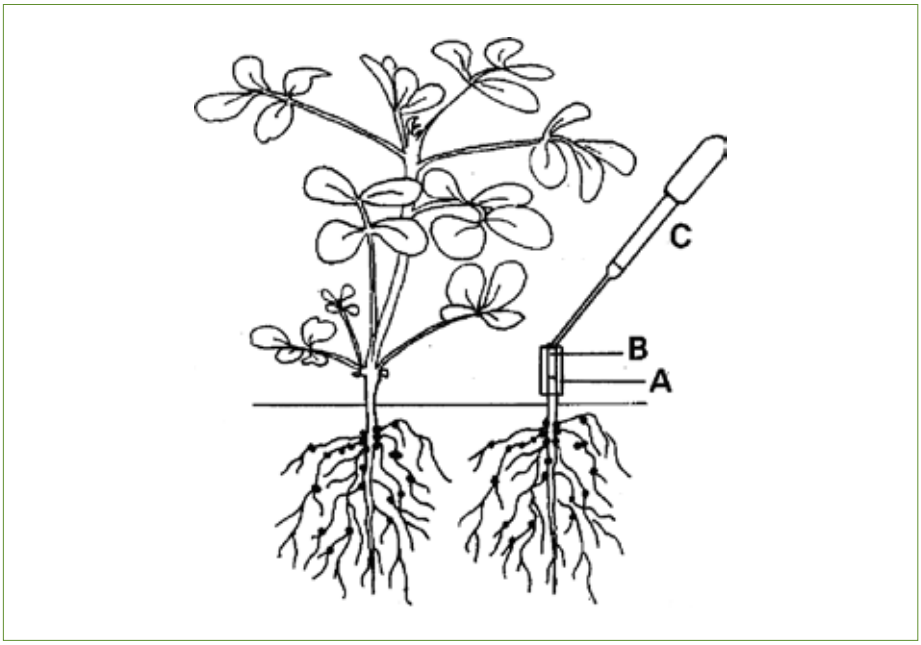


Figure 21. Sampling of root-bleeding xylem sap. A length of silicon rubber tubing (A) is placed over the root stump following decapitation of the shoot. The sap (B) collects in the tubing, out of which it can be drawn using a Pasteur pipette or syringe (C).

6.6.2 Vacuum-extracted sap

Sampling should be done between 0900 and 1600 hours because of diurnal fluctuations in relative ureide-N (Herridge et al. 1988). Effects of source or strength of vacuum on relative ureide-N are insignificant. With most species, sufficient (0.1–0.3 mL/plant) xylem sap can be recovered from each whole stem or stem section of field-grown crop legumes for complete N-solute analysis. Thus, the method may also be used as a non-destructive assay on individual plants (Herridge et al. 1988).

Collecting root-bleeding sap from glasshouse-grown chickpea. Although chickpea is an amide exporter rather than a ureide exporter (see Table 9), the methodology for collecting root-bleeding sap is the same for chickpea and other amide plants as it is for the ureide exporters.

The root-bleeding sap exudes under root pressure and collects in the silicon tubing sleeve over the exposed root stump.

Equipment requirements

Items needed for vacuum-extraction of xylem sap are:

- sharp secateurs or pruning shears
- syringe needles (19 or 20 gauge)
- silicon- or latex-rubber tubing of a range of internal diameters (3–15 mm)
- appropriate-size fittings or adaptors (e.g. disposable micropipette tip cut to size)
- 5 mL Vacutainers
- a vacuum source, which may be a hand-held vacuum pump (e.g. Nalgene, Sybron Corp., Rochester, New York, USA, as depicted in Figure 22), a foot pump (Figure 23a) or a laboratory vacuum pump powered by a petrol-run generator or a car battery (e.g. a Waters/Millipore DOA-V130-BN vacuum pump with a Kawasaki GA1000A portable generator, or a Thomas diaphragm pump model 907 cDc 18 with a 12-volt battery as depicted in Figure 23b). The advantage of the laboratory vacuum pump is that a manifold can be used to provide more than one vacuum line so that sap may be sampled from several plants simultaneously (Figure 23c).

Procedure

1. For crop legumes, using secateurs or razor blade cut a stem >3 mm in diameter (to ensure collection of reasonable volumes of sap) close to ground level. If the nodes at the base of the stem are compacted, it may be preferable to cut the stem above the lowest nodes, or to use laterals for subsequent vacuum extraction, as sap recovery is often restricted by the high xylem resistance that occurs at the vascular junctions. For tree or shrub legumes, 1.0 m long stems or canes are cut from the main plant and used for sap sampling (Herridge et al. 1996; Peoples et al. 1996).
2. The detached stem is immediately inserted into a sleeve of silicon- or latex-rubber tubing with an internal diameter slightly smaller than the stem (Figure 22), and fitted onto a syringe needle using an adaptor of appropriate size.
3. The needle is then pushed through the rubber stopper of a 5-mL Vacutainer that has been linked to a vacuum pump via another syringe needle connection and a flexible plastic-tubing line.
   Note: The base of the two syringe needles must not be level, otherwise sap may be sucked directly into the vacuum pump line rather than falling to collect in the bottom of the Vacutainer.

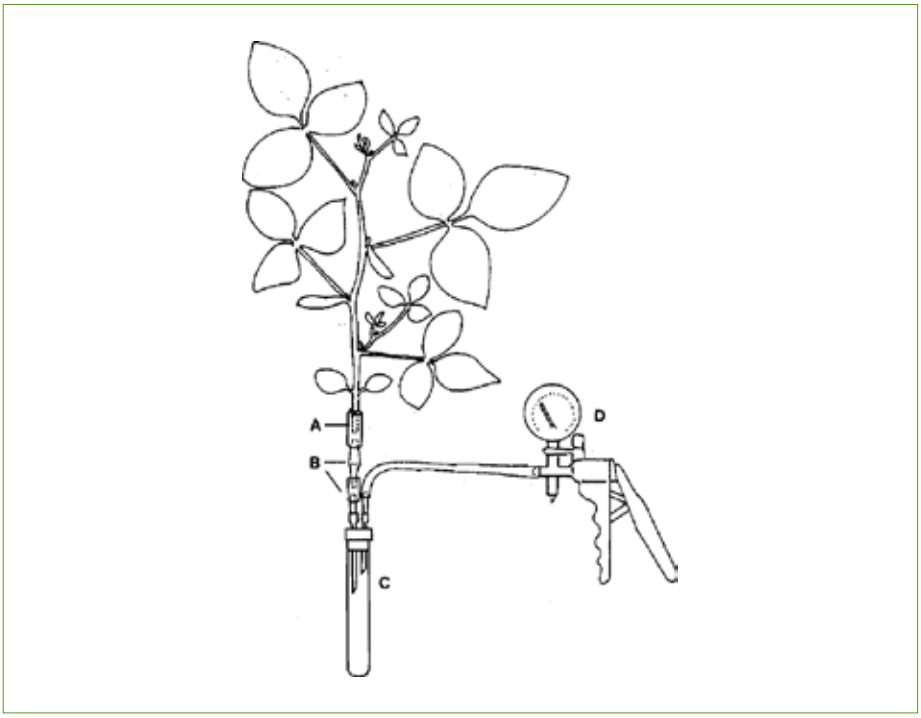


Figure 22. Apparatus for vacuum-extraction of xylem sap using a hand-held vacuum pump. The base of a freshly detached stem is placed into a sleeve of silicon-rubber tubing (A) of appropriate size fitted onto a syringe needle (using an adaptor (B) if necessary). The syringe needle is then inserted through the rubber stopper of a Vacutainer® (C) connected to a vacuum pump (D) via a second syringe needle connection.

4. A vacuum (60–70 kPa) is applied and short, 3–4 cm segments of the stem are then cut with secateurs successively from the top to the bottom of the shoot to allow entry of air at the cut surface, thus displacing the xylem sap from the base of the stem to be collected within the Vacutainer. It is essential that vacuum extraction of the stem xylem contents commence *immediately after detachment of stems* from the root. A delay of more than 5 minutes can introduce errors because of changes in concentrations of N solutes (see Herridge et al. 1988; Peoples et al. 1989a). The technique will not work if the crop is infested with pests such as stem borers since it will not be possible to maintain a vacuum on the stem. Care should be taken that the needle does not become blocked with debris during extraction. The debris should be cleared frequently and needles changed periodically.

Vacuum-extracting xylem sap from tree legumes using a hand-held vacuum pump in Malaysia

Close-up of the hand-held vacuum pump and Vacutainer® for collecting the xylem sap. A vacuum (60–70 kPa) is applied and short, 3–4 cm segments of the stem are then cut with secateurs successively from the top to the bottom of the shoot. The sap collects in the bottom of the Vacutainer.

Note: Total shoot N can be determined on the same samples if the researcher works over a large sheet of plastic or bin so that the leaves and stem cuttings can be collected after each replicate sampling and retained for drying, weighing and grinding.

(5) Sap samples should be kept chilled on ice until frozen at –15 °C for long-term storage, or stabilised immediately after extraction by adding an approximately equal volume of ethanol to the sap collected in the Vacutainer if ice is unavailable.

6.6.3 Stems and stem segments

The stem, because it is involved in transport and, to a lesser extent, storage of N solutes, is the most suitable plant part for extraction and analysis. Leaves are not suitable because they rapidly metabolise incoming N compounds, and variation in tissue N solutes can result from changes in plant metabolism that are not related to N_2 fixation. Relative ureide-N in stems is insensitive to diurnal fluctuations and is unchanged by storing the stems at ambient temperatures of 20–30 °C for up to 24 hours after harvest before being placed in an oven for drying (Herridge 1982a; Alves et al. 2000a).

The advantages of using dried and ground stems or stem segments for extraction of N solutes are the ease of sampling and the 24-hour stability of the samples. The stems or stem segments may also be the only means of field sampling N solutes from very small plants or from legumes that do not readily yield vacuum-extracted or root-bleeding xylem sap. The disadvantages are the additional steps in drying and grinding the stem segments and in the solute extraction.

It should also be noted that calibrations of the ureide method for stems have been based on whole stems (stems + petioles, in fact), large stem segments and petioles (see Table 10). Purcell et al. (2004) sampled petioles from the top of soybean canopies for extraction and analysis, and showed that the relative ureide-N and %Ndfa relationship for the petiole extracts was different to the full stem + petiole extracts (see Herridge and Peoples 1990). Thus, if soybean petioles were to be used as the source of N solutes, the calibrations of Purcell et al. (2004) should be used (Table 10).

Extraction of solutes from stems and stem segments

1. Harvest stems, stem segments or petioles and remove leaves. (Note: retain leaves in a separate labelled bag if total crop N determinations are required.)
2. Place samples in clearly labelled bags and dry at 65–80 °C in a forced-air oven for 2 days.
3. Record total dry weight if total N analysis is required later, and grind tissue to pass a 60-mesh (1.0 mm) screen. Store in a dry place until extraction.
4. Weigh 0.5 g subsamples of dried and ground material and transfer to 100 mL beakers or Erlenmeyer flasks.
5. Add 25 mL distilled water to each subsample and boil for 1–2 minutes. An electric frying pan half-filled with sand can be used for this.
6. Filter while hot through a funnel and 15 cm filter paper (Whatman No. 40) into a 50 mL volumetric flask. Wash residue onto filter and rinse with a little distilled water.
7. When contents of flask are cool, make volume up to 50 mL with distilled water.
8. The extract can be stored indefinitely in a freezer in small vials or flasks until analysis of N solutes.

Analysis of xylem saps and extracts

6.7

The following items are required for analysis of xylem sap or extracts:

- weighing balances (accurate to 0.1 and 1 mg)
- test-tube racks and glass test tubes to match (e.g. 85 × 15 mm)
- micropipettes and tips, and/or dispensers to cover 2–20 µL, 50–200 µL, 0.2–1 mL, 1–5 mL ranges
- vortex mixer
- boiling-water bath
- cold-water ice bath (e.g. ice in a foam box), or refrigerated water bath
- spectrophotometer or colorimeter.

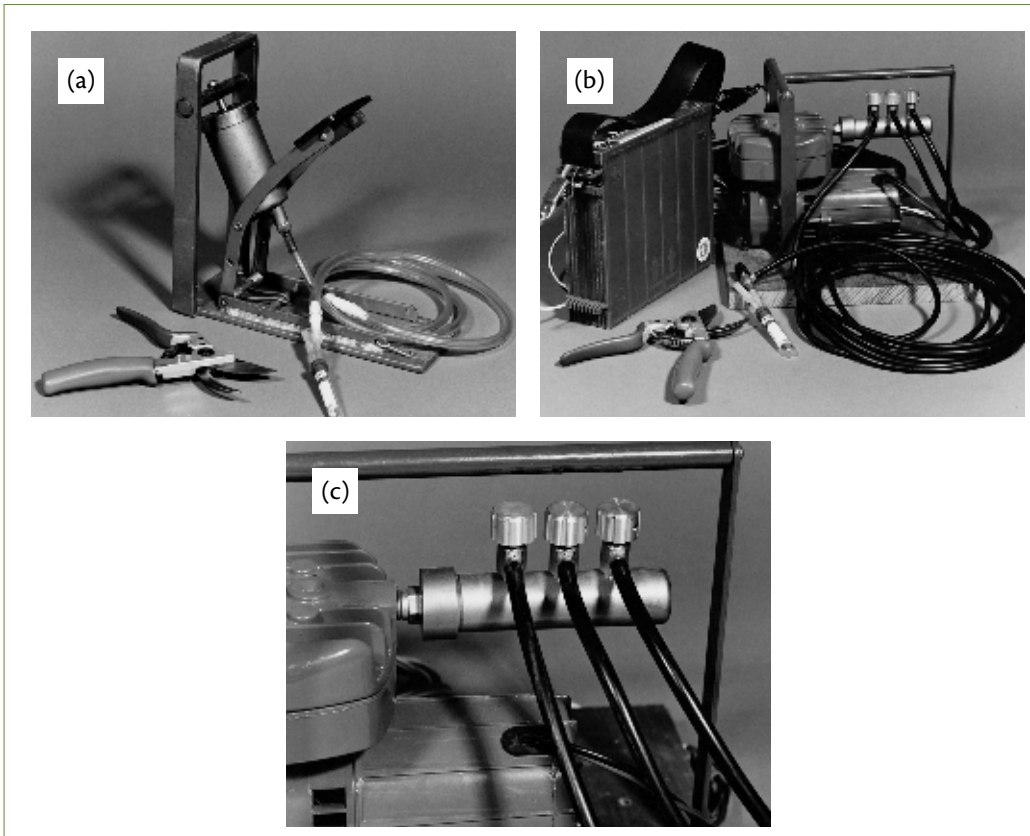


Figure 23. (a) Foot vacuum-pump, secateurs, Vacutainer® and fittings used to extract xylem sap from plant stems. The pump is a modified car-tyre pump with the plunger reversed and a one-way valve fitted in the plastic tube. (b) Battery-powered electric diaphragm vacuum-pump, fitted with a three-port manifold, for extracting xylem sap from plant stems. (c) Detail of manifold for vacuum pump.

6.7.1 Ureide assay

Reference: Young and Conway (1942)

Reagent preparation

(a) Sodium hydroxide (0.5 N NaOH)
Add 20 g NaOH to 1 L distilled H_2O and pour into **Dispenser 1**.

(b) Hydrochloric acid (0.65 N HCl)
Add 6.5 mL concentrated HCl to100 mL distilled H_2O.

(c) Phenylhydrazine hydrochloride (0.33%)
Add 0.33 g phenylhydrazine to 100 mL distilled H_2O.
Take great care with the phenylhydrazine, as it is toxic. Make fresh daily.

(d) The 0.65 N HCl and the phenylhydrazine are now mixed together—total volume 200 mL. (Pour the HCl and phenylhydrazine mix into **Dispenser 2**.)

(e) Potassium ferricyanide (KFeCN – 1.67%)
Add 1.67 g to 100 mL distilled H_2O. Make fresh daily.
Caution: KFeCN is highly toxic.

(f) Concentrated hydrochloric acid (10N HCl)
Decant 400 mL concentrated HCl from the bottle.

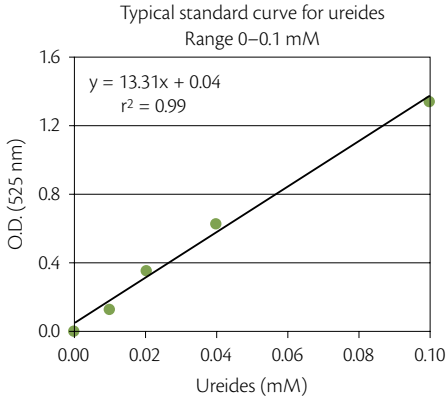
(g) The concentrated HCl and 1.67% KFeCn are mixed together—total volume 500 mL, and placed in a freezer (if possible) or a fridge/ice bath. (Pour the concentrated HCl and KFeCn mix into **Dispenser 3**; leave in fridge.) Make fresh daily.

(h) Ureide (allantoin) standard—1 mM
Add 39.53 mg allantoin to 250 mL distilled H_2O.

(i) Distilled H_2O (**Dispenser 4**)

The 1 mM ureide standard (g) is used to make the following concentrations for a standard curve determination:

Concentration (mM)	1.0 mM allantoin (mL)	Water (mL)
0.00	0.0	100.0
0.01	1.0	99.0
0.02	2.0	98.0
0.04	4.0	96.0
0.10	10.0	90.0



Analysis

1. A standard curve describing the optical density (O.D.) response to increasing concentrations of allantoin should be constructed within the range 0–0.10 mM allantoin. Add 2.5 mL of each of the five concentrations to duplicate test tubes.

2. Add 0.5 mL 0.5N sodium hydroxide (NaOH) using Dispenser 1. The NaOH can be dispensed in such a way that additional mixing is unnecessary.

3. Place rack of tubes in a boiling water bath for 10 minutes. Make sure the level of the boiling water is above the contents of the tubes.

4. Remove from water bath and place on bench.

5. Add 1.0 mL 0.65 N HCl/phenylhydrazine mix using Dispenser 2. Again the dispenser should be used in such a way that additional mixing is unnecessary.

6. Place rack of tubes in the boiling water bath for exactly 2 minutes. Make sure the boiling water is higher than the contents of the tubes.

7. Remove from boiling water bath and immediately plunge rack of tubes into an ice bath (plastic tub containing ice is okay) and leave for 15 minutes.

8. Remove rack of tubes from the ice bath and add 2.5 mL HCl/KFeCN using Dispenser 3. The HCl/KFeCN should be cold. It is important that the contents of the test tubes and the HCl/KFeCN are thoroughly mixed for uniform development of colour and accurate results. This can be achieved with careful use of the dispenser. If the colour develops in the test tubes in a layered fashion, then the contents have been inadequately mixed. The red colour that develops with high ureide concentrations should be uniform.

9. Leave on the bench for 10 minutes and read the O.D. at 525 nm on a spectrophotometer.

10. It is important to read the optical densities as quickly as possible because the colour will fade after a further 15 minutes.

The xylem sap samples together with internal allantoin standards and water blanks are best analysed in a batch. A convenient batch size is 26 tubes, consisting of:

3 water blanks	2.5 mL
3 internal standards (0.04 mM)	2.5 mL
20 sap samples	0.1 mL + 2.4 mL distilled H_2O (1:25 dilution)
or	
20 stem extracts	0.5 mL + 2.0 mL distilled H_2O (1:5 dilution)

6.7.2 Amino-N (ninhydrin method)

Reference: Yemm and Cocking (1955) with adaptations described in Herridge (1984)

Reagent preparation

(a) Ninhydrin reagent
Add 4.79 g (Merck) ninhydrin plus 167 mg ascorbic acid dissolved in 16 mL distilled H_2O to 500 mL methoxyethanol slowly, without aerating the methoxyethanol. Store in a dark bottle in the fridge (pour into **Dispenser 1**). Can be stored for up to 2 weeks.

(b) Citrate buffer
Add 67.2 g citric acid plus 25.6 g NaOH to 400 mL distilled H_2O (pour into **Dispenser 2**).

(c) Ethanol (60% v/v)
Add 300 mL absolute ethanol to 200 mL distilled H_2O (pour into **Dispenser 3**).

(d) Asparagine (Asn) / glutamine (Gln) standard—2 mM
Add 66 mg Asn and 73 mg Gln to 500 mL distilled H_2O.

(e) Distilled H_2O (fill **Dispenser 4**)

Vacuum-extracting xylem sap from field-grown soybean using a foot vacuum pump.

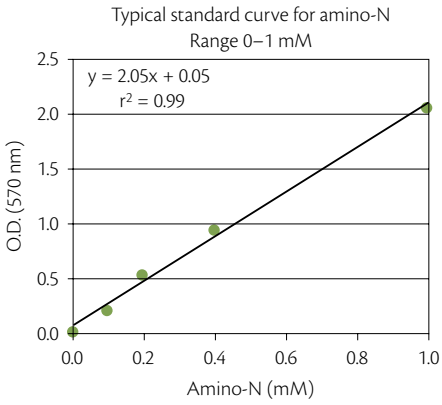
Vacuum extracting xylem sap from field-grown soybean using an electrical vacuum pump powered by a generator (foreground). Note that a manifold in the vacuum line allows at least two people to collect xylem sap at the same time.

Vacuum-extracting xylem sap from field-grown tree legumes using an electrical vacuum pump (blue in wooden box) powered by a 12-volt battery (red, behind wooden box).

The vacuum-extracted xylem sap is collected in a Vacutainer®.

The 2 mM standard is now used to make the following concentrations for a standard curve determination:

Concentration (mM)	2.0 mM amino-N (mL)	Water (mL)
0.00	0.0	100.0
0.10	5.0	95.0
0.20	10.0	90.0
0.40	20.0	80.0
1.00	50.0	50.0



Analysis

1. A standard curve describing the O.D. response to increasing concentrations of amino-N should be constructed within the range 0–1.0 mM. Add 1.0 mL of each of the five concentrations to duplicate test tubes.

2. Add 0.5 mL citrate buffer using Dispenser 1. The buffer can be dispensed in such a way that additional mixing is unnecessary.

3. Add 1.2 mL ninhydrin reagent using Dispenser 2. The ninhydrin can be dispensed in such a way that additional mixing is unnecessary.

4. Place in a boiling water bath for 15 minutes.

5. Remove from water bath and place on bench.

6. Add 3.0 mL 60% ethanol using Dispenser 3. Again the dispenser should be used in such a way that additional mixing is unnecessary. It is important that the contents of the test tubes and the ethanol are thoroughly mixed for uniform development of colour and accurate results. This can be achieved by using the dispenser appropriately. If the colour develops in the test tubes in a layered fashion, then the contents have been inadequately mixed. The blue colour should be uniform.

7. Read O.D. at 570 nm on a spectrophotometer.

The xylem sap samples together with internal amino standards and water blanks are best analysed in a batch. A convenient batch size is 26 tubes, consisting of:

3 water blanks	1.0 mL
3 internal standards (0.20 mM)	1.0 mL
20 sap samples	0.1 mL + 0.9 mL H_2O (1:10 dilution)

6.7.3 Salicylic acid method for nitrate determination

Reference: Cataldo et al. (1975)

This method has been found suitable for all legume xylem sap samples tested to date except pigeon pea (because of colour interference). Other recommended methods include Cu-hydrazine reduction (Kamphake et al. 1967) and an automated flow injection technique described by Alves et al. (2000b).

Reagent preparation

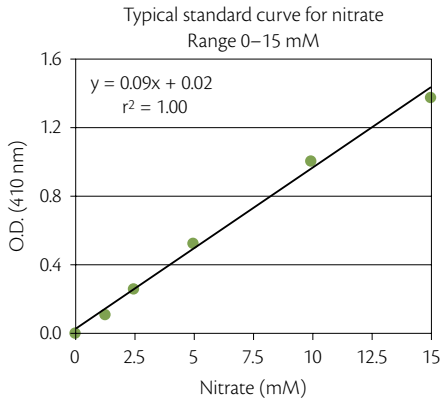
(a) Salicylic acid in concentrated sulfuric acid (H_2SO_4) (5% w/v). Leave for 24 hours before use.
Add 5 g salicylic acid to 100 mL concentrated H_2SO_4 (beaker).

(b) Sodium hydroxide (2N NaOH)
Add 40 g NaOH to 500 mL distilled H_2O (**Dispenser 1**).

(c) Nitrate standard (25 mM)
Add 632 mg KNO_3 to 250 mL distilled H_2O.

The 25.0 mM standard is now used to make the following concentrations for a standard curve determination:

Concentration (mM)	25 mM KNO_3 (mL)	Water (mL)
0.00	0.0	100.0
1.25	5.0	95.0
2.50	10.0	90.0
5.00	20.0	80.0
10.0	40.0	60.0
15.0	60.0	40.0



Analysis

1. A standard curve describing the O.D. response to increasing concentrations of nitrate should be constructed within the range 0–15.0 mM nitrate. Pipette 0.05 mL of each of the six concentrations into duplicate test tubes.
2. Pipette 0.20 mL salicylic/sulfuric acid into the tubes. Mix and leave on the bench for 20 minutes. Make sure that the solution in the tube is clear. Mix further if cloudy.
3. Add 4.75 mL NaOH using Dispenser 1. The dispenser should be used in such a way that additional mixing is unnecessary.
4. Leave on the bench for 10 minutes and read O.D. at 410 nm on a spectrophotometer.

Note that a white precipitate will sometimes form with the addition of salicylic acid. It is very important that the test tubes are shaken well to dissolve this precipitate. The precipitate will form sometimes when the salicylic acid is freshly made up and often when it is cold. Therefore, it is best to make it up the day before analysis and make sure it is at room temperature when used.

The xylem sap samples, together with internal amino standards and water blanks, are best analysed in a batch. A convenient batch size is 26 tubes, consisting of:

3 water blanks	0.05 mL
3 internal standards (2.5 mM)	0.05 mL
20 sap samples or stem extracts	0.05 mL

6.8 Calculations

6.8.1 Calculating concentrations of ureides, nitrate and amino-N

For the purposes of this exercise, the hypothetical sample for analysis is xylem sap vacuum extracted from soybean harvested during early pod-fill, i.e. R3. The sample was analysed using the methods described above, with the following results:

Analysis	Sample O.D.	Dilution	Internal standard		Sample conc.[a] (see below)
			Conc.	O.D.	
Ureide	0.50	×25	0.04	0.57	0.88
Amino	0.85	×10	0.20	0.46	3.69
Nitrate	0.14	×1	2.50	0.24	1.46

[a] Sample conc. = standard conc. × (O.D. sample / O.D. standard) × dilution

Ureide = 0.04 × (0.50/0.57) × 25 = 0.88 mM	Amino = 0.2 × (0.85/0.46) × 10 = 3.69 mM	Nitrate = 2.5 × (0.14/0.24) × 1 = 1.46 mM

6.8.2 Calculating the relative abundance of ureide-N

Now, the relative ureide-N value (RU-N) can be calculated as follows:

RU-N (%) = [4 × ureide / (4 × ureide + nitrate + amino-N)] × 100
= [4 × 0.88 / (4 × 0.88 + 1.46 + 3.69)] × 100
= 41%.

6.8.3 Calculating %Ndfa

The standard curve relating %Ndfa to RU-N for sap vacuum extracted from soybean during pod-fill is:

RU-N (%) = 0.64 %Ndfa + 16.9 (equation in Table 10)

Thus: %Ndfa (%) = 1.56*(%RU-N – 16.9)
= 1.56 × (41 – 16.9)
= 38%.

6.9 Interpretation of a typical dataset

A typical experiment usually involves quantifying amounts of N fixed by legumes in different environments or with different treatments. The example presented here utilises data from Herridge and Peoples (2002b). 'Bragg' soybean was grown at a site with high soil mineral N (260 kg N/ha at sowing, 0–1.2 m depth) and the xylem ureide technique used to quantify N_2 fixation.

Scientists inspecting a pigeon-pea crop in Myanmar. Pigeon pea is a ureide exporter and the plant's long straight stem makes it ideal for vacuum-extraction of xylem sap (see section 6.6.2).

Plants were sampled for biomass, biomass N and xylem sap for N solute analysis at regular intervals throughout growth. Biomass was determined on 15-plant samples selected at random from 8 replicate plots; sap was collected from 10 of those plants. Assessments were also made of nodulation (142 mg nodule dry weight/plant at R2.5), grain yield (3.5 t/ha) and %Ndfa using ^{15}N natural abundance with unnodulated 'Hardee' as the non N_2-fixing reference (20%).

The N solute data, including %Ndfa, are presented in Table 12 (means only, not the individual replicate values). During early growth, nitrate and amino concentrations were high and ureides low, with the reverse occurring during mid to late pod-fill. Thus, the relative ureide-N and calculated %Ndfa values increased progressively during pod-fill.

Accumulation of shoot N during the growing season is plotted in Figure 24. Maximum measured shoot N was 385 kg N/ha at R7 (Day 147). A line of best fit was calculated for the data and is also shown. Shoot N at R7, calculated using the regression equation in Figure 24, was 360 kg N/ha.

Table 12. Xylem sap N solutes, relative ureide-N (RU-N) and %Ndfa for soybean at each time of sampling

Days after sowing	Stage	Xylem sap				%Ndfa
		Ureides	Amino	Nitrate	RU-N	
		(mM)			(%)	(%)
49	V8	0.36	7.46	5.23	10	4
56	V10	0.24	5.33	3.88	10	3
72	R1	0.23	3.41	3.66	12	7
84	R2.5	0.21	2.16	2.08	17	1
100	R3.5	0.38	2.10	1.12	33	27
118	R5.5	0.62	1.86	0.60	50	53
136	R6.5	0.82	2.92	0.42	49	52

Below-ground N (i.e. roots, nodules and exudates) is virtually impossible to accurately determine without using a technique such as ^{15}N labelling (see section 3.1.1). Nonetheless, it is imperative that below-ground N be included in calculations of legume N_2 fixation. To ignore below-ground N is to ignore a substantial proportion of plant-biomass N and the major source of N-rich residues that remain in, or are returned to, the soil following grain harvest. To convert shoot N to whole-plant N in this soybean experiment, a multiplication factor of 1.5 was used (assumes one-third of total plant N is below ground, based on the ^{15}N-labelling experiment of Rochester et al. 1998). Thus, total crop N was 540 kg/ha. Total N fixed can now be determined using one or other of two approaches:

- Partition accumulation of whole plant N, derived from the line of best fit in Figure 24, for each incremental period of growth between fixed N and soil-derived N using the %Ndfa values in Table 12, as presented in Table 13. Estimated crop N fixed is 140 kg N/ha and %Ndfa of 26% (i.e. (140/540)×100).

- Use the %Ndfa value at the critical sampling at R3.5 of 27% (see section 6.2.1.) and apply to the calculated crop N of 540 kg/ha. Estimated crop N fixed using just the R3.5 %Ndfa value is 146 kg N/ha

Clearly, both approaches provided very similar estimates (26% and 27%) and are similar to the ^{15}N natural abundance derived %Ndfa value of 20%.

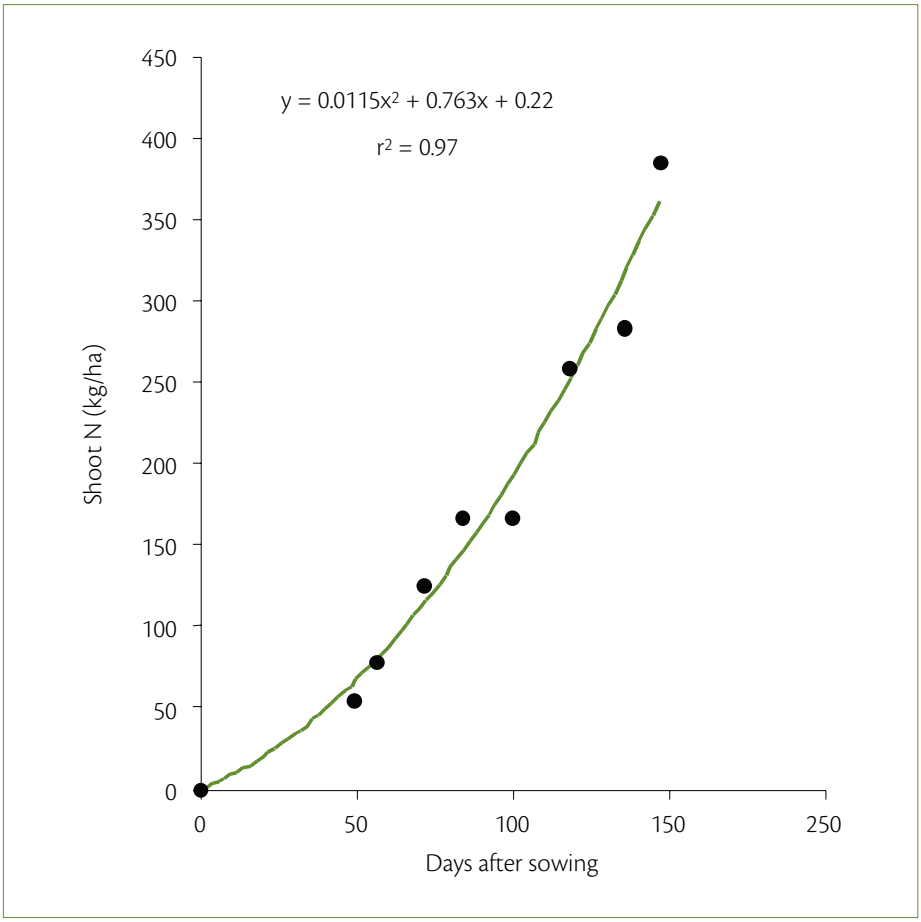


Figure 24. Accumulation of shoot N during growth of soybean in a high mineral N soil. The line of best fit is shown. Values are the means of four replicates.

6.10 Final word

For laboratories that do not have ready access to ^{15}N methodologies, the ureide technique can provide an alternative measure of N_2 fixation for the tropical and subtropical ureide-producing legumes. The great advantage of the method is that it is technically simple and within reach of any laboratory with a supply of glassware, chemicals and a simple colorimeter. The relative abundance of ureides in xylem sap and stem segments has been used to study factors regulating N_2 fixation and to quantify seasonal inputs of fixed N by legumes growing in experimental trials and farmers' fields. The results compare favourably with ^{15}N estimates. The use of a single sampling, or possibly two samplings, of xylem sap/stem segments during key periods in early reproductive growth to estimate seasonal %Ndfa simplifies the protocols and may encourage expanded use. Unfortunately, the method is not applicable to most temperate legumes or to non-leguminous N_2-fixing plants.

Table 13. Calculating crop N fixed for each incremental period of growth and total growth of soybean. Crop N was estimated using the line of best fit in Figure 24 describing the accumulation of shoot N during growth and multiplying by 1.5 to account for below-ground N (Rochester et al. 1998).

Days after sowing	Crop N (kg/ha)		N_2 fixation		
	Cumulative	Increment	%	Increment (kg/ha)	Cumulative (kg/ha)
0	0				
25	40	40	2	0.8	1
53	109	69	4	2.8	4
64	144	35	3	1.1	5
78	195	51	7	3.6	8
92	252	57	1	0.6	9
109	330	78	27	21.1	30
127	424	94	53	49.8	80
147	540	116	52	60.3	140
0–147	**540**		**26**		**140**

7

^{15}N-isotopic methods —general principles and $^{15}N_2$ feeding

Stable isotopes are elements that have variations in the number of neutrons in their atoms, but these atoms do not decay as with radioactive isotopes. For example, nitrogen has an atomic weight of 14 (^{14}N), with seven protons and seven neutrons, but other N atoms may have six (^{13}N) or eight (^{15}N) neutrons (and more in some cases). Nitrogen atoms with six neutrons (^{13}N) are unstable, emitting a positron (β+). With a half-life of less than 10 minutes, the practical application of ^{13}N is limited for anything other than short-term physiological experiments (see Meeks 1993). Of the two stable isotopes of N, the lighter isotope ^{14}N is naturally much more abundant than ^{15}N.

The isotopic abundance of the minor isotope (^{15}N) is usually expressed as a percentage of the total N present (atom% ^{15}N) (equation (7)) (see also Table 14):

$$\text{atom\% } ^{15}N = (^{15}N / (^{15}N + ^{14}N)) \times 100 \quad (7)$$

The abundances of the stable isotopes of N are routinely measured by mass spectrometry. Emission spectrometry is also possible, but only for enrichments of ^{15}N >0.05 atom% excess.

7.1 The ^{15}N abundance of atmospheric N_2, units and terminology

The largest pool of N in the environment is atmospheric N_2 and it has a constant abundance of 0.3663 atom% ^{15}N (Table 14; Mariotti 1983). Only very slight natural variations around this value occur in other N fractions within the biosphere (Hogberg 1997). Natural variations in ^{15}N abundance are usually expressed in terms of δ units, which are the parts per thousand (‰) deviation relative to the nominated international standard of atmospheric N_2 (Table 14). The δ units are normally calculated from isotopic ratios obtained from mass spectrometry but can also be approximated from atom% ^{15}N values (equation (8)):

$$\delta^{15}N\ (‰) = \left(\frac{\text{sample atom\%}^{15}N - 0.3663}{0.3663} \right) \times 1000 \quad (8)$$

The above approximation of $\delta^{15}N$ is adequate for N_2 fixation measurement using natural variations in ^{15}N. Strictly accurate calculations are provided from raw isotope ratio data during instrumental analysis (see Dawson et al. 2002).

The natural abundance of atmospheric N_2 will by definition have a $\delta^{15}N$ of 0‰ as the standard is not different from itself (Table 14). Values can either be positive (have more ^{15}N than in atmospheric N_2) or negative (have less ^{15}N than in atmospheric N_2).

To illustrate natural abundance calculations, let us consider a plant sample with 0.36784 atom% ^{15}N. It will have a $\delta^{15}N$ of:

$$\delta^{15}N = \left(\frac{0.36784 - 0.3663}{0.3663} \right) \times 1000 = +4.2‰$$

Table 14. Terms associated with ^{15}N stable isotope methods

Term	Definition
Atom% ^{15}N	Abundance of ^{15}N atoms as a percentage of the total $(^{15}N / (^{14}N + ^{15}N)) \times 100$
Natural abundance	Atom% ^{15}N naturally present in materials
^{15}N abundance of atmospheric N_2	0.3663 atom% ^{15}N
$\delta^{15}N$ (‰)	Sample natural abundance expressed as parts per thousand relative to atmospheric N_2 1000 × (sample atom% ^{15}N – 0.3663) / (0.3663)
$\delta^{15}N$ of atmospheric N_2	0‰
^{15}N-enriched nitrogen	Nitrogen with artificially elevated ^{15}N content
Atom% ^{15}N excess	A measure of a sample's ^{15}N content above the atmospheric N_2: sample atom% ^{15}N – 0.3663
Labelled nitrogen	Material generated with a specific ^{15}N enrichment
%Ndfa	The percentage of plant N derived from atmospheric N_2

Conversely, atom% ^{15}N values can be calculated from $\delta^{15}N$ via the following formula ($\delta^{15}N$ value of +6.5‰ in the example):

$$\text{atom\%}^{15}\text{N} = \left(6.5 \times \frac{0.3663}{1000}\right) + 0.3663 = 0.36868 \text{ atom\% } ^{15}\text{N}$$

Background reading for stable isotope applications in biology can be found in Dawson and Brooks (2001) and Dawson et al. (2002).

7.2 The ^{15}N abundance of soil N

Extraction and analysis of soil total N and mineral N (NO_3^- and NH_4^+) shows that soils are often slightly higher in ^{15}N abundance than is atmospheric N_2 (e.g. Table 15). These small differences in ^{15}N abundance between soil N and atmospheric N_2 result from fractionations between ^{14}N and ^{15}N that occur during almost all soil N transformations. Gaseous losses of N via denitrification, for example, generally favour the lighter isotope ^{14}N. Similarly, ^{15}N depleted NO_3^- is more readily leached, resulting in a small elevation of ^{15}N abundance in the residual surface soil N (Hogberg 1997). However, the $\delta^{15}N$ of soil total N will not be a reliable guide to the $\delta^{15}N$ of plant-available soil mineral N (e.g. Table 15), as the $\delta^{15}N$ of soil total N will be dominated by the isotopic signature of stable forms of N that are not likely to change rapidly with time. Aside from the slight elevation of $\delta^{15}N$, there seems to be little consistency in observed trends in the $\delta^{15}N$ of mineral N in agricultural soils across soil types and environments. This reflects the complex range of transformations affecting soil inorganic N, and the proportion of each of the NO_3^- and NH_4^+ pools involved in those transformations.

There is evidence that some plants may be capable of assimilating N from the soil or decaying organic matter as forms of N other than NO_3^- and NH_4^+ via ericaceous-, ecto- or endomycorrhizae (Boddey et al. 2000). These different forms of N in soil may also differ in $\delta^{15}N$ signature in time and space. Such differences can have consequences for the natural abundance methodology, particularly if the major sources of soil N assimilated by N_2-fixing plants differ from the forms of N taken up by non N_2-fixing reference plants. This scenario is more likely to be a problem in natural than agricultural ecosystems where plant-available N tends to be dominated by NO_3^- and

NH_4^+. Given that the roots of most plant species appear not to discriminate between ^{15}N and ^{14}N when assimilating mineral N (Mariotti 1983), reference plants are likely to reflect the $\delta^{15}N$ of the soil inorganic N pool in agricultural systems (Table 15).

Table 15. Examples of the $\delta^{15}N$ of total N and mineral N of agricultural soils and non N_2-fixing plants growing in them[a]

Location	Average annual rainfall	Soil total N	Soil mineral N	Non N_2-fixing plants
	(mm)	(‰)	(‰)	(‰)
Indonesia	2,580	6.8	12.3	12.1
Kenya	1,600	7.7	nd[b]	5.0
Australia	927	6.0	nd	8.8
Kenya	900	8.9	nd	5.8
Australia	780	nd	6.6	6.6
Australia	633	6.0	8.2	8.5
Australia	625	8.4	10.0	10.0
Australia	479	8.1	5.3	5.5
Australia	395	8.4	18.7	nd

[a] Sources: Turner et al. (1987); Peoples et al. (1992); Gault et al. (1995); Peoples et al. (1996); Boddey et al. (2000); Cadisch et al. (2000); Ojiem et al. (2007)

[b] nd = not determined.

$^{15}N_2$ feeding

7.3

Definitive proof of N_2 fixation was first provided by exposing ^{15}N-labelled N_2 gas to bacterial cultures and to a nodulated pea plant, followed by recovery of ^{15}N from within the bacterial cultures and plant (Burris et al. 1942). While use of $^{15}N_2$ proved vital during early studies of the process of N_2 fixation, it is limited to systems that can be enclosed in a $^{15}N_2$-labelled atmosphere, and this generally restricts its use to small-scale experiments over short time

frames (hours to a few days). However, $^{15}N_2$ methodology can provide critical insight in N translocation studies and in asymbiotic N_2 fixation systems, as it is still the only highly sensitive and direct measure of N_2 fixation. Labelled $^{15}N_2$ gas can be purchased in steel cylinders or glass breakseal flasks, or can be readily made in the laboratory or field from $^{15}NH_4^+$ salts using the Rittenberg reaction (see Bergersen 1980a).

However, logistic problems (use of closed systems) restrict its broad application in field studies of N_2 fixation. Practical guides to this technology are detailed in Bergersen (1980a), Focht and Poth (1987) and Warembourg (1993) and will not be dealt with here.

7.4 ^{15}N analysis by mass or emission spectrometry

Stable isotope analysis is a specialised field requiring sophisticated, well-maintained equipment and highly skilled technicians. Generally, we recommend sending samples for analysis to a reputable, established laboratory (see Appendix 9). It is important to consult with the laboratory prior to preparing your samples for analysis as each analysis and laboratory will have very specific requirements.

If samples are to be sent by mail or courier to another country for weighing and analysis, ensure that they are dry and enclose them in heat-sealed or 'ziplocked' plastic bags. Although small paper envelopes may appear to seal well, the shaking that samples experience during transport means that they tend to leak, with consequent risk of cross-contamination.

8

^{15}N natural abundance method

8.1 How it works

Where an effectively nodulated legume is growing in a medium free of combined N (i.e. mineral N and/or organic N) and is completely reliant upon symbiotic N_2 fixation for growth, the isotopic composition of the legume would be expected to be similar to that of atmospheric N_2 ($\delta^{15}N$ 0‰, Figure 25, pot A). Conversely, if a non N_2-fixing plant is grown in a soil containing mineral N, its $\delta^{15}N$ value should resemble that of the soil mineral N taken up by the plant ($\delta^{15}N$ +6.2‰ in Figure 25, pot C).

In the case of a nodulated legume (or other N_2-fixing plant, see Chapter 10 for details) that is using a combination of atmospheric N_2 and soil mineral N for growth, the $\delta^{15}N$ of the legume should lie between the values of the two possible N sources, soil and atmospheric N_2 (Figure 25, pot B). The %Ndfa of the legume can then be calculated from its $\delta^{15}N$ value using equation (9):

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of N}_2\text{ fixing legume}}{\delta^{15}\text{N of soil N} - \delta^{15}\text{N of N}_2} \times \frac{100}{1} \qquad (9)$$

This is an example of a simple two-source mixing model (see Dawson et al. 2002). To undertake such calculations, the $\delta^{15}N$ of N of the soil and N_2 from air need to be sufficiently different and the variability in $\delta^{15}N$ for each should be small compared to the difference between them.

While estimates of N_2 fixation could theoretically be derived from such direct measures of the ^{15}N abundance of soil mineral N, it is technically less challenging, and often more convenient, to use a non N_2-fixing 'reference' plant such as an unnodulated legume or a non-legume (e.g. Figure 25, pot C) to sample the $\delta^{15}N$ of soil mineral N for us. Therefore, equation (9) can be re-written as (equation (10)):

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of N}_2\text{-fixing legume}}{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of N}_2} \times \frac{100}{1} \qquad (10)$$

Inserting data into equation (10) from the hypothetical example presented in Figure 25, where the $\delta^{15}N$ of the legume was +4.2‰, the $\delta^{15}N$ of plant-available soil N determined by analysis of the non N_2-fixing reference plant was +6.2‰, then %Ndfa is estimated at 32%:

$$\%\text{Ndfa} = \frac{(6.2 - 4.2)}{(6.2 - 0.0)} \times \frac{100}{1} = \frac{2.0}{6.2} \times 100 = 32\%$$

The ^{15}N natural abundance method has a number of advantages over other methodologies. While it can be applied in glasshouse or field experiments like other techniques described in this handbook, it also allows N_2 fixation to be assessed in almost any situation where both N_2-fixing and non N_2-fixing plants are present at the same location. The technique can therefore be applied to farmers' fields, or to experiments not originally designed with the measurement of N_2 fixation in mind but where legumes and non N_2-fixing plants (usually non-legumes) conveniently coexist. However, the technique has a number of important limitations that should be recognised before it is used. These are related to:

- the need to adjust for isotopic fractionation within the legume (in the example in Figure 25 data are for the whole plants. In practice, shoots, rather than whole plants, are almost always harvested and the isotopic fractionation within the legume becomes important (see section 8.2).
- the choice of non N_2-fixing reference species to provide a measure of plant-available soil N (section 8.4).
- the magnitude of, and variability in, the ^{15}N abundance of plant-available soil N (section 8.5).
- the potential complication of seasonal relocation and recycling of N within either the N_2-fixing or non N_2-fixing species. This relates particularly to perennial plants, or if the plants are grazed or hedgerowed and analyses are undertaken on the foliar regrowth (section 8.8.2).

Each of these will be examined in the following sections. Both annual and perennial systems will be discussed, but much of the focus will be on annual legumes. Applications of the method to perennial legumes are explored in greater detail by Boddey et al. (2000) and to non-legumes in Chapter 10.

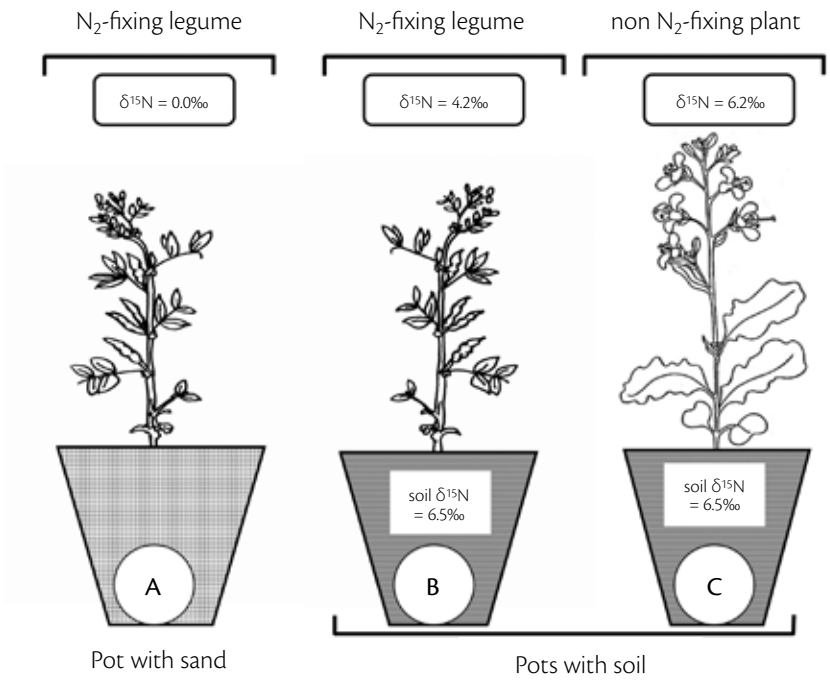


Figure 25. Illustration of the $\delta^{15}N$ natural abundance method for estimating N_2 fixation, highlighting that the $\delta^{15}N$ of a legume completely dependent on atmospheric N_2 (A) should have a $\delta^{15}N$ in shoots very close to that of its N source, atmospheric N_2 (0‰); that a plant totally dependent on soil N (C) will have $\delta^{15}N$ close to that of soil N; and that a plant able to utilise both soil and atmospheric N_2 (B) will have a $\delta^{15}N$ which reflects the relative contributions from these two N sources

8.2 Adjusting for isotopic fractionation within the legume—the 'B' value

If effectively nodulated legumes are grown in systems where all plant N is derived from symbiotic N_2 fixation, the $\delta^{15}N$ of whole plants is usually close to the $\delta^{15}N$ of atmospheric N_2 (0‰) (e.g. Table 16, Figure 25). With the possible exception of soybean nodulated by Brazilian strains of *Bradyrhizobium elkanii* (Table 16), the data collectively suggest that discrimination between ^{14}N and ^{15}N during N_2 fixation at the whole plant level is relatively small.

Table 16. Examples of the ^{15}N of nodules or nodulated roots, shoots and whole plants for a number of nodulated legumes growing in sand culture and provided with all nutrients except combined N

Legume	Variable	^{15}N of plant part (‰)		
		Nodules	Shoots[a]	Whole plant
Temperate species				
Chickpea	Laboratories	+7.00	−2.00 to −1.34	+0.28
	Rhizobial strains		−2.82 to −1.31	
Lupin	Laboratories	+2.00 to +2.40	−0.70 to −0.44	−0.05
	Rhizobial strains	−0.80 to +7.10	−1.16 to +0.10	
Subterranean clover	Laboratories		−0.78 to −0.58	
	Rhizobial strains	+0.47 to +2.71	−1.37 to +0.06	−0.16 to +0.30
Vetch	Laboratories and varieties		−0.98 to −0.70	
Subtropical/tropical species				
Green gram (mung bean)	Varieties	+5.46 to +7.67	−2.20 to −1.14	−0.35 to +0.17
Pigeon pea	Laboratories and rhizobial strains	+5.35 to +10.60	−1.45 to −0.90	−0.22 to +0.29
Soybean	Varieties		−1.40 to −1.15	−0.50
	Thai rhizobial strains	+4.70 to +8.80	−0.90 to −1.50	
	B. japonicum #1[b]	+6.30 to +8.0	−1.7 to −1.0	
	B. japonicum #2[b]	+6.70 to +12.5	−2.6 to −1.0	
	B. japonicum #3[b]	+7.40	−1.31	−0.43
	B. elkanii	+6.82	−2.58	−1.28

[a] More comprehensive lists of estimates of shoot 'B' values are provided in Appendixes 2–6.

[b] *Bradyrhizobium japonicum* strain trials undertaken by different laboratories (#1 Australian data reported in Boddey et al. (2000), #2 New Zealand data by Steele et al. (1983) and #3 by Okito et al. (2004) in Brazil)

While the $\delta^{15}N$ of whole plants fully dependent upon N_2 fixation is close to 0‰, the $\delta^{15}N$ of the shoots is commonly less than zero (i.e. lower in ^{15}N abundance than atmospheric N_2, Table 16). This is important because it is difficult, if not impossible, to recover entire root systems for field-grown legumes. Consequently, most estimates of N_2 fixation rely on analysis of shoots rather than the whole plant, and a correction needs to be made for the within-plant fractionation of ^{14}N and ^{15}N between shoots and nodulated roots. If the $\delta^{15}N$ of shoots of legumes fully dependent upon N_2 fixation for growth is used to account for this, equation (10) can then be rewritten as (equation (11)):

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N of reference plant} - \delta^{15}\text{N of } N_2 \text{ fixing legume}}{\delta^{15}\text{N of reference plant} - \text{B}} \times \frac{100}{1} \qquad (11)$$

Where 'B' is the $\delta^{15}N$ of shoots of legumes that are fully dependent upon N_2 fixation and sampled at the same growth stage as the field plants.

The data presented in Table 16 indicate that there is often a relatively small impact of cultivar or variety on shoot $\delta^{15}N$ for a particular legume species, and that estimates of 'B' can be similar in studies on the same legume undertaken in different laboratories. The 'B' values of most temperate legume shoots tend to lie between 0 and –1‰, but chickpea, white clover and many tropical and subtropical species are generally less than –1‰ (see Table 16 and Appendixes 2 and 3). The rhizobial strain involved in the symbiosis can also influence the 'B' value (e.g. see the range of values for lupin and soybean shoots in Table 16). It is not fully understood why this occurs, but it could be through impacts on nodule mass (Figure 26). Whatever the reason for the observed differences, a 'B' value should ideally be prepared for each new species under study and include the specific rhizobial strain – legume host association to be examined. 'B' values must also be determined at the same growth stage as when the field-grown plants are sampled for N_2 fixation assessment, as they tend to decrease with time until N_2 fixation ceases (see Unkovich et al. 1994b and Figure 27). Normally, this would be just before physiological maturity (i.e. late pod-fill) for crop legumes.

It may be possible to predict the rhizobial strain responsible for nodulation where an inoculated legume is being sown for the first time and there are no effective, native rhizobia present in the soil. In most situations, however, legume samples are likely to be collected from field sites where many unknown rhizobial strains contribute to nodulation. Unkovich and Pate (2000) suggested that this uncertainty might be lessened by inoculating sand-

cultured legumes with mixed rhizobial strains isolated from a diverse range of field sites. While such studies utilising multiple rhizobial strains in the glasshouse may not necessarily mimic or predict plant responses in the field, they may provide a more useful 'B' value than one based on a single strain.

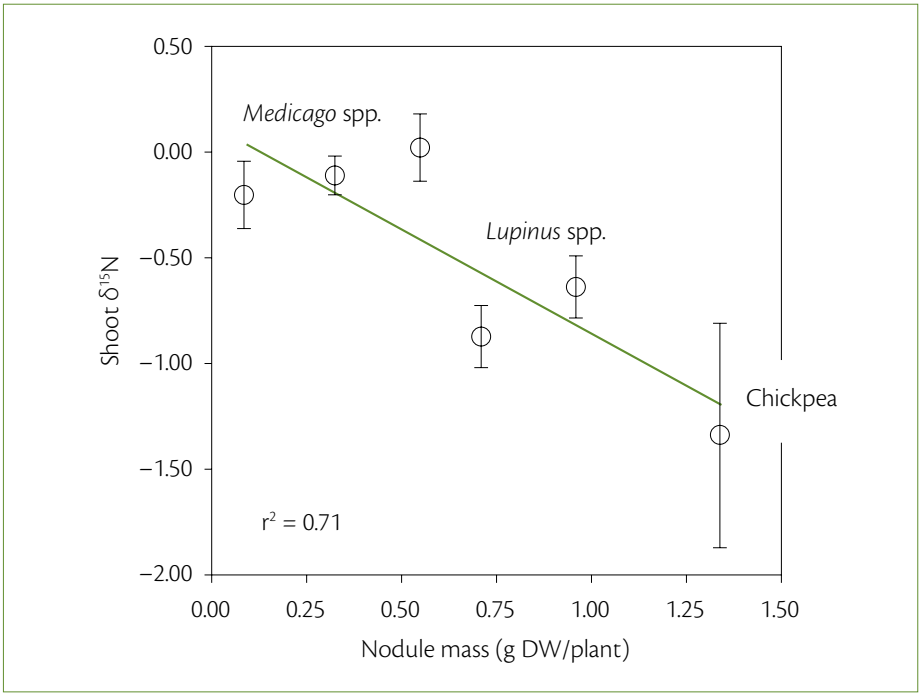


Figure 26. The relationship between legume shoot $\delta^{15}N$ and nodule mass for glasshouse-grown plants relying solely upon N_2 fixation for growth (DW, dry weight). Source: M. Unkovich, unpublished data

8.2.1 The impact of 'B' value on estimates of %Ndfa of legumes

Table 17 presents $\delta^{15}N$ data for chickpea and reference plants collected from a number of different field experiments in Australia to illustrate the effect of adjusting for isotopic fractionation by using different 'B' values when calculating %Ndfa. The different shoot 'B' values are:

- 0‰, which assumes no isotopic fractionation occurs
- −1.5‰, determined from the glasshouse experiment described in section 8.3 (Figure 27b)
- −2.1‰, calculated indirectly in the field as proposed by Doughton et al. (1992) (see section 8.3.1).

Table 17 shows that the 'B' value (0, –1.5 or –2.1‰) is of relatively little importance at low %Ndfa, but becomes important when %Ndfa is high ($\delta^{15}N$ of the N_2-fixing legume approaches and falls below 0‰). In the case of this particular dataset, the estimates of %Ndfa diverge more strongly when %Ndfa is >60%. Slight differences in 'B' value (i.e. –1.5 compared with –2.1‰) have only small effects on calculated %Ndfa (4–6% units).

Table 17. The impact of adjusting for isotopic fractionation when using ^{15}N natural abundance to calculate %Ndfa of chickpea

Material analysed		Estimate of %Ndfa		
Reference	Chickpea	'B' = 0 ‰[b]	'B' = –1.5‰[c]	'B' = –2.1 ‰[d]
$\delta^{15}N$[a]	$\delta^{15}N$			
(‰)	(‰)	(%)	(%)	(%)
8.7	8.2	6	5	5
6.7	5.9	12	10	9
6.8	5.4	21	17	16
10.0	7.0	30	26	25
12.2	6.4	47	42	40
10.1	3.6	64	56	53
5.1	1.0	80	62	57
7.7	0.3	96	80	76
6.3	–0.1	100[e]	82	77
6.3	–0.8	100[e]	91	85

a The individual reference ^{15}N values differ, as data were collated from different field experiments in Australia to provide a wide range in measures of chickpea %Ndfa.

b If no fractionation occurred the ^{15}N of a fully symbiotic legume shoot would be the same as atmospheric N_2.

c Calculated using a 'B' value determined for effectively nodulated chickpea grown in sand culture in the absence of combined N in the glasshouse.

d Calculated using a 'B' value derived from field data comparing estimates of %Ndfa calculated from ^{15}N enrichment and natural abundance plots (Doughton et al. 1992).

e Designated 100%, although using equation (11) would generate physiologically impossible estimates of %Ndfa >100%.

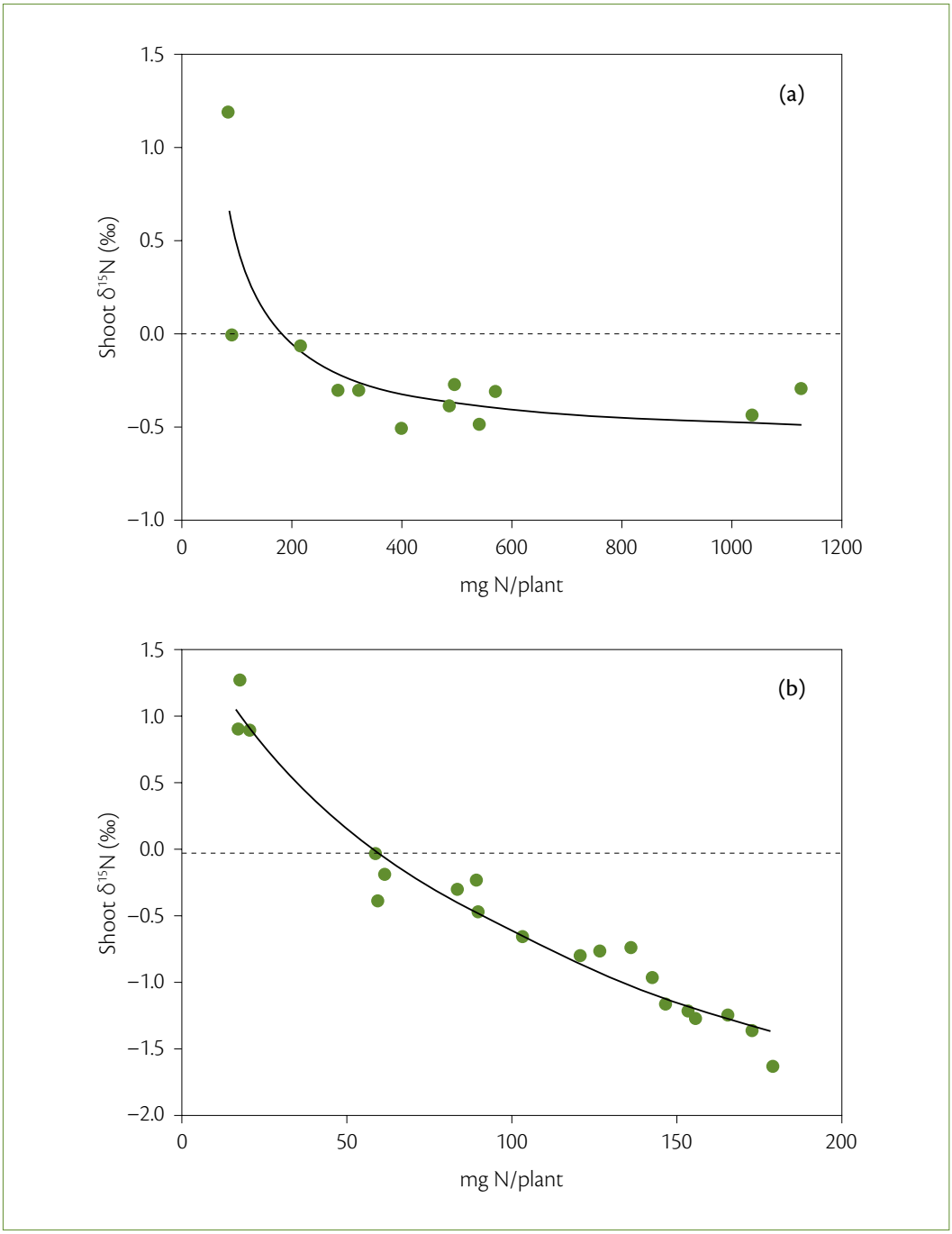


Figure 27. Changes during development in the $\delta^{15}N$ composition of shoots of effectively nodulated (a) faba bean and (b) chickpea growing in sand culture in the absence of combined N in a glasshouse and totally dependent on N_2 fixation

8.2.2 What if the $\delta^{15}N$ of the legume is less than that of the presumed 'B' value?

A quandary can arise when the $\delta^{15}N$ values of legume samples are less than that of the estimated 'B' value. Under such circumstances, %Ndfa would be incorrectly estimated using equation (11) to be >100%. These estimates could be redesignated as 100%, but another approach is to assume that samples exhibiting the low $\delta^{15}N$ values had a high reliance upon N_2 fixation and that the indigenous rhizobial strains were responsible for differences in isotopic fractionation between shoots and roots. One solution to this problem, proposed by Peoples et al. (1997, 2002), is to collate and examine all the $\delta^{15}N$ data collected in a district and designate a %Ndfa value of 100% (i.e. 'B' value) to those legume samples exhibiting the lowest $\delta^{15}N$.

8.2.3 Recommendations on what 'B' value to use

Table 18 provides a systematic way of working through the suitability of published 'B' values for different situations, and identifies conditions under which you may need to determine your own 'B' values (see also Appendixes 2–6 for a listing of 'B' values for shoots and foliage for a range of legumes).

The recommendations in Table 18 consider the two main factors that determine whether or not the 'B' value will substantially influence calculations of %Ndfa using equation (11). The first is whether the legume has a high or low reliance upon N_2 fixation (%Ndfa): the size of the 'B' value will be less important when %Ndfa is <50% (see Table 17). The second contributing factor is the magnitude of reference plant $\delta^{15}N$ (surrogate for $\delta^{15}N$ of soil mineral N): the smaller the reference $\delta^{15}N$, the greater the potential impact the choice of 'B' value has on the final estimate of %Ndfa. Unkovich et al. (1994b) provide a graphic illustration of the sensitivity of %Ndfa estimates to 'B' values and reference plant $\delta^{15}N$.

8.3 'B' value determination

The 'B' value is best determined on plants grown in a glasshouse in sand culture, and using the same strain(s) of rhizobia responsible for N_2 fixation at the field site(s) under study. Free-draining coarse sand is the preferred medium for such studies and, if possible, should be steam sterilised before use to eliminate resident rhizobia. The rooting media must not contain any N. Vermiculite should be avoided as it may release N (Giller et al. 1986).

Table 18. Recommendations on the 'B' value to use in various situations

Level of knowledge	Action
If reference plant $\delta^{15}N$ > 4‰, but legume $\delta^{15}N$ is greater than half reference value:	
For legume with no previously published 'B' values	use average of data in Appendixes 2–6 for relevant legume group.
If no local 'B' value for legume	use mean of previously published 'B' values from Appendixes 2–6.
If previously published 'B' values	use regionally explicit 'B' value.
If reference plant $\delta^{15}N$ > 4‰, but legume $\delta^{15}N$ is less than half reference value:	
If no local 'B' value for legume	use mean of previously published 'B' values from Appendixes 2–6.
If previously published 'B' values for region	use regionally explicit 'B' value.
If no regionally specific 'B' value	determine 'B' value in pot study.
If infecting strains are unknown	use mixed or soil inocula to determine 'B' value.
If specific strains are likely to infect plants	use specific strain inocula to determine 'B' value.
If reference plant $\delta^{15}N$ < 4‰:	
If no local 'B' value for legume	use mean of previously published 'B' values from Appendixes 2–6.
If previously published 'B' values for region	use regionally explicit 'B' value.
If no regionally specific 'B' value	determine 'B' value in pot study.
If infecting strains are unknown	use mixed or soil inocula to determine 'B' value.
If specific strains are likely to infect plants	use specific strain inocula to determine 'B' value.
If $\delta^{15}N$ of legume is less than available 'B' value:	
If <10% of legume samples fall in this category	assume %Ndfa approximates 100% for those samples.
If surveying legumes in farmers' fields and $\delta^{15}N$ of >10% of legume samples less than 'B' value	average the lowest values observed in the field dataset and assume this approximates 'B' value.

A naturally lit, temperature-controlled glasshouse is ideal for 'B' value determinations, but pots can also be placed outside, providing that N deposition in rainfall or dust is not a problem. Any size of pot can be used, with 10 L being a good size. Pots should be sterilised prior to filling with sand. This can be achieved with household disinfectant, but ensure that pots are rinsed thoroughly with distilled water after disinfecting and before sowing seeds. Pots can be leached with N-free water after they are filled. An absolute minimum of three replicate pots/inoculant, species, variety or sampling time must be used.

Seeds should be surface sterilised prior to sowing or inoculation, and this can be achieved by immersing seeds in a solution of 2% hypochlorite for 2–5 minutes followed by five or six rinses with sterile water. More rigorous seed-sterilisation methods can be found in Gibson (1980). After sterilisation, seeds may be pre-germinated before sowing, or sown directly into the prepared pots. If direct sowing, sow more seeds than required and thin out the seedlings once they have all emerged. Remove ungerminated seeds from the pot.

Inoculation can be with a prepared inoculant or a soil suspension from a field site. If using a soil suspension, soil should be collected from the top 10 or 20 cm layer of soil from many points in the target area. The soil should be mixed thoroughly and then passed through a 2 mm screen. If the soil has been dry for an extended period (weeks–months), it may be necessary to wet it and allow the rhizobial numbers to build up for 1 week prior to the next step. Mix 10 g soil with 1 L sterile water, decant or filter through cloth, then add 100 mL inoculant solution to each pot after the seeds have been sown. Ten days after sowing the number of seedlings can be reduced to four or five per pot, and thereafter supplied with adequate volumes of dilute nutrient solution containing all nutrients except N (see Appendix 1).

Whole shoots should be harvested from replicate pots at intervals between early vegetative growth and late pod-filling, just before leaf yellowing or leaf drop. Shoot material from each pot should be placed into paper bags and immediately oven-dried, weighed, ground and analysed for total N and $\delta^{15}N$ (see Chapter 3). Nodulated roots can also be harvested from pots and analysed for total N and $\delta^{15}N$, but should be kept and analysed separately from shoots.

Typical data from a series of sequential samplings are presented in Figure 27. Shoot $\delta^{15}N$ was high initially, reflecting the $\delta^{15}N$ of seed reserves, but declined progressively as the shoots accumulated fixed N. Although there was some variation between individual pots, shoot $\delta^{15}N$ levelled out at –0.4‰ for

Field pea is an amide exporter. As a consequence, the ureide technique cannot be used to quantify N_2 fixation by this species. The most appropriate measurement method, for both experimental plots and farmer crops, is the ^{15}N natural abundance (see Table 3).

faba bean and –1.5‰ for chickpea. Both these values were similar to previous determinations of shoot 'B' values for faba bean and chickpea from other experiments or laboratories (see Table 16 and Appendix 2).

Herbaceous and woody perennial species are ideally grown in much larger (30 L) pots for longer periods (at least a year). This is clearly a compromise as it is impossible to expect to be able to grow, in a glasshouse, perennial legumes that resemble plants in the field. However, the $\delta^{15}N$ of the glasshouse-grown plants should provide a reasonable 'B' value for subsequent use in field studies. It is also possible to derive a 'B' value for perennial species growing over an extended period by calculating the $\delta^{15}N$ of the shoot or foliage N accumulated during growth between sequential harvests. Examples of 'B' value determinations for perennial species can be found in Unkovich et al. (2000) and Boddey et al. (2000), and are given in Appendixes 4 (temperate forage legumes), 5 (tropical forage and covercrop legumes) and 6 (woody perennials).

An indirect means of determining 'B' values under field conditions using a combination of natural abundance and ^{15}N enriched methodologies has also been proposed. Details of the methodology can be found in Doughton et al. (1992) and Okito et al. (2004). However, the method is not generally recommended for use by those less experienced with the use of ^{15}N.

8.4 Non N_2-fixing reference plants

An assumption of the method is that the $\delta^{15}N$ of non N_2-fixing reference plants is identical to the $\delta^{15}N$ of soil N utilised by the legume. To increase the likelihood of this, reference species should:

- exploit the same soil N pool as the legume (Note that for this reason no N-fertiliser should be supplied to the reference plant unless it is a specific treatment that is also applied identically to the legume.)
- have a similar duration of growth and pattern of N uptake as the legume
- receive no significant transfer of fixed N from the legume if they are growing in close association (Note that this is not necessarily a problem if the fixed N is released by the legume into the soil matrix, and is available for subsequent uptake by roots of both the legume and reference plant. Errors will arise only if there is a direct transfer of fixed N to the reference plant, such as might be mediated via a mycorrhizal hyphal bridge.).

Reference plants used in natural abundance studies have included:

- non-nodulating legumes
- uninoculated legumes
- non-legume crop, pasture and tree species
- broadleaf and grass weeds.

Non-nodulating isolines of soybean, groundnut and chickpea have proven to be valuable reference plants in a number of ^{15}N natural abundance studies with annual legume crops (e.g. Herridge and Peoples 1990; Peoples et al. 1992; Schwenke et al. 1998; Cadisch et al. 2000), and species incapable of forming N_2-fixing nodules such as *Senna* have been used for tree and shrub legume studies (e.g. Table 19; Ladha et al. 1993). However, non-nodulating isolines do not always show equivalent $\delta^{15}N$ to alternative non-legume references (Peoples et al. 1997). Partial breakdown of the non-nodulating character occasionally occurs. Routine examination of the root systems of putative 'non-nodulating' legume lines or species for the presence of nodules is recommended, even if the plants appear stunted and yellow compared with the N_2-fixing legume.

Uninoculated legumes have also been used as reference plants (e.g. Bergersen et al. 1989; Herridge et al. 1998), but their use is restricted to sites where the soil is known to be free of effective rhizobia (e.g. where the legume species has never been grown before). If planning to use uninoculated legumes as reference plants, soils from potential field sites should be tested for their rhizobial status before commencing a trial. Just as with the use of non-nodulating legume lines, it is essential that the roots of the uninoculated legume be examined at the time of sampling to confirm the absence of nodules. It is particularly important to look for late nodulation on lateral roots. If nodules are found, the plants must be discounted as a reference plant, no matter how small the nodules may appear. Never rely on an uninoculated treatment as the sole reference material.

In Asia and Africa where legumes are commonly grown in association with other crop species, or in agroforestry systems where crops are grown in 'alleys' between tree-legume hedgerows, an intercropped cereal or broadleaf crop could be utilised as the non-fixing reference. Similarly, in grazing systems where it is common for many species to be growing together, the non-legume component of the pasture provides convenient reference material. In both of these situations, most of the potential reference plants are likely to have been growing under the same conditions and for a similar duration as the legume.

In plot studies, specific reference treatments are routinely included in the experimental design. Broadleaf or grass weeds can also be used as additional reference species (e.g. Rerkasem et al. 1988; Unkovich et al. 1994b). However, in farmers' fields where legumes are monocropped, weeds may be the only reference species available. Some care is required when relying on weeds as reference plants since there can be several germination events and it may be difficult to judge the duration of their growth relative to the legume under study. Errors may be introduced if the weed reference plants assimilate soil N over a markedly different period to that of the legumes. For this reason, it is best to avoid collecting small weeds. Another strategy to use in commercial legume crops where there is not likely to be companion non-legumes other than weeds is to specifically hand-sow small areas of reference species early in the growing season (e.g. Schwenke et al. 1998: this option is discussed in more detail in section 8.8). Something like an oilseed *Brassica* species (canola or mustard) may be a good choice as they can be easily found for later sampling in the midst of a legume field and often have similar rooting patterns.

If the $\delta^{15}N$ of mineral N is relatively uniform down a soil profile, and/or the $\delta^{15}N$ values of reference plants change only gradually over time (e.g. Table 19, see also Herridge et al. 1990; Boddey et al. 2000), the impact of choice of

reference species is likely to be small. A number of studies involving both annual and perennial legumes have reported almost identical $\delta^{15}N$ values for different reference plants (e.g. Peoples et al. 1997; Boddey et al. 2000), even in situations where the reference species differ markedly in rooting depth (e.g. Table 19, Ladha et al. 1993). However, if the $\delta^{15}N$ of soil mineral N changes markedly with depth or fluctuates during the growing season (Turner et al. 1987; Bremer and van Kessel 1990), measurable differences in $\delta^{15}N$ of reference species may occur if they differ in rooting depth or N uptake characteristics (Ojiem et al. 2007; Pate et al. 1994).

Table 19. $\delta^{15}N$ of regrowth of two different non N_2-fixing plants growing at the same location in northern Queensland, Australia[a]

Time after sowing (months)	*Senna spectabilis*	Guinea grass
	mean $\delta^{15}N$ ± SE	mean $\delta^{15}N$ ± SE
	(‰)	
6	4.4 ± 0.22	4.3 ± 0.21
10	4.3 ± 0.18	6.0 ± 0.44
12	3.6 ± 0.14	3.7 ± 0.19
16	4.3 ± 0.26	4.4 ± 0.24
24	4.2 ± 0.12	not done
48	4.1 ± 0.32	4.2 ± 0.32

[a] The non-nodulating shrub legume *Senna spectabilis* had lateral roots to a soil depth of 1.5 m with many fibrous roots in the top 30–50 cm. The majority of the guinea grass (*Panicum maximum*) roots, on the other hand, appeared to be in the top 15–30 cm. All plant foliage above a height of 75 cm in the case of *Senna*, and 5 cm for the guinea grass, was removed at 2–4-monthly intervals for the duration of the experiment. The mean $\delta^{15}N$ values detected in the subsequent regrowth differed significantly between species at only one sampling (10 months after sowing). Sources: Peoples et al. (1996) and additional unpublished data

We strongly recommend use of more than one reference species (see Table 20). Preference should be given to including a non-nodulating or unnodulated legume if available, and a broadleaf (dicotyledonous) non-legume, as a dicot's taproot system might be expected to more closely resemble that of a legume than the fibrous root systems typical of cereals and grasses. However, cereal

or grass species can also be considered. There is some advantage in including reference species with differing rooting patterns as it provides an indication of likely variation in $\delta^{15}N$ with soil depth and time. If $\delta^{15}N$ differs between reference species, calculate %Ndfa using each reference species to show the range of estimates, and also calculate a mean %Ndfa, as illustrated in Table 20.

The key points from Table 20 are that:

- the impact of reference species on the calculations of %Ndfa is generally most important when the %Ndfa of the legume is low (<30%), or where the $\delta^{15}N$ of the reference is less than about 4‰
- the choice of reference species has less influence when the legume %Ndfa is high (>70%), and/or the $\delta^{15}N$ of the reference is >6‰.

Magnitude and variability of soil ^{15}N abundance

8.5

The accuracy of the natural abundance technique ultimately depends upon the magnitude and uniformity of $\delta^{15}N$ in the plant-available soil N pool. Since the relationship between legume $\delta^{15}N$ and %Ndfa is linear for a given reference $\delta^{15}N$, the sensitivity of the final estimate will also be proportional to the reference $\delta^{15}N$ value (Unkovich and Pate 2000). The higher the reference $\delta^{15}N$, the more precise the estimate of N_2 fixation. Unkovich et al. (1994b) suggested that, given an analytical precision of ± 0.2‰, a reference $\delta^{15}N$ of at least 2‰ (about 10 times the precision of measurement) would be required to detect a theoretical change in %Ndfa of around 10%. This is also the lowest reference $\delta^{15}N$ recommended here unless you have considerable experience with the $\delta^{15}N$ natural abundance technique and %Ndfa is likely to be higher than 65–70%.

Some studies have shown that the $\delta^{15}N$ of reference plants can be relatively uniform across a field (see Australian data in Table 21), but others have shown short-distance variability in soil $\delta^{15}N$ (e.g. data from Pakistan in Table 21; also Bremer and van Kessel 1990; Holdensen et al. 2007). Reasons for this heterogeneity in the $\delta^{15}N$ of plant-available soil N are not always obvious. Heterogeneity in $\delta^{15}N$ (and N_2 fixation) might be associated with applications of farmyard manures, urine deposition by grazing animals (Eriksen and Hogh-Jensen 1998), residual fertiliser or slight variations in topography that induce differences in water content, soil mineral N, waterlogging and denitrification (Stevenson et al. 1995). Strategies to reduce the impact of such variations are discussed under sampling protocols in section 8.8.

Table 20. Impact of different non N_2-fixing reference species on estimates of legume %Ndfa[a]

Location	Reference species	^{15}N (‰)	Legume	^{15}N (‰)	Ndfa (%)[b]
Brazil	Maize	4.4	Common bean	2.9	23
	Grass	4.3	'B' value: –1.98‰		22
	Dicot weed	5.9			38
			Mean		(28)
Australia	Uninoc chickpea[c]	7.9	Chickpea	5.7	22
	Barley	8.9	'B' value: –1.67‰		30
	Wheat	9.9			36
			Mean		(29)
Australia	Wild radish	3.7	Faba bean	0.7	73
	Milk thistle	3.6	'B' value: –0.36‰		73
	Grass	2.9			61
			Mean		(69)
Australia	Non-nod. soybean	8.0	Soybean	3.0	53
	Uninoc. soybean[c]	7.4	'B' value: –1.35‰		50
			Mean		(52)
Kenya	Maize	3.6	Soybean	1.5	40
	Dicot weeds	6.3	'B' value: –1.65‰		60
			Mean		(50)
Philippines	Grass	5.9	Gliricidia	3.3	36
	Maize	7.3	'B' value: –1.45‰		45
	Senna spectabilis	7.7			48
			Mean		(43)

[a] Sources: Bergersen et al. (1989), Ladha et al. (1993), Unkovich et al. (1994a), Herridge et al. (1998), Ojiem et al. (2007) and unpublished data

[b] Individual estimates of %Ndfa are calculated for each reference species at each location. Values in parentheses represent the mean across all reference species at that specific location.

[c] The experiment was undertaken in a soil that had no native rhizobia capable of nodulating soybean. Treatments included ± inoculation with *Bradyrhizobium japonicum*.

Table 21. ^{15}N of non N_2-fixing weeds collected at 10 sampling points from within farmers' legume crops in New South Wales, Australia, and in the North West Frontier Province, Pakistan[a]

Farm location:	Australia	Pakistan
Crop:	Chickpea	Lentil
Crop area (ha):	50 ha	0.66 ha
Distance (m)[b]:	225m	30m
Sample point	**^{15}N (‰)**	**^{15}N (‰)**
1	6.5	4.1
2	7.4	2.5
3	6.6	4.1
4	7.2	1.1
5	6.3	4.5
6	7.5	1.9
7	6.4	1.1
8	6.2	0.0
9	7.1	−0.1
10	5.8	0.8
Mean ± SE	6.7 ± 0.18	2.0 ± 0.55

[a] Data derived from Peoples et al. (2002).

[b] Distance between sampling points

Glasshouse application of ^{15}N natural abundance

8.6

The natural abundance technique has been used successfully in glasshouse studies of N_2 fixation. A suitable soil is one in which reference plants have a relatively high $\delta^{15}N$ (ideally >4‰), and this can be assessed beforehand by sampling some plants from the soil in the field. In this way several sites can be sampled and the site with the highest $\delta^{15}N$ then selected for use in a pot study. However, it should be noted that the $\delta^{15}N$ of the plant-available soil N pool may change once the soil has been disturbed and placed into pots and subjected to regular watering. If this happens, the $\delta^{15}N$ measured in the glasshouse-grown material will not be identical to that previously observed in plants growing in the field. The use of soil cores, rather than disturbed soils, may reduce this difference.

Non N_2-fixing reference species need to be included and sown into their own pots, and all treatments should be replicated at least three times (preferably 4–6 replicates). Pot sizes and plant densities should be the same for both legume and reference plants. Treatments and legume and reference pots are normally randomised on the bench in order to avoid bias that results from temperature gradients or differences in light quality (shading) within the glasshouse. Cigarette smoke is a significant source of NH_3 contamination of plants and smoking should not be permitted in glasshouses where plants are grown for $\delta^{15}N$ analysis.

Legume and reference plants should be sampled at the same time, and preferably before either reach maturity or have significant leaf drop. Do not apply N to reference plant pots if it is not also applied to the legume pots under study. Only whole shoots should be sampled. Do not sample individual shoot components (leaves, stems, pods) separately and calculate %Ndfa for each fraction. Differences in $\delta^{15}N$ within plants are not due to differences in N sources, but to other fractionation processes within the plant. If you do need to separate components, then the dry weight, N content and $\delta^{15}N$ of each will need to be measured, and the $\delta^{15}N$ for whole shoots calculated. The %Ndfa can be calculated for each treatment based on the average shoot $\delta^{15}N$ for the reference and legume pots. If roots are also harvested from pots, a whole plant $\delta^{15}N$ and %Ndfa can be calculated (Table 22).

Table 22. Calculation of aggregate $\delta^{15}N$ (or atom% ^{15}N) from two plant parts

	Parameter	Shoots	Roots
Measured	Dry weight (g)	3.52	1.25
	N content (%)	2.40	1.67
	$\delta^{15}N$ (‰)	+0.66	+1.23
Calculated	Total N (mg)	84.48	20.87
	Atom% ^{15}N	0.36654	0.36675
	Total mg ^{15}N	0.30965	0.07656
	Shoot+root ^{15}N (mg)	(0.30965 + 0.07656) = 0.38621	
	Shoot+root total N (mg)	(84.48 + 20.87) = 105.35	
	Shoot+root atom% ^{15}N	100 × (0.38621)/105.35 = 0.3666	
	Shoot+root $\delta^{15}N$ (‰)	1000 × (0.3666–0.3663)/0.3663 = +0.82	

8.7.1 Pre-experiment information

Nitrogen fixation of legumes can be influenced by the previous cropping sequence, periods of fallow, cropping intensity, reduced tillage, the use of 'starter-N' and the carryover of unused fertiliser from previous crops. Previous crops can also influence N_2 fixation in the absence of major effects on soil nitrate through impacts on legume growth resulting from changes in soil structure, soil water and the incidence of pests or disease. Therefore, while in the process of selecting a potential site for experimentation, it is useful to know:

- what species were grown in the past year, what fertiliser was used (type and rate), and what herbicide may have been applied (to identify potential carryover of herbicide residues that might affect legume growth during the experiment and/or place any restrictions on future weed control options)
- the extent of insect damage, disease incidence and weed control in previous crops (to identify whether there may be any specific agronomic challenges at a particular location)
- how the prior crop performed and how variable it was—this will provide information about specific areas of soil with underlying nutritional or structural constraints across a potential field site that either need to be accounted for or avoided
- what legumes have been grown previously and what inoculants might have been applied, if any
- whether the $\delta^{15}N$ of plant-available soil N is high or low. This can be assessed by selecting some weeds from prospective sites, analysing them for $\delta^{15}N$ and choosing the site with the highest value. A very rough guide can also be obtained from analysis of non-legume grain harvested from the site in the previous season.

Sites with high N fertility or a recent history of N fertiliser applications will reduce N_2 fixation and mask treatment effects. Soil nitrate-N above 10 mg/kg could reduce nodulation, with values >20 mg/kg delaying establishment of a functioning symbiosis.

8.7.2 Experimental design

The recommended design of a field trial is typically randomised plots of both legumes and non N_2-fixing reference plants. However, as the natural abundance method relies on a comparison of the ^{15}N contents of legume and non N_2-fixing plants, and because they will vary spatially, the relative positions of the legume and non N_2-fixing plants can substantially affect results. Alternative designs such as those shown in Figure 28 can be accommodated statistically and may reduce errors in estimating N_2 fixation due to soil heterogeneity. Consult your biometrician during the experimental design phase, show them the designs in Figure 28 and highlight the points raised above.

More than one reference species is recommended, and there should be at least three replicate plots (preferably 4–6) for each experimental treatment. The same rates of N fertiliser should always be applied to both the legume and reference species because of effects on plant $\delta^{15}N$. An example of the effects of applied fertiliser N on the $\delta^{15}N$ of soil mineral N, assessed as $\delta^{15}N$ in the harvested non N_2-fixing reference plant, is shown in Figure 29. Clearly, applying different rates of fertiliser N to the legume and reference plants would invalidate a major assumption of the natural abundance method that legume and reference plant use soil N of similar ^{15}N abundance.

The size of each plot will depend on the objective of the experiment, the species under study, the number of harvests during the experiment, and land and labour resources available. If the objective of the experiment is simply to provide a comparative assessment of treatment effects on %Ndfa, the individual plot size does not need to be exceptionally large, as measures of legume biomass will not be required. If estimates of kg N fixed/ha are required, it will be necessary to allow adequate sampling areas to ensure plant biomass can be accurately determined (see section 3.1.1). Additional plot space should also be included for crop legumes so grain yield data can be collected from previously unsampled areas. The area required will depend upon whether the final yield determinations are to be based on hand-harvested material, the use of a small mechanised harvester, or the use of a farmer's harvesting equipment. A stout fence around the outside of the experimental area to keep out domestic animals, rabbits etc. is always a good investment.

Regardless of whether or not biomass determinations are necessary, plots need to be large enough so that plant sampling can occur without being influenced by edge effects. Plants at the edge of a plot are often noticeably larger than those growing in the centre of a plot. Such edge effects result from access to additional soil water, nutrients and light, because of reduced plant-to-plant

(a) Classical randomisation

legume 2
reference
legume 5
legume 1
legume 3
legume 4

legume 4
legume 1
legume 5
legume 3
legume 2
reference

(b) Reference plant strips

reference A	legume 4	reference B
	legume 1	
	legume 5	
	legume 3	
	legume 2	

reference A	legume 3	reference B
	legume 4	
	legume 2	
	legume 1	
	legume 5	

(c) Reference plants interspersed

legume 4
reference
legume 1
reference
legume 5
reference
legume 3
reference
legume 2

legume 3
reference
legume 4
reference
legume 2
reference
legume 1
reference
legume 5

Figure 28. Examples of plot layouts for quantifying legume N_2 fixation using ^{15}N techniques, compared with classical randomisation (a). The strips of non N_2-fixing reference plants (b) and interspersed reference plots (c) can reduce effects of spatial heterogeneity. The above designs cover some possible reference plant strategies but do not cover other important statistical considerations such as randomisation and phase replication.

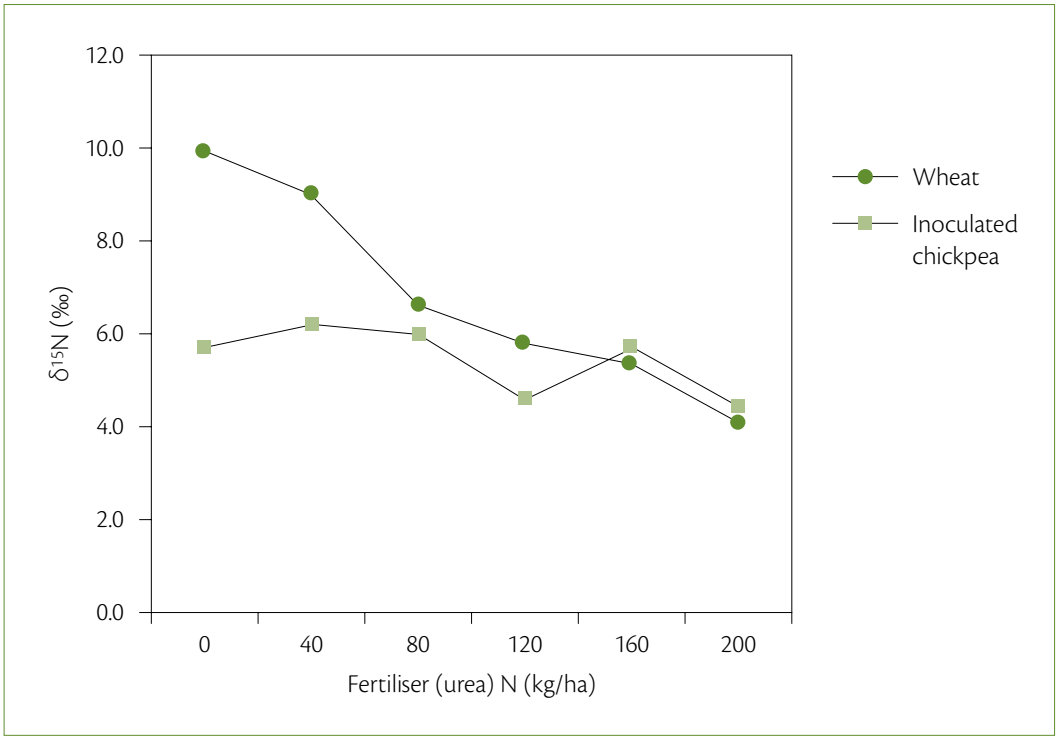


Figure 29. Effects of applied fertiliser (urea) N on the $\delta^{15}N$ of soil mineral N, assessed as the $\delta^{15}N$ of the non N_2-fixing wheat, and on $\delta^{15}N$ and calculated %Ndfa of chickpea, grown in the field in Australia. Source: Herridge et al. (1998)

competition. The plants at the edge can also be smaller, usually because of pest damage. Either way, edge effects will bias estimates of both %Ndfa and plant biomass. As a rough guide, crop legumes should have a buffer of 1 m at the end, and two rows at the edge, of plots, and forage legumes 0.5 m at the edge of plots. In the case of trees and shrubs, the sampled plants should be surrounded on each side by at least one tree of the same species receiving the same treatment. Where experiments involve different tree species in hedgerows, Rowe and Cadisch (2002) recommend inter-plot spacings of 8 m to ensure no mineral N is taken up by lateral roots from neighbouring plots.

Plant-available soil $\delta^{15}N$ is highly sensitive to rapid changes in soil N forms. Urine patches, manure heaps, high rates of fertiliser application and soil disturbance through tillage can impact on the $\delta^{15}N$ values of plant-available N. When setting up trials make a note (map) of any evidence of the above disturbances as this may be highly useful in assisting with interpretation of raw $\delta^{15}N$ data.

Sampling farmers' fields

8.8

Companion non-legume species, or weeds growing immediately beside the sampled N_2-fixing legumes, make convenient reference material in farmers' fields. However, depending upon the agronomic skills of the farmer, it can be difficult to find weeds in some commercial crops. To ensure the presence of non N_2-fixing reference material when undertaking surveys of farmers' chickpea and faba bean crops in Australia, Schwenke et al. (1998) hand-sowed small areas of non-nodulating chickpea and/or canola in the fields when the farmer sowed the main crop. These additional references were sown at each of 10 predetermined sampling points at regular intervals along fixed transects crossing each field in a 'W' pattern. Plant harvests were made in the immediate vicinity of these sampling points and the non-nodulating chickpea and

A farmer's crop of narrow-leafed lupin in Australia. The ^{15}N natural abundance method can, with appropriate sampling of both the legume and non N_2-fixing reference plants, provide accurate estimates of seasonal N_2 fixation.

canola collected separately. If weeds were found growing in farmers' crops, they were also collected as additional references along with the sown reference species, but some discretion was required. Very small dicot or grass weeds were avoided as it was possible that they may not have grown for the same length of time as the legume.

8.8.1 Sampling procedures—annual legumes

With annual species, $\delta^{15}N$ can differ between plant parts of both N_2-fixing legumes and reference plants (e.g. Figure 30). Thus, it is recommended that whole shoots be harvested and analysed. When combining the collected reference and legume data to calculate %Ndfa, it may not always be possible to avoid variability resulting from 'noise' in plant-available soil $\delta^{15}N$. Various protocols have been suggested involving increased replication, bulking replicates, larger sampling areas or strategic sampling to improve precision of measurements (e.g. Unkovich et al. 1994b; Peoples et al. 2002; Holdensen et al. 2007).

Variability in plant-available soil $\delta^{15}N$ may remain a problem where non N_2-fixing reference and N_2-fixing plots are physically separated in experimentation. It follows that errors should be minimised if the reference plants are close to the N_2-fixing species. This is usually possible in pasture systems where legumes are generally growing in association with a range of non-legume species, and for crop legumes where they are intercropped with cereals or interspersed with weeds (see above). It is clear from experience that sampling legumes together with 'paired' reference material from neighbouring non-legume plants or weeds can reduce problems of variability in soil $\delta^{15}N$ between replicates and result in more accurate estimates of %Ndfa (Table 23).

8.8.2 Sampling procedures—perennial legumes

There are obvious difficulties in obtaining for ^{15}N and total N analysis representative samples from large, woody perennials. When it is not practical to sample whole trees, an alternative approach is to subsample leaves or regrowth. Collections of 20–60 leaves at prescribed intervals, sampling of most recently formed leaves or the use of regrowth from trees that are regularly pruned and maintained as hedgerows are all approaches that have been used to estimate %Ndfa (e.g. Peoples et al. 1996; Unkovich et al. 2000).

From the limited evidence available, it seems that variation between plant organs in perennials is less than in annual species, except perhaps in tree seedlings. For example, a $\delta^{15}N$ range of only 0.3–0.6‰ was found in young

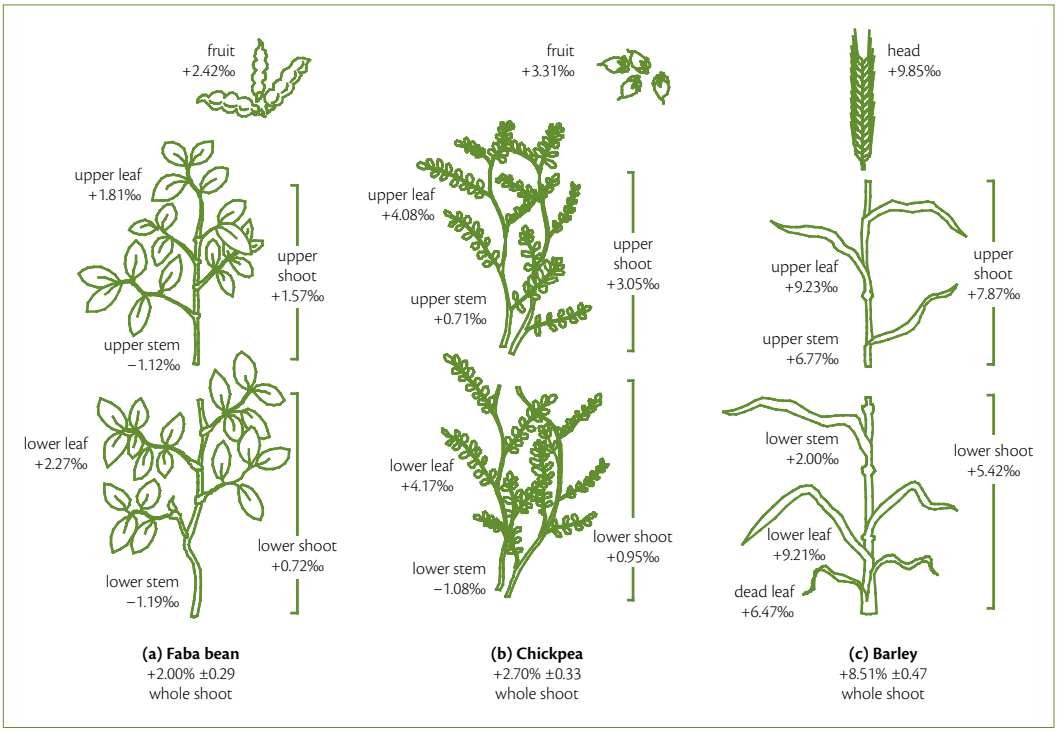


Figure 30. $\delta^{15}N$ in different leaf and stem strata, shoot fractions, reproductive parts and whole shoots of field-grown (a) faba bean, (b) chickpea and (c) barley. Data are from Peoples et al. (2002).

and old leaflets and branches and leaves taken at different heights from 4–5 m high *Desmodium rensonii* trees growing in Indonesia (Peoples et al. 1991). Other data indicate that the differences in the $\delta^{15}N$ of old and new growth, and of the trunk, branch and leaf material in prunings, can be relatively small (e.g. Table 24). This suggests that subsampling of the woody perennials is a realistic option. In these situations, potential errors associated with the relocation and recycling of N, either during the growing season or following regrowth after cutting, appear insignificant.

However, differences in $\delta^{15}N$ between different tissues of perennials are not always trivial. For example, the $\delta^{15}N$ in the 3–10 cm apical region of coppiced shoots of the perennial shrub legume tagasaste was found to be >1‰ higher than measured in the whole coppiced dry matter (Unkovich et al. 2000). Data from Kenya showed that the new growth of calliandra had $\delta^{15}N$ values of 3.7‰ compared with 1.4‰ for the old growth (Gathumbi et al. 2002). Differences of up to 3‰ between leaves and branches have also been reported

Table 23. Estimating %Ndfa of legumes where there is variability in the ^{15}N among replicate samples of non N_2-fixing reference plants and legumes

Location	Reference ^{15}N	Legume ^{15}N	%Ndfa[a]
Species and replicate	**(‰)**	**(‰)**	**(%)**
Banke, Nepal			
Chickpea 1	2.8	–0.6	77
2	3.2	–0.9	85
3	4.7	–0.3	79
4	6.2	0.0	79
5	7.5	0.2	80
Mean ± SE			80 ± 1.7
Chiang Mai, Thailand			
Soybean 1	1.7	–0.5	72
2	2.1	–0.2	67
3	7.8	0.9	76
4	4.1	0.3	70
Mean ± SE			71 ± 1.9
Victoria, Australia			
Lucerne 1	13.9	4.4	64
2	11.5	3.7	65
3	7.0	2.2	64
4	5.0	1.6	63
Mean ± SE			64 ± 0.4

[a] 'B' values used for calculations were: –1.5‰ (chickpea), –1.3‰ (soybean) and –0.44‰ (lucerne).

for leucaena growing in plantations (van Kessel et al. 1994). Therefore, at least a preliminary investigation should be undertaken of the different tissues (leaves, twigs, branches, trunks and roots) of trees, for both the nodulated legumes and the potential non N_2-fixing reference plants, to assess potential errors in using only readily harvested foliar samples.

Table 24. Comparisons of the ^{15}N natural abundance (‰) of various plant parts of perennial legumes growing in plantation or agroforestry systems[a]

Location and species	Uncut trees		Pruned hedgerows			
	Old growth	New growth	Regrowth	Leaf	Stem	Trunk
Australia						
Calliandra	–	–	2.1[b]	2.1	2.0	
Calliandra	2.5	2.3				
Senna spectabilis	–	–	4.3[b]	4.8	3.8	
Indonesia						
Desmodium rensonii	0.6	0.5				
Calliandra	1.1	1.4	1.1[c]			
Leucaena	5.3	6.2	5.8[c]			
Philippines						
Acacia mangium	0.5	0.6				
Albizia falcataria	–0.9	–1.0				
Senna spectabilis	–	–	6.5[b]	6.8	6.2	5.1
Gliricidia	–	–	3.7[b]	4.0	3.1	3.4

[a] Sources: Ladha et al. (1993), Peoples et al. (1996), Boddey et al. (2000)

[b] Regrowth 3 months after pruning trees growing in hedgerows or alleys. The leaf and stem data in the neighbouring columns represent the $\delta^{15}N$ of the leaf and stem component parts of the regrowth.

[c] One-third of the branches of individual trees were cut and the $\delta^{15}N$ of the regrowth after 3 months was compared with the old and young growth on the remaining uncut portion of the same trees.

While it is likely that leaves and regrowth represent the largest single pool of above-ground N in many agroforestry systems, it should be recognised that the omission of roots from the calculations will underestimate the amounts of N_2 fixed by trees. Even where roots are harvested from soil and included in the analysis, the resulting measures of plant N and N_2 fixed are likely to be conservative since it is virtually impossible to recover all live roots and nodules, let alone senesced materials. However, what such data can provide are estimates of the minimum inputs of fixed N into the agroforestry system under study.

A further issue when estimating inputs of fixed N for a given period concerns the measurement of annual dry-matter accumulation (i.e. net primary productivity). With perennial pasture legumes, this can be achieved by combining (rotating) animal exclusion cages and sequential harvests (e.g. Unkovich et al. 1998), or by timing harvests to coincide with hay cuts, when all above-ground material can be collected. However, this is more problematic in agroforestry systems.

There are also challenges in preparing material for ^{15}N analysis. While it is relatively easy to grind leaves, the preparation of woody trunks, branches and roots for analysis can be more difficult. One approach is to subsample the woody components using a saw and collecting the sawdust to be ground for N and ^{15}N analysis (Peoples et al. 1996). Once the ^{15}N abundance has been determined for each part, a 'weighted' ^{15}N composition based on the ^{15}N abundance and proportional amounts of N in each organ can be used to calculate total above-ground ^{15}N and total N content (see Table 22 and Chapter 3).

8.9 Evaluation of ^{15}N natural abundance data and calculations

It is important to be familiar with the calculation of $\delta^{15}N$ from atom% ^{15}N data since it may be necessary at times to calculate $\delta^{15}N$ or atom% ^{15}N for an aggregate, and the values cannot simply be added because neither atom% ^{15}N nor $\delta^{15}N$ values are absolute amounts. To calculate atom% or $\delta^{15}N$ values for aggregate samples, you must calculate the fraction of the total N as ^{15}N, and to do this you must know the total amount of N in the sample and the total amount of ^{15}N.

For example, assume you have harvested plants whose shoot material had $\delta^{15}N$ of 0.66‰ and the $\delta^{15}N$ of the nodulated roots was 1.23‰. What is the $\delta^{15}N$ of the whole plant? To calculate this you need to know the dry matter and %N of the shoots and roots as well (see example workings in Table 22). From this you can calculate the total N in the plant (shoot + root). Then, from the $\delta^{15}N$ values calculate the atom% ^{15}N of the shoot and the root, and multiply this by the N in each to get the total ^{15}N in each. This can then be summed (0.38621 mg ^{15}N) and divided by the total N (105.35 mg N) to get the atom% ^{15}N of the whole plant (0.3666 atom% ^{15}N), from which a $\delta^{15}N$ value can then be recalculated (0.82‰).

A farmer's crop of faba bean in Australia. The farmer's control of weeds in the crop can sometimes be so effective that sampling of non N_2-fixing reference plants becomes virtually impossible. In such cases, hand sowing of reference plants at the time of sowing the legume can provide a solution.

Application of the ^{15}N natural abundance method is not always straightforward. It does not work if the $\delta^{15}N$ of the legume does not fall between the 'B' value and the $\delta^{15}N$ of the reference plant. This problem will be indicated by %Ndfa being <0% or >100%. If this happens, a greater investigation into 'B' values or reference plants may be warranted. Unkovich et al. (1994b) and Pate et al. (1994) provide fuller discussions of these issues.

Analysis of $\delta^{15}N$ requires highly sophisticated and well-maintained equipment and skilled operators. Do not take all data at face value but check to see that the data make sense. Check the %N as well as $\delta^{15}N$ data and look for irregularities, especially outlying data points that may indicate a problem with analysis. The cost of analysis is high but it is always worthwhile to include a few replicates of individual samples to check for within-sample variability.

$\delta^{15}N$ values rarely fall outside the range of –2‰ to 12‰ (Letolle 1980), and values >15‰ should be carefully examined. If soil NH_3 volatilisation or denitrification rates are thought to be high, this could lead to such high values, otherwise re-analysis may be warranted. Shoot N concentrations are usually within the range 1–4%. It is always worthwhile plotting out raw data of each replicate and checking for outliers. The outliers should not be discarded, but considered carefully before inclusion.

8.10 Final word

The $\delta^{15}N$ natural abundance technique is a powerful means of assessing N_2 fixation in the field. Confidence in the technique is strengthened by similarity in $\delta^{15}N$ values among reference plants and treatment replicates. Large shifts in reference plant $\delta^{15}N$ should be accompanied by some change in legume $\delta^{15}N$, unless the legume is very highly dependent on N_2 fixation. If estimates of %Ndfa change, but the $\delta^{15}N$ of legume does not, the $\delta^{15}N$ of the reference plant may not be reflecting the $\delta^{15}N$ of soil N taken up by the legume. As with all other methods for estimating N_2 fixation, collate supporting data on nodulation and soil mineral N.

9

^{15}N isotope dilution method

Section 7.1 gives an introduction to stable isotopes and to ^{15}N terminology and notation. The ^{15}N isotope dilution method was most widely used in the 1970s–1990s, prior to improvements in mass spectrometry, beginning in the 1980s, that led to the development of the ^{15}N natural abundance technique. Where the $\delta^{15}N$ of plant-available N is <2‰ and the $\delta^{15}N$ methodology should not be used, or you do not have access to high-precision mass-spectrometry analysis ($\delta^{15}N \pm 0.3$‰, ± 0.0001 atom% ^{15}N), plant-available soil N can be artificially enriched with ^{15}N-labelled fertilisers. This enables estimation of legume (and other N_2-fixing plants—see Chapter 10) uptake of N from the soil, and calculation of N_2 fixation by difference.

9.1 How it works

Legume and non N_2-fixing reference plants are grown in soil receiving the same amount of ^{15}N-labelled N fertiliser. The total N in shoots is then analysed for ^{15}N, and the percentage of N derived from the atmosphere (%Ndfa) by the legume is calculated using equation (12):

$$\%\text{Ndfa} = \left(1 - \frac{\text{atom\%}^{15}\text{N excess } N_2\text{-fixing plant}}{\text{atom\%}^{15}\text{N excess reference plant}}\right) \times 100 \qquad (12)$$

The derivation of equation (12) can be found in McAuliffe et al. (1958) or IAEA (2001). The term 'atom% ^{15}N excess' reflects the ^{15}N enrichment above background (see Table 14, section 7.1)

The basis of the method is essentially the same as for ^{15}N natural abundance, except that the soil is artificially enriched in ^{15}N above the background ^{15}N abundance. No account of isotope fractionation is needed (i.e. 'B' value) since the enrichments used greatly exceed natural variations in ^{15}N.

The principal assumption is that the ^{15}N enrichment of the non N_2-fixing reference plants accurately reflects the ^{15}N enrichment of soil N taken up by the legume. The amount of N taken up from the soil by the reference plants does not have to be the same as the for legume.

For this to be valid, the ^{15}N enrichment of the soil N would need to be relatively constant over time and space, or the time course and depth of soil N uptake by the reference and N_2-fixing plants the same. When applying the ^{15}N isotope dilution methodology, most effort is focused on these two aspects.

Labelling plant-available soil N with ^{15}N

Compounds labelled with ^{15}N are expensive and it is thus standard practice to mimimise the amount of ^{15}N used. Some possible sources of stable isotope-labelled materials are given in Appendix 8. There is no single correct method for the addition of ^{15}N to label plant-available N in the soil. The method used will depend on the scale and nature of the experiment and the resources available. However, the most common method is addition of ^{15}N-labelled inorganic salts (e.g. $(NH_4)_2SO_4$, NH_4Cl, KNO_3, NH_4NO_3) or ^{15}N-labelled urea fertiliser. These can be added as a liquid by using a watering can, spraying or injecting into soil, or as a solid by broadcasting, banding or mixing with soil. As the efficiency of fertiliser N uptake is commonly 30–60% and plants will also use indigenous (native) soil mineral N, the enrichment of plants growing in labelled soil is much less than the enrichment of the label applied. For example, in an experiment where $(^{15}NH_4)_2SO_4$ at 9.9 atom% ^{15}N excess was applied to field crops, the resulting enrichments were only 0.02–0.99 atom% ^{15}N excess (Table 25). For these crops, little more than 2% of the labelled N was recovered in the legume shoots and 6% in the case of the wheat, highlighting the typical, significant contributions of native soil N to plant growth.

Table 25. Atom% ^{15}N excess of shoots of two grain legumes and wheat grown in the field. Each crop was supplied with $(^{15}NH_4)_2SO_4$ at 9.9 atom% ^{15}N excess at 10 or 30 kg N/ha.

	10 kg N/ha	30 kg N/ha
Lentil	0.0182	0.0826
Chickpea	0.0224	0.2191
Wheat	0.2495	0.9950

Source: McNeill et al. (1996)

9.2.1 Dealing with the problem of plant-available soil ^{15}N changing in space and with time

Following addition of $^{15}NH_4^+$ or $^{15}NO_3^-$ to a soil, the mineralisation of N from unlabelled soil organic N constantly dilutes the added label, causing a continual decrease in the ^{15}N enrichment of the plant-available soil N pool (Figure 31). This can result in differences in the ^{15}N enrichment of the N taken

up from the soil by N_2-fixing and reference plants, particularly if N uptake patterns of the N_2-fixing and reference plants are different. Thus, the principal assumption of the ^{15}N isotope dilution technique (Chalk 1985)—that the N_2-fixing legume and the non N_2-fixing reference plant obtain N of the same or similar ^{15}N enrichment from the soil—can be undermined.

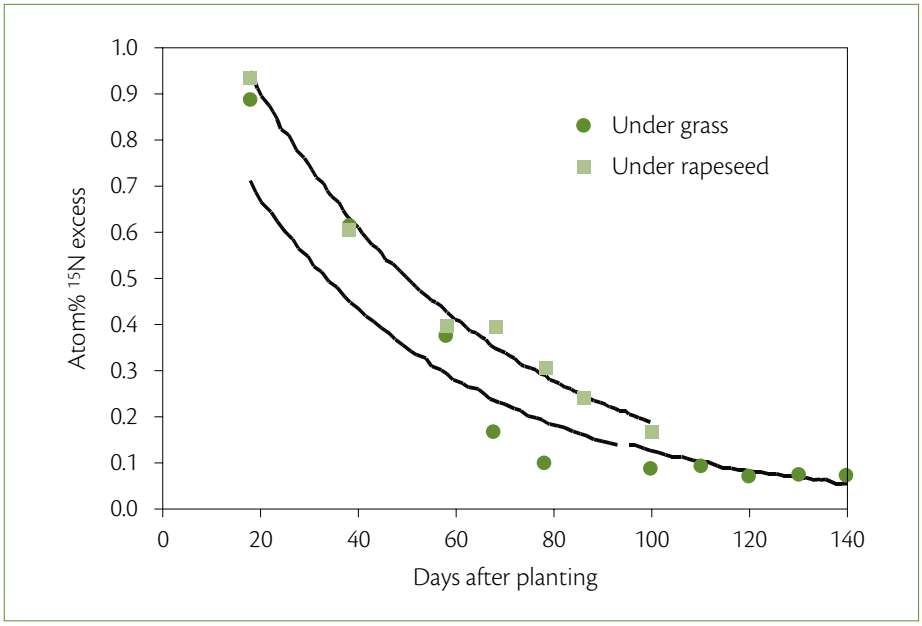


Figure 31. Changes in the ^{15}N enrichment of soil mineral N under grass or rapeseed following application to soil of 3.85 atom% ^{15}N excess KNO_3 at 30 kg N/ha. Data are from Witty (1983).

Furthermore, the added ^{15}N is not distributed evenly with depth when applied to the soil surface (Figure 32). Thus, ^{15}N of extractable NH_4^+ and NO_3^- can change with both time and depth during plant growth.

Figure 33 illustrates possible differences in ^{15}N distribution with depth and the interaction with the rooting depth of different N_2-fixing and reference plants. While the scenarios highlight the magnitude of changes in ^{15}N enrichment with depth, they paint an exaggerated picture of the potential impact, as most annual plants will take up the bulk of their N from the near-surface layer (0–30 cm), after which changes in ^{15}N tend to be less. Exceptions would include some perennial and deeper-rooted species, particularly during periods when the surface soil is too dry for effective nutrient uptake but sufficient subsoil moisture remains available.

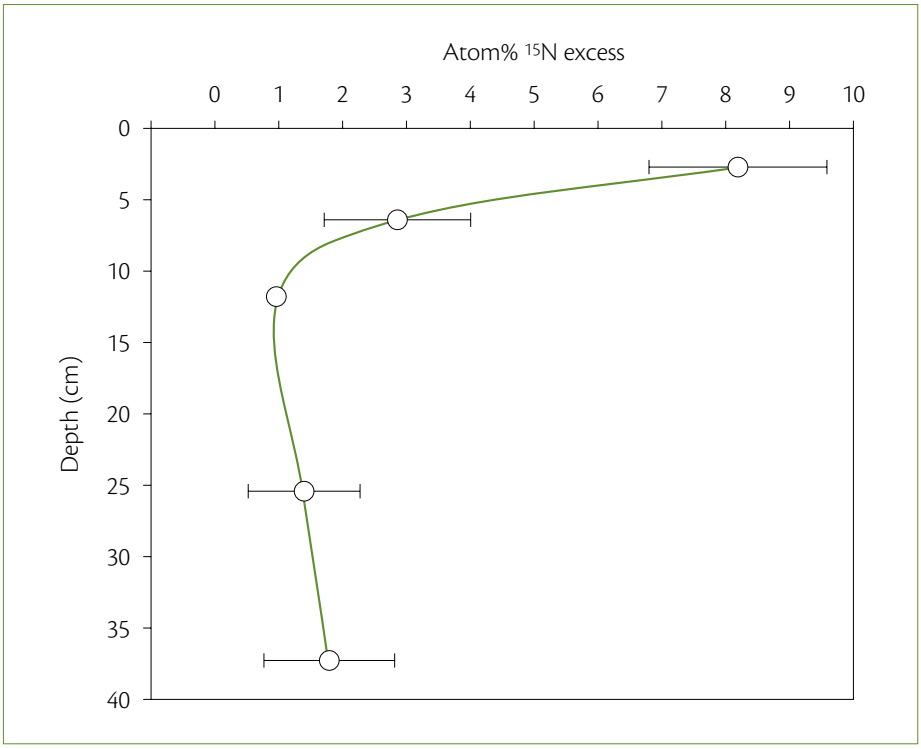


Figure 32. Variation in ^{15}N enrichment of total soil N with depth after labelling with 10 atom% ^{15}N excess $(^{15}NH_4)_2SO_4$. Error bars are s.d. of the means. Data are from Witty (1983).

Practices to reduce the impact of uneven distribution of ^{15}N in space and time include:

- regular additions of ^{15}N-labelled inorganic N to the soil (Viera-Vargas et al. 1995b)
- addition of soluble C to immobilise mineral N (Boddey et al. 1995a)
- the use of ^{15}N-labelled plant residues to provide mineralised N of a more constant value (Watanabe et al. 1990)
- the use of residual ^{15}N fertiliser carried over in soil from a previous experiment (McNeill et al. 1998)
- the use of a series of different reference plants to capture variations in ^{15}N enrichments in space and time (Viera-Vargas et al. 1995b)
- repeated sampling and analysis of extractable soil N over time (Chalk et al. 1996).

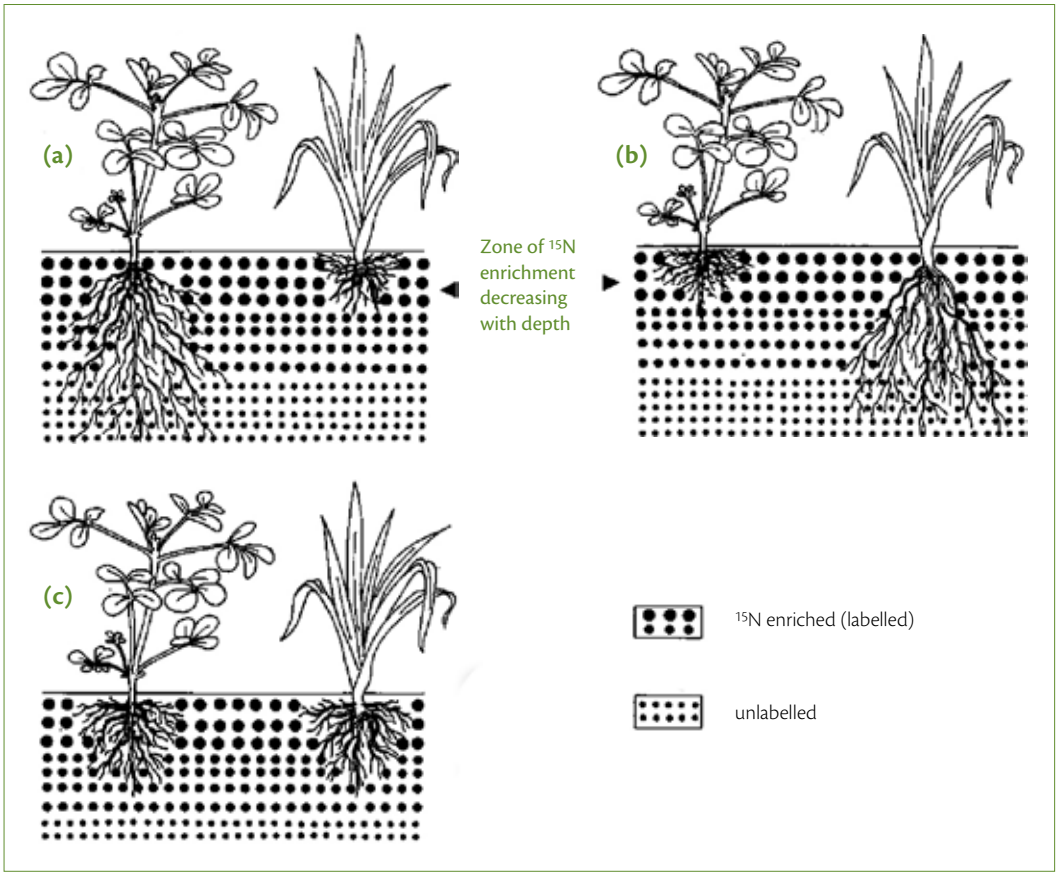


Figure 33. Scenarios that can arise in field experiments using ^{15}N labelling. The three examples show differences in root growth of N_2-fixing plants and non N_2-fixing reference plants (a and b), and differences in the distribution of the ^{15}N enrichment with depth (a, b and c). Scenarios (a) and (b) show that the N_2-fixing plants and non N_2-fixing reference plants would take up soil mineral N of different enrichments, thereby compromising the isotope dilution methodology. The ideal scenario is shown in (c). All three scenarios, however, lack the additional dimension of time, which may also provide for differential ^{15}N uptake between N_2-fixing and non N_2-fixing plants, even in the case of (c).

In some cases combinations of these procedures have been used. All highlight the fact that using non N_2-fixing reference plants to assess legume ^{15}N uptake from the soil is the principal weakness of the isotope dilution methodology. However, the potential to provide a yield-independent and time-integrated estimate of %Ndfa makes it worthwhile to persist with this potentially powerful technique.

Some researchers have applied a higher rate of fertiliser N (sometimes at different ^{15}N enrichment) to the reference plant than the legume. This so called 'A-value' technique, first proposed by Fried and Broeshart (1975), was thought to allow the non N_2-fixing reference plants to grow at a comparable rate to the legume in soils of low N status, thereby providing a more appropriate reference. Implicit in this approach is the assumption that a plant with more than one source of available N will utilise each of these in proportion to their relative amounts (the 'A-value'). This assumption has been shown to be invalid (e.g. Smith et al. 1989) and such a modification of the isotope dilution methodology has little to recommend it, adding complexity but not reducing any of the other deficiencies (Chalk 1996b).

9.2.2 Applying ^{15}N-labelled materials to soil

The best way to evenly mix ^{15}N with the soil is to spread the soil out on a plastic sheet and spray with a fine mist of very dilute ^{15}N solution, mix the soil and repeat the spraying. Such an approach is practical for a pot study, but not for field studies where the quantities (depth) of soil are much greater and minimal soil disturbance is preferable. Solutions of ^{15}N-labelled fertilisers can be applied to field soils using a watering can and, if sufficient quantities are applied, may penetrate to about 40 cm. Note that the quantity of solution required to penetrate to 40 cm would be large (about 8 L/m^2), and may impact substantially on plant-available water, resulting in significantly improved crop growth. This could be a problem and, at the least, the same volume of water would need to be applied to all plots (Table 26*)*. Liquids can also be injected into soils but only in the absence of rocks or hard layers in the soil, and gas injection systems have also been devised (Murphy et al. 1997). Regardless of the technique used, applying a ^{15}N solution evenly down a field soil profile will not result in an even profile of ^{15}N enrichment.

Another useful method for evenly spreading small amounts of ^{15}N is to spray ^{15}N-labelled solution on to clean sand, allow it to dry, and then spread the sand evenly over the soil surface (Cadisch et al. 1989) or mix it into the soil.

Solid ^{15}N-labelled fertilisers may not be as readily transported down the soil profile but can provide similar results to liquids. Solids can be applied with or without mixing. The method chosen will depend on sowing and fertiliser practices for the crops being studied, and the scale and objective of the experiment. Generally, liquid applications are easier to manage.

Table 26. Strategies to reduce variations in the ^{15}N enrichment of plant-available soil mineral N with depth in the soil and over time (the more ticks the better)

Depth	Time	Strategy	Advantages/disadvantages
✓		Fertilisers in liquid form	• easy to manage • no disturbance to soil • quantities of liquid required to penetrate to depth may be large • water may affect crop growth
✓✓		Incorporate fertilisers to depth by tillage	• mixes ^{15}N to required depth • substantial disturbance to rooting zone • may affect other soil processes
	✓	Repeated applications of ^{15}N over time	• may be useful for long-lived species • may be complicated by differences in N uptake rate for annual species • requires modelling technique
	✓	Pelleted/coated fertilisers	• a small advantage over unpelleted fertilisers • difficult to produce • not readily available
	✓✓	Add soluble C to reduce short-term availability of the N fertiliser	• increases stability of ^{15}N enrichment over time • may reduce available N in soil
	✓✓	Use ^{15}N-labelled organic matter	• greater stability of ^{15}N enrichment over time • requires incorporation into soil and significant soil disturbance • may impact more generally on soil N mineralisation and hence N_2 fixation • can be used only where organic matter addition is part of treatment, OR at least 6 months before sowing
	✓✓✓	Label soil 6–12 months before experiment	• effective but long lead-in time required

Some workers have advocated repeated applications of ^{15}N in an attempt to stabilise soil ^{15}N enrichment. However, differences between the reference and N_2-fixing crops in the amount of N extracted can still result in differences in the dilution of the ^{15}N label in the soil. Such a technique may nevertheless be useful in woody perennial systems (see Peoples et al. 1996 and section 9.7).

9.2.3 Slow-release formulations

Immobilised forms of N can be used to reduce the magnitude of shifts in the ^{15}N enrichment of plant-available N over time. Immobilised forms of N include ^{15}N-labelled organic matter and inorganic ^{15}N mixed with a soluble C source. The most commonly used soluble C source is sucrose. This is cheap, readily available and highly soluble, and can be effective in temporarily immobilising added N. When using sucrose or other readily available C sources to immobilise the ^{15}N, aim to add about 10 times more C than N, i.e. a C:N ratio near 10:1 for the added N. If a soluble C source is used, label the soil well before crop planting to allow the soil ^{15}N pool to stabilise. Generally, the longer the soil has been labelled, the greater will be the stability of the plant-available ^{15}N pool. A period of 6 weeks of stabilisation before sowing is recommended, with an absolute minimum of 2 weeks.

Labelled organic matter should be used only if addition of organic matter is part of your experimental treatment or normal crop husbandry. This is because the addition of organic matter is likely to impact on a range of soil physical, chemical and biological properties, especially mineralisation and immobilisation of N, which may affect N_2-fixation rates.

Isotope-labelled organic matter can be produced in two ways. The first is by growing plants in ^{15}N-labelled soil, then harvesting those plants, drying them and incorporating the dried, ^{15}N-labelled plant material into the soil. In this case the ^{15}N enrichment of the N released from the labelled material should be very close to that of the plant material used. Residual plant material from previous ^{15}N-labelling experiments can be used for this purpose and, if pot grown, both root and shoot material can be obtained.

The second approach is to mix ^{15}N-labelled salts with plant material or other organic matter, or with a readily soluble C source such as glucose. Ground crop residues are usually used for this purpose and mixed with $(^{15}NH_4)_2SO_4$ or $^{15}NH_4Cl$ to produce a final enrichment of about 5–10 atom% ^{15}N. Organic material such as sugarcane mill waste has also been used in combination with ^{15}N-labelled salts (Viera-Vargas et al. 1995b). ^{15}N-labelled organic matter should be incorporated into the soil at least 6 months before the start of the experiment.

9.2.4 How much ^{15}N to add?

The total amount of N applied in the ^{15}N-labelled material should not be sufficient to influence N_2 fixation, unless you are specifically studying the effects of N inputs on N_2 fixation. High concentrations (>10 mg/kg) of mineral N reduce N_2 fixation. Low rates of mineral N application are therefore preferable (e.g. < 5 kg N/ha), and certainly <10% of the plant's total N should be provided by the added N. If a C source is being used to immobilise added ^{15}N, or if ^{15}N-labelled organic matter is being used, it is also important that there is not a stimulation of N_2 fixation due to these amendments that reduces mineral N availability. As ^{15}N is expensive, the objective is to add no more than is necessary to measure the process under study.

Concentrations of ^{15}N of >5 atom% in the soil mineral N pool are rarely required, and a concentration of 10 times background (about 4 atom%) should be sufficient for most studies where samples are analysed by mass spectrometry. With soil mineral N of 4 atom% ^{15}N, a legume fixing 90% of its N (only 10% from the soil) would have a ^{15}N concentration of about 0.4 atom%, which is easily detected by both mass and emission spectrometry. In terms of total soil N, the enrichments are usually no more than 0.1% ^{15}N excess. When purchasing ^{15}N-labelled material, it is less expensive per gram of ^{15}N to buy 10 atom% ^{15}N than 99 atom% ^{15}N. In only a few situations would enrichments of >10 atom% ^{15}N excess be required.

To calculate the amount that you should add, measure or estimate the amount of mineral N in the soil, and add to this an estimate of how much additional N will be mineralised from soil organic N over the period of study (in the absence of any other information, 2% of total soil N per year is a rough guide). Then calculate how much ^{15}N you would need to give this pool the required ^{15}N enrichment. For those with access to high-precision 'natural abundance' mass spectrometers, a soil N pool of only 1 atom% ^{15}N would be sufficient (consult your mass spectrometry laboratory). The actual amount of N added will depend on the enrichment of the material that you have available. If you have 99 atom% ^{15}N salts, these can be diluted by mixing with a solution of unlabelled N salts. Solutions of about 5 atom% ^{15}N are usually sufficient. A very low-level, broad-scale ^{15}N enrichment technique has been described (Reiter et al. 2002), but this is not recommended for those inexperienced with ^{15}N.

The areas of crop to be labelled with ^{15}N need not be large, and are usually much smaller than those sampled for dry matter and total N assessment. This is because the isotope dilution technique gives an estimate of the proportion of N_2 fixed, independent of the crop yield. Only the estimate of the amount of

N_2 fixed (i.e. kg/ha) is dependent on total N yield. Actual areas labelled with ^{15}N depend on plot dimensions (width) and row spacing, but (microplots) are typically only 1–2 m^2 per replicate, with plant and soil sampling confined to the central area of the microplot or inner rows of crop. Steel boxes are often placed into the soil around the ^{15}N-labelled area to reduce lateral movement and run-off of label, and prevent the scavenging of unlabelled N by the roots of plants that might otherwise have grown beyond the labelled area of soil (Figure 34). Sanchez et al. (1987) detail constraints on microplot size and construction. Separate plant biomass samples are usually taken from larger areas near to the ^{15}N-labelled microplots for estimation of crop dry matter, but plant ^{15}N and %N are determined from samplings within the microplots.

In situations where you are also interested in assessing soil ^{15}N, soil from the whole microplot can be excavated and thoroughly mixed prior to subsampling and analysis. Samples below the depth of the steel microplot may be taken with a soil sampler or auger as shown in Figure 35. Such microplots are suitable for annual species only.

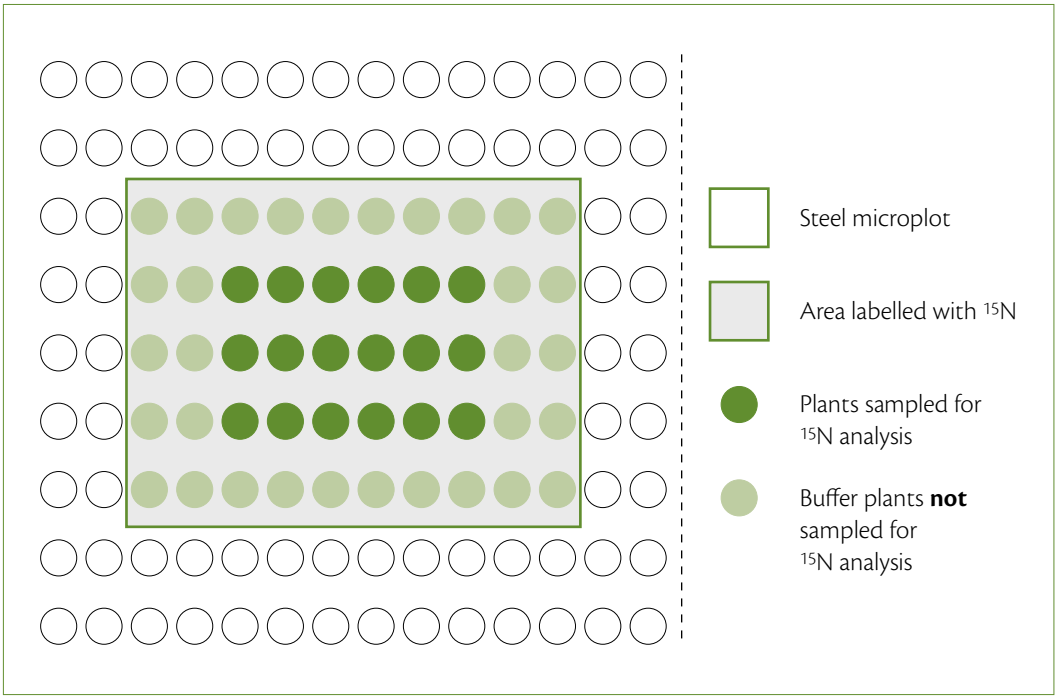


Figure 34. Typical microplot and sampling strategy for ^{15}N-labelling experiments. The ^{15}N application and sampling strategy could be the same with or without the steel microplot.

Figure 35. Field experiment showing (a) chickpea plants growing within a steel microplot for ^{15}N labelling and outside the microplot (unlabelled), (b) soil sampling from within a faba bean microplot and (c) deeper soil coring from within the faba bean microplot

It should be noted that such confined microplots may influence root growth of crops and this can change crop growth and N_2 fixation compared with unconfined roots (e.g. Kumar Rao et al. 1987).

9.2.5 Preliminary calculations

Regardless of the labelling method used, it is wise to estimate the approximate enrichment of the soil mineral N pool and make a preliminary calculation of potential plant total N uptake and N_2 fixation based on the range of things that you think could happen. Use these data to then make an estimate of final plant ^{15}N enrichment. Calculations such as those in Table 22 and equation (13), the universal mixing equation (Dawson et al. 2002), can be used to ensure that the enrichment of your final plant samples is sufficiently high to quantify N_2 fixation.

$$\text{Pool atom\% } {}^{15}\text{N} = fA \times \text{atom\% } {}^{15}\text{N A} + (1-fA) \times \text{atom\% } {}^{15}\text{N B} \quad (13)$$

where 'fA' is the fractional contribution of the added N to the total pool.

For example, if we were to add 5 kg N/ha of N-fertiliser at 5 atom% ^{15}N and the soil already had 60 kg mineral N/ha, the ^{15}N of the soil mineral N would then be, according to equation (13):

$$= (5/65) \times 5 + (1-(5/65)) \times 0.3663$$
$$= 0.385 + 0.338$$
$$= 0.723 \text{ atom \% } {}^{15}\text{N}$$

Pot experiments

9.3

If you have not worked with ^{15}N before, it could be useful to do a preliminary pot experiment with the same soil you are proposing for your field study, to familiarise yourself with the technique. Pots containing 5–10 kg of soil are suitable. Try to collect soil from your field site to the same depth as your pot is high. Soil can be collected either with a shovel and mixed uniformly, or intact cores can be taken by forcing open-ended steel cylinders into the ground, then taking them with the soil to the glasshouse to grow plants in a less disturbed situation. An analysis of the total N and mineral N concentration of your soil provides valuable background to determining how much ^{15}N you might need to add. For disturbed soils, the ^{15}N can be mixed with the soil on a plastic sheet by misting the ^{15}N solution onto the soil using a hand-pumped aerosol bottle while carefully turning the soil over with a shovel. This should result in an even distribution of ^{15}N throughout the soil profile in the filled pots. For undisturbed cores the ^{15}N will need to be added as a solution to the surface of the pot, taking care not to add the solution too rapidly or with too much water, which might result in bypass flow down the side of the core. N_2-fixing and non N_2-fixing plants can then be grown in different pots/cores, and the shoots and perhaps roots harvested for ^{15}N analysis.

Such experiments should have sufficient replications (4–6) to determine the variation between pots, and sufficient pots for sequential harvests if desired. Simple initial experiments may assist in familiarising yourself with ^{15}N calculations, the likely magnitude of ^{15}N addition required in the field, and

possible differences in ^{15}N enrichment between reference plants growing in the same soil. Uninoculated and inoculated legumes might also make valuable treatments and provide some insight into likely field inoculation responses. It should be noted that, while such experiments have merit in enabling you to familiarise yourself with the ^{15}N methodologies and providing insight into possible N_2 fixation responses to various treatments, they cannot be used for quantification of N_2 fixation with any relevance to field conditions. Thus, results should not be extrapolated from g/pot to field kg N/ha.

9.4 Choice of reference plants

Choose plants that have a similar life form to that of the legume under study (e.g. annual or perennial, herbaceous or woody) and that normally grow under the same set of environmental conditions (e.g. temperature, soil moisture, salinity and waterlogging tolerance). They should grow to a similar size as the legume, and preferably flower, set seed and senesce at about the same time. Try to choose reference plants with similar rooting characteristics (depth and architecture) as the legume, rooting specialisations (mycorrhizal status), nutrient uptake characteristics and susceptibility to root diseases. The more of these characteristics that the legume and reference plants have in common, the more likely are they to have similar patterns of soil mineral N uptake. Because it is very difficult to find a single species that fulfils all of the above requirements, it is essential to use more than one reference species in any given experiment. It is likely that they will have different ^{15}N enrichments and thus provide different estimates of legume N_2 fixation. Whether or not this is a major problem will depend on differences in ^{15}N enrichments between reference plants and the extent to which the legume is dependent on N_2 fixation (see Figure 36).

Reference plants should not be restricted to cereal (grass) species only, as these are known to have a different root architecture to most broadleaf species (Gregory 2006) and have often been shown to extract more N from the soil than broadleafed species (Barley 1970). The following criteria should be used to assist in initial selection of reference plants.

9.4.1 Absence of N_2-fixing ability

Reference plants should take up N from the soil only, and not have access to fixed N_2, either symbiotically or asymbiotically. For this reason care should be taken in the use of C4 grasses, some of which have demonstrated potential in

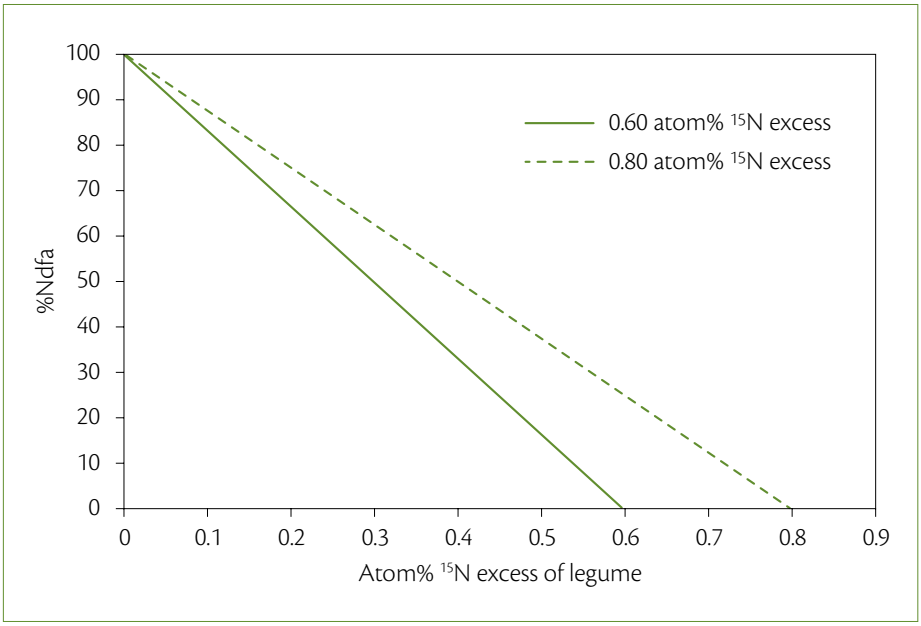


Figure 36. Relationships between N_2-fixing legume atom% ^{15}N excess (X axis) and estimates of %Ndfa (Y axis) calculated using two different atom% ^{15}N excess values (0.60 and 0.80) for the reference plants. Clearly, differences in reference plant ^{15}N have a much greater impact on estimates of N_2 fixation at lower %Ndfa values.

associative N_2 fixation under conditions of low N availability (e.g. *Brachiaria*, *Panicum*, *Pennisetum*, see Reis et al. 2001). Non-nodulating isolines have been used for some crops (see Table 7) but may not be readily available for many crops or users of isotope dilution technology. In situations where competent N_2-fixing rhizobia are not widespread in the soil, uninoculated legumes may be used as reference plants. However, in this case you must be certain that the reference plants are not nodulated. Plants with nodules must be discarded.

9.4.2 Exploitation of the same soil rooting zone

Nitrogen uptake patterns of the reference plant and legume should be similar. Witty (1983) described how to examine this, and the conditions under which such errors can be minimised. If N uptake constants cannot be determined for the crops being tested, the reference plant and legume should at least start to accumulate N and reach their maximum N contents at about the same time.

Note that the N_2-fixing and non N_2-fixing reference plant do not need to take up the same amount of soil N, only soil N of the same ^{15}N enrichment. However, large differences in the pattern of N uptake may confound the methodology since the ^{15}N-labelled mineral N pool is constantly being diluted with N mineralised from the soil organic matter. Thus, if the mineral N pool is drawn down by one crop and not the other, the result will be that the ^{15}N enrichment of the plant-available N pool will likely be different. Reference plants with similar total N uptake to the N_2-fixing legumes are likely to have extracted more N from the soil than the legume.

9.4.3 Duration of growth

Since the ^{15}N enrichment of the plant-available soil N pool can change with time (especially immediately following ^{15}N application), non N_2-fixing reference plants should be harvested at the same time as legume plants, regardless of growth stage. In this way they will have integrated plant-available N over the same period as the N_2 fixation measurement period. The non N_2-fixing reference plant should not reach maturity before the legume. In situations where reference plants flower before the legumes under study, it may be possible to remove the flowers from the reference plants and extend the vegetative period for a short while.

Figure 37 shows the time course of total N accumulation for two non N_2-fixing crops fed 2 kg N/ha of 21 atom% $(^{15}NH_4)_2SO_4$. While both crops took up about the same amount of labelled N from the soil, the sorghum had a lower ^{15}N enrichment because it took up much more unlabelled N. Using reference crops with similar total N uptake should reduce such discrepancies.

9.4.4 N transfer between species

In some situations legumes have been shown to 'transfer' N to companion species directly via a mycorrhizal bridge (Bethlenfalvay et al. 1991). In this case the reference plant might obtain N of a lower ^{15}N enrichment than that available to the legume. However, this has been shown to have no significant impact on estimates of N_2 fixation (Chalk 1996a). Similarly, where N transfer from the legume to a companion non N_2-fixing reference plant is due to legume root exudation and turnover, or through grazing and litterfall, the N would be equally available to both the legume and non N_2-fixing reference plant and thus not confound the isotope dilution methodology.

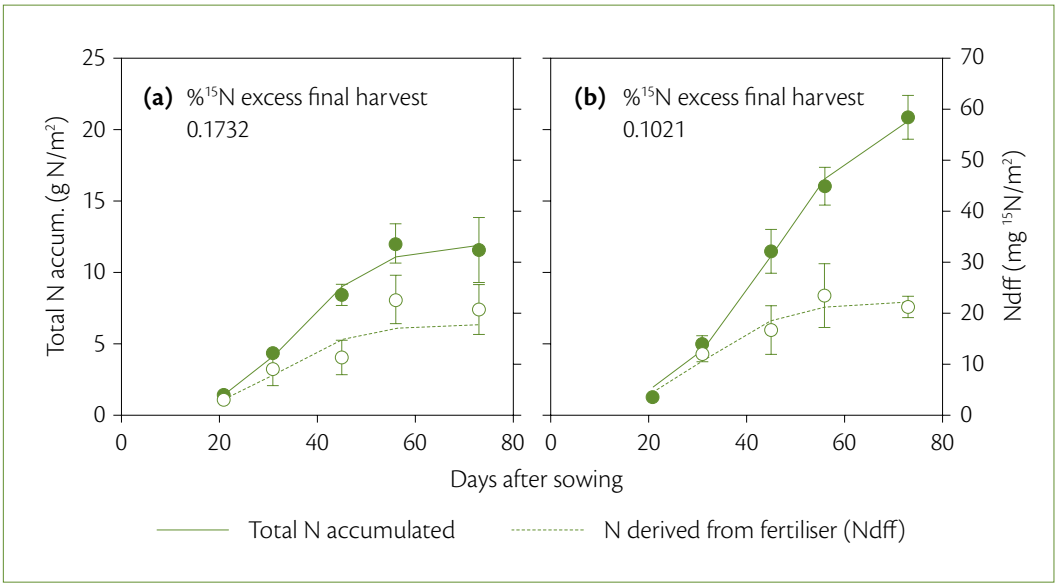


Figure 37. Total N accumulation and uptake of ^{15}N-labelled fertiliser for two non N_2-fixing reference plants fed 2 kg N/ha of 21 atom% ^{15}N-labelled ammonium sulfate: (a) non-nodulated soybean; (b) sorghum. Source: Boddey et al. (1995a)

Modelling soil ^{15}N enrichment

9.5

9.5.1 Background

Fifty years ago, McAuliffe et al. (1958), in their seminal paper on assessing %Ndfa by ^{15}N dilution, considered the possibility of using a soil extract as a reference for comparison with the isotope ratio of the legume, but abandoned the idea in favour of a non N_2-fixing reference plant. The stated rationale was that:

> It is extremely difficult to follow the ratio in the soil since an extract permits analysis only at the time the extraction is made, whereas the plant integrates the ratio over the entire growth period. Thus changes in isotopic ratio of the nitrogen in the soil cannot be adequately traced by extraction procedures.

However, two models that allow extracts of soil mineral N or nitrate to be used as reference criteria have subsequently been developed: a yield-dependent model (Smith et al. 1992) and a yield-independent model (Chalk et al. 1996). The yield-independent model will be described here as it is conceptually simpler and requires less sampling and analysis. More importantly, it provides a yield-independent and time-integrated estimate of legume %Ndfa. This is the significant

advantage of all ^{15}N dilution estimates of %Ndfa, whether or not a reference plant is used—it allows the effect of any variable to be separated into plant-growth-mediated and N_2-fixation-mediated components. For example, Smith et al. (1993) and Chalk (2000), using ^{15}N dilution and reference plants, were able to differentiate the effects of two abiotic stresses (salinity and plant nutrition, respectively) on legume growth from the effects on symbiotic dependence.

9.5.2 Yield-independent model

In a soil that has been labelled with ^{15}N-enriched fertiliser, the ^{15}N enrichment of the mineral N or nitrate pools decreases exponentially with time (Witty 1983), as described by equation (14).

$$E_t = E_0\, e^{-\mathrm{k}t} \qquad (14)$$

where E_t and E_0 are the ^{15}N enrichments (atom% excess) of the KCl-extractable mineral N or nitrate pools at times t and zero, respectively, k is the decline or first-order rate constant (days^{-1}) and t is the time (days). It is worth noting that the decline in the ^{15}N enrichment of the KCl-extractable available N pools is due to the addition of unlabelled N through mineralisation of soil organic N, and not to processes that remove N from the available N pool such as plant uptake and immobilisation (Barraclough 1991). While abstraction processes may alter the *size* of the pool, they do not in themselves alter its *isotopic composition*, since both labelled and unlabelled N are removed in proportion to their respective concentrations in the pool, provided both forms of N are uniformly mixed.

The decline in the ^{15}N enrichment of the mineral N and nitrate pools (0–15 cm) under a lupin crop between 28 and 190 days after sowing (DAS) is illustrated by the data of Smith et al. (1992). The measured ^{15}N enrichments and the fitted curves (Figure 38) were used to interpolate the daily ^{15}N enrichment of the soil N assimilated by the lupins.

The integrated pool enrichment (E^*) during the time interval t_1 to t_2 is obtained by mathematical integration of equation (14) as follows (equation (15)):

$$E^* = \int_{t_1}^{t_2} E_t \,\mathrm{d}t \,/\, (t_2 - t_1)$$

$$E^* = E_0\, (e^{-\mathrm{k}t_1} - e^{-\mathrm{k}t_2}) \,/\, [\mathrm{k}\,(t_2 - t_1)] \qquad (15)$$

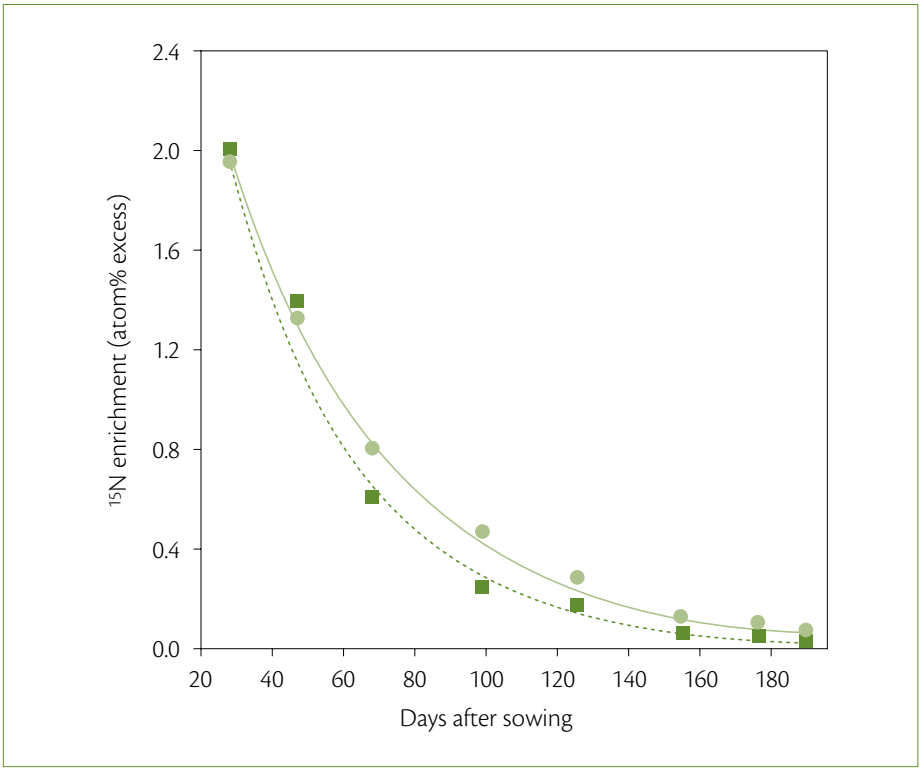


Figure 38. Temporal decline in the ^{15}N enrichment of the KCl-extractable soil nitrate (●) or mineral N (■) pools (0–15 cm) under lupin. Labelled fertiliser was applied at sowing at the rate of 2.5 g N/m^2 at 10 atom% ^{15}N. Curves represent the relationships described by equation (14). For the NO_3^- pool (—) E_t = 3.582 (±0.142) exp [−0.02187 (±0.00089) t] r^2 = 0.97. For the mineral N pool (– – –) E_t = 3.962 (±0.396) exp [−0.02663 (±0.0024) t] r^2 = 0.85.

where E^* represents the integrated ^{15}N enrichment of the mineral N or nitrate that would be acquired by plant roots growing exclusively in the depth of soil sampled (e.g. 0–15 cm) during the time interval t_1 to t_2. The symbiotic dependence (%Ndfa) is then calculated as (equation (16)):

$$\%\text{Ndfa} = 100\,[1 - (\text{atom \% } ^{15}\text{N excess}_{\text{legume}} / E^*)] \qquad (16)$$

A comparison of the %Ndfa values obtained using the yield-independent model and three reference plants was made by Chalk et al. (1996) for lupin sampled on six occasions after sowing (Table 27).

Table 27. Estimates of %Ndfa of lupin using a yield-independent model and three reference plants

Time (days)	Yield-independent model[a]	Reference plants		
		Canola	Ryegrass	Wheat
68	49	48	55	37
99	58	59	71	53
126	66	70	73	64
155	76	78	79	76
176	82	77	79	78
190	82	74	71	77

[a] Based on the ^{15}N enrichment of the nitrate pool (0–15 cm)

Estimates of %Ndfa differed for different reference plants, particularly at 68 and 99 days after sowing (DAS), but the yield-independent model and canola gave very similar estimates at each sampling time. Use of the modelling approach as an alternative to using reference plants for estimating %Ndfa was therefore vindicated.

9.5.3 How it works

The key requirement is to define the decline in the ^{15}N enrichment of the mineral N or nitrate pools that are being accessed by the legume roots. Sufficient samples need to be taken from replicated labelled plots to define this relationship from the late seedling stage to maturity. A bulk sample consisting of three or four random cores should be taken from each labelled plot at each sampling time. Sampling every 2–3 weeks is recommended, with the first samples being taken 4 weeks after isotope application at or before sowing (t_1). It is suggested that the exponential relationship (equation (14)) that gives the best fit to the data (i.e. either nitrate or mineral N data) be used to determine k, the decline or first-order rate constant.

A variable sampling depth is preferable to a fixed depth, as it is important to match the depth of measurement with the zone where soil N uptake is taking place. Visual observation of legume root distribution is the simplest way to define sampling depth at any one time. Visual observations should be done outside the labelled plots so as to cause minimum disturbance within.

The legume plants can either be sampled as frequently as desired to estimate %Ndfa during crop development or, if this is of no interest, samples need only be taken at physiological maturity. However, there is little point in taking plant samples too early (i.e. in the seedling stage) before active N_2 fixation is established, and when the error due to the contribution of unlabelled N from the seed is still significant. For example, Smith et al. (1992) took the first lupin samples at 68 DAS and the first soil samples at 28 DAS.

The replicated labelled plots should each be of sufficient area to accommodate the sampling intensity (both soil and legume) that is contemplated. As a rule of thumb, it is recommended that the area of labelled plot be such that less than 20% of the standing crop is harvested during the experiment. Soil sampling using a corer (e.g. 32 mm diameter) will normally disturb only a small part of the plot area. Sample holes should be backfilled with unlabelled soil and clearly marked to avoid resampling.

Care must be taken to preserve the integrity of fresh soil samples by keeping them at a low temperature (e.g. 5 °C) before KCl extraction, and only KCl that is free of NH_4^+ and NO_3^- contamination should be used as the extractant (Chen et al. 1991).

9.5.4 Attributes of the yield-independent model

The advantages and disadvantages of the modelling approach were summarised by Chalk and Ladha (1999). The following are the main attributes of the yield-independent model:

- It provides a yield-independent, time-integrated estimate of %Ndfa.
- Fewer ^{15}N-labelled plots are required when the reference plant is abolished. Potential savings are proportional to the number of reference plants that would otherwise have been used and the degree of replication employed. For example, it is common for three or four replicate plots to be employed, and use of 3–5 reference plants is recommended. Thus, potential savings on isotope, fertiliser, seed, fuel, labour, pest control, plant sampling, sample preparation and analysis can be considerable if 9–20 labelled plots are eliminated for only one harvest in just one experiment.
- The model can accommodate the temporal decline in soil ^{15}N enrichment, one of two major sources of error encountered using reference plants. Thus, strategies to minimise the rate of decline (e.g. slow release formulations) are not required, and the experiment can begin within a few weeks after the plots are labelled. The potential problem of the decline constant (k) differing under the legume and reference plants is overcome.

- The model has the potential to accommodate the non-uniform vertical distribution of the isotope, the second major source of reference-plant-derived error, through sampling a variable depth commensurate with the growth of the legume roots.
- The model can provide an estimate of %Ndfa in situations where it is impossible to have a reference plant available when needed. This can be particularly important in crop rotation experiments involving legumes and non-legumes, where the main consideration in experimental design is that each treatment should be present in each phase of the rotation. Rotations are seldom, if ever, designed with the idea of estimating legume %Ndfa, but the model can provide a strategic opportunity to accomplish this goal.

9.6 Experimental plots

The design and layout of experimental plots are discussed in section 8.7.2 (see also Figure 28). In experimental plots where you have some flexibility in choosing the reference plant, it is recommended that at least two, and possibly three, reference plant species are used. A non-nodulating genotype and one other dicotyledonous species should be included if at all possible. Where non-nodulating legumes are used they must be diligently checked for nodulation status. Do not assume that they will always be nodule free and be non N_2-fixing. In exceptional cases where there are no compatible rhizobia in the soil, uninoculated legumes or another unnodulated legume may be used as non N_2-fixing references. In either case the absence of nodulation must be confirmed.

It is important that reference plants are grown in exactly the same soil as the legumes under study since differences in soil history will affect N mineralisation rates, plant-available N and the dilution of added ^{15}N. For example, in crop rotation experiments you should not use a reference crop that follows a different crop to that of the legume under study. An example of a system for maintaining the integrity of the reference plant is illustrated in Figure 39, where it can be seen that the reference and legume crops are always sown into soils with the same history. Additional rotation plots may thus need to be installed at the commencement of a rotation experiment for later sowings to legume and reference plants.

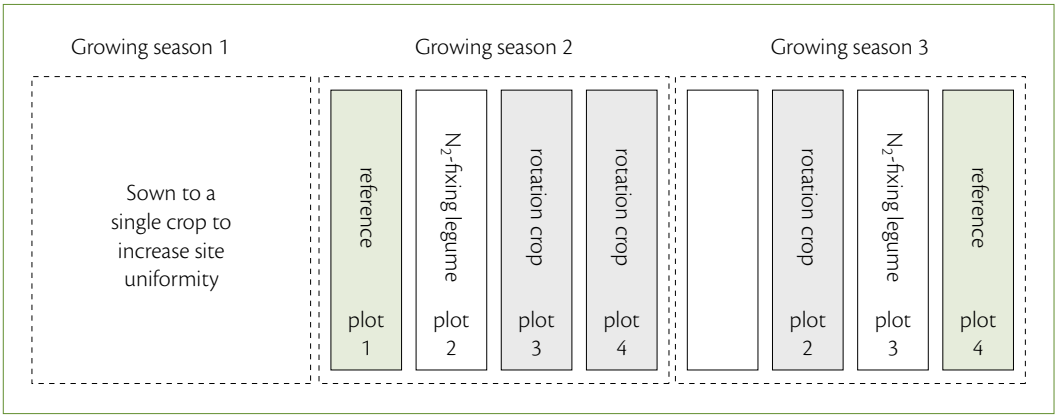


Figure 39. Layout of field plots through a two-crop sequence to ensure that non N_2-fixing reference plants are grown in soil with exactly the same history as the N_2-fixing legume under study. In this case plot 1 is of no use (redundant) for the final crop sowing. Replication of crops and rotational phases should also be included in your experimental statistical design as a matter of course.

Whenever an estimate of N_2 fixation is required, both legume and reference plants will need to be sampled. If only one annual estimate of N_2 fixation is required, then plants can be sampled near maturity, being sure to include all fallen leaf and litter material. It is preferable to take samples prior to leaf drop, reducing the likelihood of sample contamination and loss of ^{15}N material. Sampling for N_2 fixation measurement is best done on whole shoots rather than just seeds as there can be substantial differences in ^{15}N distribution between plant parts (Table 28). However, if you are comparing a number of lines or cultivars and just need a relative measure, then seed ^{15}N would enable a robust ranking of %Ndfa.

Table 28. ^{15}N enrichments of different plant parts of soybean following application of 5 atom% ^{15}N excess $(NH_4)_2SO_4$

Plant part	Atom% ^{15}N excess
Shoots	0.4609
Pods	0.3151
Roots	0.3988
Nodules	0.0681

Source: Danso and Kumarishinge (1990)

9.7 Perennial tree systems

The size, longevity and deep-rootedness of established woody tree legumes provide extra challenges for quantifying N_2 fixation. These problems are detailed in Baker et al. (1995). Due to the larger area of root exploration by woody perennials, small (1–2 m^2) microplots, such as those used for annual legumes, are not suitable, as the plants would obtain mineral N from outside the labelled area. To reduce the effect of this, many researchers install barriers to root growth around the plants under study. Barriers are typically made by digging a 60–80 cm deep trench around the labelling area and lining it with impermeable (5–8 mm) plastic (e.g. Ovalle et al. 1996). An alternative approach is to apply the ^{15}N over a wider area of trees, but sample only trees in the centre for ^{15}N analysis (see e.g. Peoples et al. 1996).

It is difficult to apply ^{15}N label evenly across a large area and down the soil profile, and to maintain soil ^{15}N enrichment for the life of a tree. Most applications of the isotope dilution method to trees involve repeated additions of ^{15}N to maintain ^{15}N enrichment over time. Intervals between applications vary depending on tree management, but applications would typically be made 2–4 times per year, or at each cutting (e.g. Peoples et al. 1996).

On sandy soils, ^{15}N-labelled fertilisers can be leached down the profile with additional water after labelling (e.g. Busse 2000), and/or a series of thin PVC tubes can be inserted into the ground and ^{15}N solution poured down them as well as on the soil surface. These tubes can be left in the ground for later ^{15}N additions if necessary.

Because experiments on woody perennials are likely to continue for months or years, and because trees are larger than annual crops, the amount of added ^{15}N is greater than that for annual crop species. It is thus advisable to conduct a preliminary application of ^{15}N to spare trees and examine the ^{15}N enrichment of these after some period of time. In this way you will get an estimate of how much ^{15}N you might need to add. Whereas no more than 5 kg N/ha is recommended for crop legumes (section 9.2.4), 10–20 kg N/ha is usually required in studies of tree legumes. Because of the risk of 'contamination' with ^{15}N from neighbouring plots, it is important to have adequate distance between ^{15}N microplots, particularly when they are unbounded (e.g. Rowe and Cadisch 2002).

Evaluation of ^{15}N data and calculations

Check all your primary ^{15}N data, particularly comparing data from replicate plots. Are the legumes lower in ^{15}N than the reference plants? Do they vary with treatment as you might expect? How similar in ^{15}N enrichment are your 2–4 reference plants? Reference plants with higher ^{15}N enrichment are likely to have taken relatively more N from the soil early in growth than later, or to have extracted relatively more from nearer the soil surface. How similar are your reference plants and legumes in terms of total dry matter and total N uptake? Generally, we would expect legumes that are highly dependent on N_2 fixation to have greater total N than non N_2-fixing reference plants.

If your data look sensible according to the above criteria, then you can calculate %Ndfa. A typical set of data from a ^{15}N isotope dilution experiment is given in Table 29, where %Ndfa is calculated using equation (12). An example calculation for the 128-day-old plants in Table 29 is:

$$\%\text{Ndfa} = \left(1 - \frac{0.0681}{0.3189}\right) \times 100 = 78.6$$

These calculations should be done for each legume – reference plant pairing in each replicate.

Table 29. ^{15}N enrichments of shoots of lupin and wheat sampled from ^{15}N-labelled microplots and estimates of %Ndfa at 128 and 193 days after sowing

Species	Days after sowing	Atom% ^{15}N	Atom% ^{15}N excess	Lupin %Ndfa
Lupin	128	0.4344	0.0681	79
Wheat		0.6852	0.3189	
Lupin	193	0.4112	0.0449	85
Wheat		0.6561	0.2898	

Source: Evans et al. (1987)

While atom% ^{15}N excess can be calculated as atom% ^{15}N – 0.3663, some workers choose to set up unlabelled plots as well as labelled plots and to use the atom% ^{15}N natural abundance for the reference plant instead of the value of 0.3663. In practice this rarely makes much difference as the range in background values is only 0.3656–0.3718 atom% ^{15}N, and other errors in the methodology are likely to be much greater.

As with all other methodologies, the collection of supporting data on nodulation, soil mineral N and total N of non N_2-fixing reference crops assists greatly with ^{15}N and %Ndfa data interpretation (see section 2.3).

9.9 Final word

The ^{15}N isotope dilution technique is a valuable tool for estimating N_2 fixation of field-grown crops. While its use has been somewhat overtaken by the ^{15}N natural abundance technique, it remains an important tool. In addition to providing estimates of N_2 fixation, ^{15}N isotope dilution also permits tracing of the fate of fertiliser and legume N in cropping systems (see Chalk 1998; Fillery 2001). The methodology is less sensitive than ^{15}N natural abundance to sample handling and management problems, and sample analysis is also easier. For this reason the isotope dilution technique may be more robust in areas remote from reliable drying ovens and well-equipped laboratories.

The two ^{15}N methodologies are commonly considered to be the benchmarks against which other measures of N_2 fixation must be judged because, like the ureide technique, they are yield independent. The isotopic techniques have the additional advantage of integrating %Ndfa over time. Both the ^{15}N natural abundance and enrichment methods have been used to study many different annual and perennial N_2-fixing species growing in a diverse range of farming systems across the globe. However, obtaining accurate estimates of N_2 fixation using these methods requires considerable attention to detail and some effort on behalf of the researcher to minimise the influence of factors that can potentially invalidate the assumptions inherent in their use.

10

Precautions when quantifying N_2 fixation associated with non-nodulating plants (associative N_2 fixation)

Since the discovery and characterisation of *Azospirillum* spp. by Dobereiner and colleagues (Dobereiner and Day 1975; Tarrand et al. 1978), and the more recent discoveries of other N_2-fixing bacteria that associate with non-legume plants as diverse as wheat, maize, sugarcane, sweet potato and coffee (Dong et al. 2003; Boddey et al. 2006), there have been many hundreds of publications describing various aspects of these associative N_2 fixation systems. Quantifying associative N_2 fixation is essentially the same as for nodulated legumes and actinorhizal plants. However, as rates are typically much lower than for symbiotic systems, the methods available may not always provide reliable estimates, or even unequivocal evidence, of N_2 fixation activity. Indeed, the question of whether there are significant inputs of biologically fixed N to such associations remains controversial (e.g. Giller and Merckx 2003), and it is thus often necessary to have much more rigorous experimental protocols than in the case of nodulated legumes. Because of the inherent uncertainties of current methodologies, defensible proof of N_2 fixation by associative N_2-fixing systems may need to be established along with quantification.

Cultivating a rice paddy in Myanmar. Rice is an N-demanding crop and about 20% of the global use of fertiliser N is for rice production. Nitrogen-fixing systems also occur in rice paddies and include cyanobacteria, either free-living or in symbiosis with the water fern *Azolla*, and other N_2-fixing bacteria associated with the rice roots.

Some of the methodologies detailed in this book are not suitable for associative N_2-fixing systems. The ureide (N solute) method (Chapter 6) cannot be used because it is applicable to only the subtropical and tropical legumes that produce ureides as a product of N_2 fixation. The N difference technique (Chapter 5) relies on the assumption that the N_2-fixing plant utilises identical soil N as the non N_2-fixing control crop. While a N_2-fixing crop will often accumulate more total N than the reference crop under field conditions, this alone is not unequivocal evidence that the extra N was derived from N_2 fixation. Thus, singular evidence from the N difference technique is not sufficient to either prove or quantify N_2 fixation in non-nodulating systems in the field.

This chapter describes the most useful techniques for (i) determining if there are statistically significant inputs of fixed N to inoculated plants, and (ii) quantifying N_2 fixation inputs to plants growing in non-sterile conditions (field or pots of soil) containing naturally associated N_2-fixing bacteria.

Nitrogen balance and nitrogen difference approaches 10.1

The N balance and N difference methods are as described in Chapters 4 and 5, and application of these methods to the measurement of associative N_2 fixation raises similar issues.

10.1.1 Pot experiments

The N balance and N difference methods can be used for plants in pots. Suitable controls can be uninoculated plants or plants inoculated with ineffective (nif–) mutant bacteria. Because N-free growth media cannot be used for plants that might be able to acquire only a small fraction of their N from the N_2-fixing association, the amount of N in pot soils will be relatively large compared with that in the plants. Thus, small changes in the N balance due to the associative N_2 fixation will be very difficult to detect. For this reason, comparison of plant–bacteria associations with uninoculated controls (N difference) may be a more useful approach than trying to measure small absolute changes in the total N of plant–soil systems (N balance). Both N balance and N difference methods can provide quantitative data on associative N_2 fixation in pots, provided all other sources of N addition are carefully controlled, analyses of N in the soil and plant pools are conducted with accuracy and precision, and experimental conditions are conducive to plant growth. An example of such data from experiments on sugarcane in Brazil is given in Table 30.

Table 30. Nitrogen balance for 21-month-old sugarcane plants in a pot study. All values are mg N/pot (source: Lima et al. 1987). Total N balance = (final N in soil + N in plant material) – (initial N in soil + N in waste + 2.65 g N in urea fertiliser).

Sugarcane cultivar	Total plant N	N in soil			Total N balance
		Initial	Final	Difference	
CB47-89	34.78	53.60	46.82	–6.79	+25.34
CB47-355	16.54	49.67	44.54	–4.91	+8.98

In experiments comparing the total N accumulation of plants with and without inoculation with N_2-fixing bacteria, results can be confounded by responses to inoculation that are not due to N_2 fixation. For example, hormone production by bacteria has been shown to have growth-promoting effects on plants. These issues are discussed in Vessey (2003) and must be carefully considered when designing experiments and interpreting results in associative N_2 fixation studies.

10.1.2 Field experiments

Given the uncertainties in measuring inflows and outflows of N in field experiments (see section 4.3), a positive N balance alone cannot provide unequivocal evidence of associative N_2 fixation. Positive N balances for cereal-cropping systems over long periods (e.g. Firth et al. 1973; Dart and Day 1975) can result from N_2 fixation by cyanobacteria (Witty 1979; Giller and Day 1985), NH_3 deposition and significant contamination by animal and human wastes (Wani 1986).

In particular, wet and dry deposition of N from the atmosphere should not be ignored in N balance calculations as they are often likely to be of a similar order of magnitude to associative N_2 fixation. For example, positive N balances for experimental plots averaging 25–30 kg/N/ha per year could be explained by annual atmospheric N deposition that ranged between 10 kg/ha per year in the late 1800s up to 43 kgN/ha per year in the late 1990s in the United Kingdom (Goulding et al. 1998)

To be more conclusive, N-balance studies on associative N_2 fixation must also include estimates of possible uptake of N from deeper soil horizons, and inputs from irrigation waters. Even when there are low concentrations of N in water, large application volumes will result in significant N inputs over time (see Giller and Merckx 2003).

Proof of associative N_2 fixation could be obtained only from long-term N balances in which detailed and accurate assessment of all inputs and losses are included. N balance usually does not provide definitive proof of associative N_2 fixation, but the technique can be used to approximate it where it is known to occur.

^{15}N-isotopic techniques

10.2

The $^{15}N_2$, ^{15}N isotope dilution and ^{15}N natural abundance methods (see Chapters 7–9) can all be applied to associative N_2-fixing systems.

10.2.1 $^{15}N_2$ feeding

Intact plant–soil (or other growth medium) systems are totally enclosed in a sealed chamber, which is then filled with ^{15}N-enriched N_2 gas. Enrichment of the plant with ^{15}N provides unequivocal proof that the plant, or plant–soil system, is obtaining N directly from the labelled N_2 gas via biological N_2 fixation.

Major problems associated with applying the $^{15}N_2$ gas technique to associative N_2 fixing systems are that:

- the ^{15}N-labelled N_2 gas must be free from any fixed N impurity
- the enclosure of the plant(s) in a sealed chamber requires at least CO_2 concentrations to be monitored and maintained at ambient levels (~370 μL/L), and O_2, light intensity, temperature and humidity may also need to be managed.

While it is possible to purchase sealed vials of $^{15}N_2$ gas, they may be contaminated with ^{15}N-enriched combined N, so the gas must be purified before use. As $^{15}N_2$ is much more expensive to purchase than, for example, $^{15}(NH_4)_2SO_4$, many researchers generate $^{15}N_2$ from an NH_4^+ salt in their laboratory. Bergersen (1980a) suggested that $^{15}NH_3$ generated from $(^{15}NH_4)_2SO_4$ with NaOH could be passed over hot Cu to reduce it to $^{15}N_2$. A simpler technique that generates $^{15}N_2$ from $(^{15}NH_4)_2SO_4$ using sodium hypobromite was described by Ohyama and Kumazawa (1981). It is beyond the scope of this book to give detailed procedures for the generation of $^{15}N_2$ and its purification but excellent advice on this can be found in Witty and Day (1979) and Warembourg (1993).

Detailed guidelines with diagrams for the construction and running of gastight chambers for exposing plants to $^{15}N_2$ gas can be found in the above references, plus those of Eskew et al. (1981) and McNeill et al. (1993) who

developed systems with automated gas control. Somewhat simpler systems are described in Ruschel et al. (1975), De Polli et al. (1977), Yoshida and Yoneyama (1980) and Warembourg et al. (1982).

A good example of the application of $^{15}N_2$ to study associative N_2 fixation in grasses can be found in De Polli et al. (1977), who collected established plants from the field in intact soil cores. The intact cores were subject to C_2H_2 reduction assay (see section 11.1) and the most active cores then incubated with $^{15}N_2$ in 1–4 L vessels for up to 70 hours.

Very low rates of N_2 fixation in associative systems are difficult to prove unequivocally. Thus, when estimating N_2 fixation of non-legumes using $^{15}N_2$, the ^{15}N enrichment of the plants after exposure must be compared statistically with the ^{15}N natural abundance of replicate plants grown under identical conditions but not exposed to the labelled atmosphere. Only in this way can you be sure that the ^{15}N enrichment of your putative N_2-fixing system is due to incorporation of $^{15}N_2$ and not natural variation in ^{15}N abundance (see section 7.1).

If the experiment involves inoculation with a single bacterial species under sterile (axenic) conditions, a control treatment with a non N_2-fixing (nif–) mutant can be of great value. If your laboratory is equipped with a good workshop where glassware can be made or modified, and/or sophisticated automatic systems for control of CO_2, O_2, light, water and temperature can be assembled, it may be possible to expose plants to $^{15}N_2$ for several days or even weeks.

10.2.1 ^{15}N isotope dilution

Application of the isotope dilution technique to associative N_2-fixing systems involves the same issues that were discussed in Chapter 9 for nodulated plants. A particular problem is that ^{15}N isotope dilution is most accurate at higher levels of %Ndfa but most associative N_2-fixing systems will be operating at low %Ndfa. Another problem is that the enrichment of soil N with ^{15}N invariably results in both spatial and temporal variations in ^{15}N enrichment of the soil mineral (plant-available) N pool, and it is impossible to verify that a non N_2-fixing reference plant is accumulating soil N with the same ^{15}N enrichment as the N_2-fixing plant. The ideal solution to this problem would be to grow the plants in a soil that is uniform in ^{15}N in space (depth and area) and time. As discussed in section 9.2, however, this is not easily done.

Threshing rice in Myanmar where, as in most of Asia, rice is the major food staple

Pot experiments

In pot experiments where all soil can be mixed uniformly, the ideal of spatial and temporal uniformity of ^{15}N enrichment may be adequately achieved. For work on non-sterile systems, where the associative plants are naturally infected with N_2-fixing micro-organisms, soil is probably the best growth medium. A free-draining, medium-textured soil will have sufficient nutrient-buffering capacity to allow healthy plant growth.

It is essential that some effort be put into producing a soil that is labelled as uniformly as possible with ^{15}N, and that the ^{15}N enrichment of the soil mineral N declines only slowly with time. If the ^{15}N enrichment of the soil mineral N only declines by one-fifth of its excess ^{15}N value during plant growth, errors due to the N_2-fixing and reference plants having different N-uptake patterns should not mask a %Ndfa value of 10%.

At least two reference plant species are desirable if the objective of the experiment is to compare plant genotypes or other treatments. If reference plants that are thought to have contrasting N-uptake patterns (e.g. a monocot and a dicot) have similar ^{15}N enrichments, and both are significantly higher in ^{15}N enrichment than the putative N_2-fixing genotype/treatment, then this will provide strong evidence of the incorporation of biologically fixed N.

Experiments to determine the effects of inoculation of cereal crops with N_2-fixing bacteria have often used sterile media labelled with ^{15}N (e.g. Giller et al. 1986; Iniguez et al. 2004). While mixtures of sand and vermiculite have been used with success with legumes (Vincent 1970), there may be particular problems with this approach in associative systems. When plants are grown under severe N stress, the N from interstitial positions in the lattice of vermiculite can be differentially accessed by plants, such that apparent ^{15}N dilution not due to N_2 fixation may occur (Giller et al. 1986). Thus, this substrate is not recommended, but perlite (an expanded silica) is appropriate and can be washed free of all soluble residues. The only disadvantage is that it is almost impossible to separate fine roots from perlite, making it difficult to assess total root mass.

With a substrate such as sand or sand–perlite, pots can be irrigated with a solution of $^{15}NH_4^+$ or $^{15}NO_3^-$. The concentration of N should be low (e.g. 5 μg NO_3^--N/mL) so as not to inhibit N_2 fixation, and the irrigation can be frequent or via a flow-through drip system. Theoretically, if the ^{15}N enrichment of the plants at harvest is significantly lower than that of the N in the nutrient solution, then the plants have obtained N from an unlabelled source. A non N_2-fixing reference control (or controls) should be included which, in the case of inoculation experiments, may be the uninoculated plants. Ideally, the uninoculated plants should be treated with the same quantity of microbial suspension as the other treatments, but with the inoculant autoclaved before addition to ensure the same quantity of N is added to all treatments. When experimenting with plants that are grown in conditions close to N-starvation, the amount of N in the bacterial inoculum may be significant (Lethbridge and Davidson 1983).

Before use, the plant growth medium (e.g. sand or sand–perlite) should be washed in hydrochloric acid and then thoroughly rinsed with large volumes of distilled water until there is no sign of Cl^- ions (check with silver nitrate solution). Rather than a single addition of a ^{15}N-labelled salt, it is recommended that a continuous, or regular, addition of a labelled N solution at a low concentration should be used (see above). The source for most plants should be nitrate and under no circumstances should $^{15}NH_4NO_3$ be used, unless both

the NO_3^- and NH_4^+ ions are equally labelled with ^{15}N. For wetland rice, an NH_4^+-N source is essential. Systems continuously supplied with NO_3^- will become somewhat alkaline with time. Conversely, where plants are supplied with NH_4^+, the rooting medium can become acidic. Hence the nutrient medium should be well buffered. One of the advantages of a continuous supply of a low concentration of a ^{15}N-labelled salt is that the outflow can carry away excess OH^- or H^+. Nitrogen-free solutions used for legumes tend to be too low in potassium (K) for use with graminaceous crops, and solutions recommended for hydroponic maize or rice will be more appropriate. It is always good practice to run a trial growth experiment without ^{15}N addition to ensure it is possible to grow robust and healthy plants.

Another potential problem is that N-starved plants exposed to atmospheric pollution (especially NH_3) may incorporate this into plant tissue and dilute the ^{15}N label, indicating apparent N_2 fixation. Clearly, it is not wise to conduct such experiments close to animal production units such as feedlots or intensive chicken or pig farms. Cleaning products commonly contain high concentrations of NH_3 and should not be used in glasshouses where studies of plant N nutrition are undertaken. Cigarette smoke can also be a significant source of gaseous N. Ammonia contamination is more likely to be a problem in developed countries (e.g. Netherlands, Belgium) where ambient NH_3 concentrations are high. Atmospheric NH_3 is likely to be much lower in ^{15}N than atmospheric N_2 (Turner et al. 1983), and consequently only a small amount of NH_3 uptake can translate into large apparent N_2 fixation. This should be less of a problem for N-sufficient than N-deficient plants.

If experiments to quantify associative N_2 fixation are undertaken in pots containing soil, precautions must be taken to label the soil N uniformly with ^{15}N. Incubating the soil with ^{15}N-labelled fertiliser or organic matter months before starting the experiment, and then mixing all the soil thoroughly, may result in reasonably uniform ^{15}N. However, even several years after labelling, soils are unlikely to be completely stable in ^{15}N with time (e.g. Boddey and Victoria 1986).

The role of non N_2-fixing reference plants is very important in such studies. It is recommended that more than one reference plant be included in the experimental design. As seed size, and hence total seed N input, can differ between plant species, it is necessary to correct for the input of unlabelled seed N in all treatments (see Hamilton et al. 1992; Okito et al. 2004).

Pot experiments with rice

Application of ^{15}N enrichment to quantify N_2 fixation in waterlogged soils is difficult, as suitable non N_2-fixing reference plants capable of growing well in waterlogged soils are not readily available. To overcome this problem, Shrestha and Ladha (1996) suggested the following approach. The authors mixed $^{15}(NH_4)_2SO_4$ with waterlogged soil in a large concrete container (6.5 × 2 m and 25 cm deep). Mixing of the whole submerged soil mass was repeated three times a week for 6 weeks. Subsequently, 350 plastic pots were filled with the wet ^{15}N-labelled soil (10 kg/pot), and 70 rice genotypes were planted. The ^{15}N enrichment of the soil mineral N in a set of unplanted pots was measured weekly. The %Ndfa was calculated for the rice genotypes using either the highest recorded ^{15}N enrichment of the rice genotypes (within a maturity group), assuming this to represent a plant obtaining all of its N from the soil, or by using the average ^{15}N of the soil mineral N from the weekly samplings. Estimates of %Ndfa from the two methods were very similar and highly correlated.

A similar approach was used by Oliveira (1992, cited by Boddey et al. 1995b) with 40 rice genotypes planted in large concrete tanks. Within the maturity groups, genotypes with significantly lower ^{15}N enrichments were regarded as obtaining significant inputs of N from N_2 fixation.

Field experiments

If it can be ascertained that the ^{15}N enrichment of the plant-available N is uniformly labelled to the maximum rooting depth of all plants in the experiment, and if the ^{15}N isotope dilution observed in the N_2-fixing plant is significantly greater than that of an array of non N_2-fixing reference plants, then this is powerful evidence for N_2 fixation being responsible for the observed isotope dilution. However, uniform labelling of field soils remains problematic (see section 9.2)

Studies conducted in concrete tanks or cylinders in the field have met with some success. The main advantage of using pots or larger containers in the open field is that the plants can be grown to somewhat near their agronomic yield potential without the potential problems of light variability or overheating that may occur in greenhouses.

For work on large plants such as sugarcane and elephant grass, large containers are almost essential. Urquiaga et al. (1992) grew sugarcane plants in a concrete tank filled with soil mixed with ^{15}N-labelled compost for three consecutive years. The large and significant differences in ^{15}N enrichment

of some of the cane varieties compared to the reference plant (tanner grass), combined with differences in total N balances, were taken as strong evidence of significant N_2 fixation. The fact that two of the commercial cane hybrids accumulated over 25 g N/m^2 (equivalent to 250 kg N/ha) with no discernible decrease in soil N for three consecutive years also constituted strong supportive evidence for significant N_2 fixation inputs.

Boddey et al. (1986) conducted a study in concrete cylinders containing soil labelled some years earlier with ^{15}N to investigate the effect of inoculating wheat with three strains of *Azospirillum brasilense* (Table 31). All three strains promoted increases in total N accumulation in the grain (average of 40%), but the ^{15}N enrichment of the wheat was not significantly changed. The conclusion in this instance was that the increased grain N was not derived from biological N_2 fixation.

Women taking a break from work in a rice paddy, Myanmar. There is scope to substantially reduce fertiliser N inputs in rice systems through more effective in-crop use of cyanobacteria and *Azolla*, and legume green manures between rice crops.

Table 31. Accumulation of dry matter and N and the ^{15}N enrichment of wheat grown in soil labelled with ^{15}N after inoculation with three alternative strains of *Azospirillum brasilense*. Values followed by the same letter within a column are not significantly different by analysis of variance ($P<0.05$).

Azospirillum inoculum	Shoot dry weight (g/cylinder)	% N	Total N (g/cylinder)	Atom% ^{15}N excess
Sp 245	46.7	2.72 a	1.27 a	0.171
Sp 107	42.3	2.83 a	1.20 a	0.190
Sp 7	45.2	2.81 a	1.28 a	0.159
Autoclaved cells	39.3	2.21 b	0.89 b	0.156
Level significance	ns	0.01	0.001	ns

Shoot dry weight, % N, total N, atom % ^{15}N excess and ^{15}N recovery showed no significant differences between the three inoculants.

Source: Boddey et al. (1986)

10.2.3 ^{15}N natural abundance

As detailed in section 7.2, soil mineral N tends to have higher natural ^{15}N abundance than atmospheric N_2, and thus associative N_2-fixing plants should have lower ^{15}N abundance than non N_2-fixing plants growing in the same soils. The basic protocols of the techniques are identical to those applied to nodulated legumes (Chapter 8). The major exception is that while free-living microbes can be grown totally dependent on N_2 fixation (e.g. *Azobacter*, Mariotti et al. 1980), the associative plants do not grow well in a glasshouse without at least some mineral N. Since no 'B' value can be determined, 'B' is assumed to be 0‰ for the calculation of N_2 fixation (see equation (11)).

In the case of associative N_2 fixation, differences in $\delta^{15}N$ between N_2-fixing and non N_2-fixing plants are usually small, and can be caused by influences of soil N fractions, mycorrhizal status, and N physiology on plant natural ^{15}N abundance (Stewart 2001), rather than by N_2 fixation. Thus, a number of known non N_2-fixing species growing in the same soil may have quite different $\delta^{15}N$ values (Table 32), making it virtually impossible to calculate %Ndfa with any certainty (Ndfa values of sugarcane are in the range 15–57%).

Table 32. $\delta^{15}N$ (‰) of sugarcane and other species at Miyako, Japan, and %Ndfa for sugarcane estimated using each reference plant

Species	Leaf $\delta^{15}N$	Calculated sugarcane %Ndfa
Sugarcane	4.5	–
Poa chinensis	5.6	20
Amaranthus gracilis	10.4	57
Euphorbia hirta	5.3	15
Senecio vulgaris	7.0	36
Oxalis corniculata	5.3	15

Source: from Yoneyama et al. (1997)

Similarly, Watanabe et al. (1987) reported that the $\delta^{15}N$ value of NH_4^+ taken from incubated soil samples increased from 8.2‰ at 0–10 cm depth to 12.6‰ at 20–30 cm, and differences in $\delta^{15}N$ of the different wetland rice genotypes in the study could have resulted from different effective rooting depths and not necessarily from N_2 fixation.

In a more rigorous application of the methodology, Boddey et al. (2001) sampled field-grown sugarcane to quantify N_2 fixation. Their approach provides a good example of a sound application of the methodology. An area of 40 × 50 m in a uniform area of each of 11 fields was 'divided' into four equal strips of 10 × 50 m. Each strip was regarded as a statistical block with two or more treatments, i.e. the sugarcane and the weeds. Thirty cane plants were sampled from each block by taking the third-emergent leaf, the usual practice for nutrient monitoring ('crop logging') for this crop. Non-leguminous species that were present in all four blocks were used as non N_2-fixing reference plants and were analysed separately. This sampling strategy allowed statistical analysis of the data to assess whether (a) there were significant differences between the mean $\delta^{15}N$ values of the weed species, and (b) the mean $\delta^{15}N$ value of the sugarcane samples was significantly lower than that of some or all of the weed species. In 10 of the 11 cane plantations sampled across four states in Brazil, the $\delta^{15}N$ values of the cane samples (mean 6.9‰) were significantly lower than those of the weeds (mean 9.0‰). At a further site, two of the three weeds sampled were significantly lower in $\delta^{15}N$ than the sugarcane. Based on these data, N_2 fixation was estimated to have ranged from zero to approximately 71% of total sugarcane N across the crops and varieties (see Table 33).

Table 33. $\delta^{15}N$ (‰) of sugarcane and other species and estimated %Ndfa from 11 sites in Brazil. The mean %Ndfa value does not include the two negative %Ndfa estimates that arose from the sampling and analysis.

Plant	No. of samples	Minimum	Maximum	Mean
Sugarcane	12	3.3	13.2	6.9
Reference species	28	5.4	26.5	9.0
Sugarcane %Ndfa	28	−12.3	71.2	37.0

Source: from Boddey et al. (2001)

When sampling only parts of plants, such as described above, some investigation into the variation of $\delta^{15}N$ within plants is required. In the above study, a sugarcane plant growing at one field site was dissected and analysed for N and $\delta^{15}N$ (Figure 40). The third-emergent leaf had a ^{15}N abundance of 4.1‰, with the different tissues ranging from 5.0‰ to 3.5‰. The weighted mean of the whole shoot was 3.9‰. These data were used to justify the sampling and analysis of the third-emergent leaf to represent the whole shoot tissue of the cane plants.

The above data provided strong evidence for significant contributions of N_2 fixation to some of the cane crops but, due to variations in reference plant $\delta^{15}N$, not unequivocal evidence (see also Table 32).

The same strategy was used in a study of N_2 fixation of elephant grass. Over a 12-month period, the different elephant grass genotypes accumulated 28–37 tonnes dry matter per ha and 162–241 kg N per ha. There was no significant response of dry matter or N accumulation to the addition of 50 kg N per ha as urea 70 days after planting. The mean $\delta^{15}N$ of all three weed species was significantly higher than those of the third-emergent leaf samples of all of the elephant grass genotypes (Table 34).

To elucidate the question of variation of $\delta^{15}N$ of plant-available N with depth, soil samples were taken at 10 or 20 cm intervals and planted separately with three weed species, a technique originally suggested by Ledgard et al. (1984). It can be seen that the $\delta^{15}N$ of the weeds increased with the depth from which the soil was taken (Table 35). If the elephant grass genotypes had accessed N from greater depth in the soil than the weeds, the genotypes should have accumulated N with a higher $\delta^{15}N$ than the weed species. Hence, in this particular case, calculations of %Ndfa for the elephant grass genotypes would

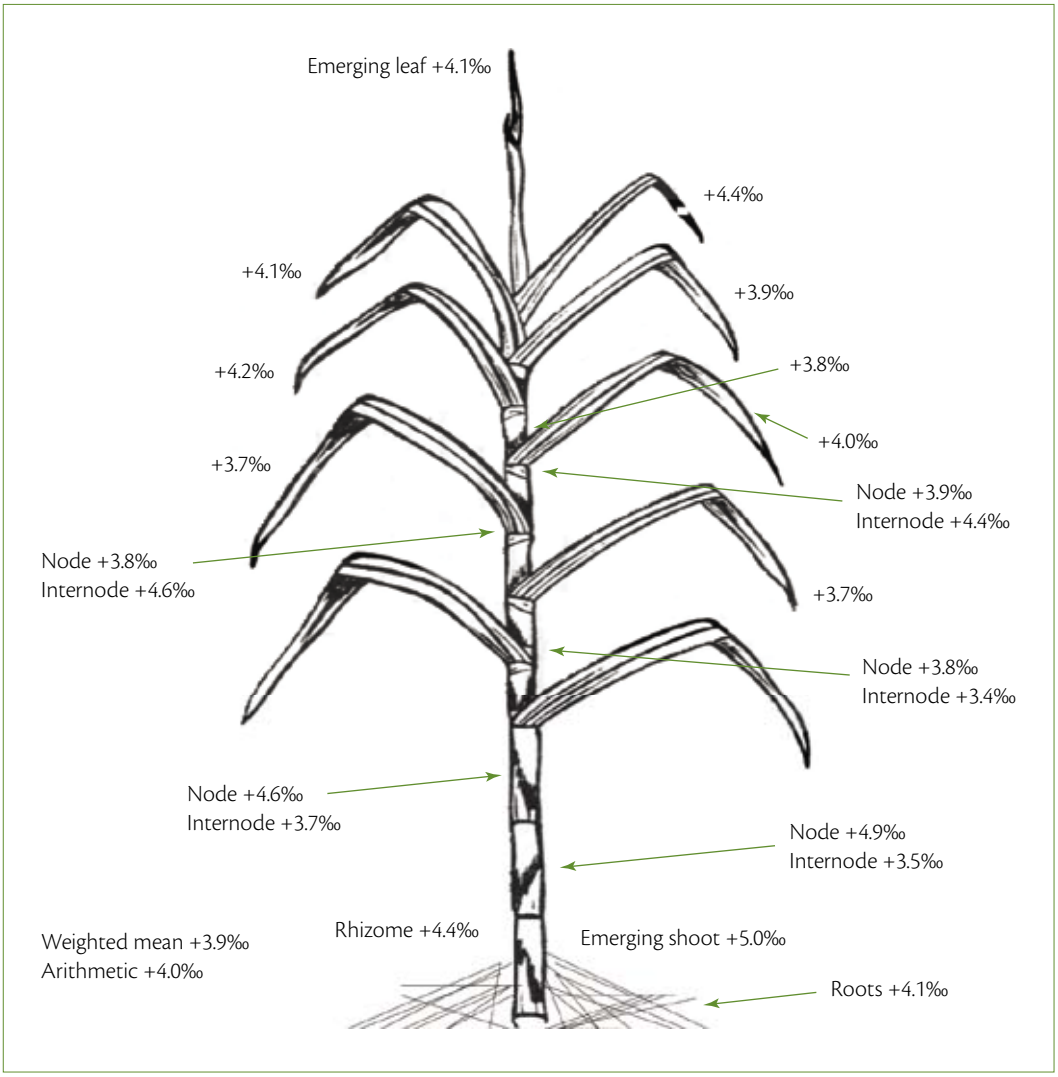


Figure 40. $\delta^{15}N$ natural abundance in leaves, stem (nodes and internodes), roots and emerging shoots of a sugarcane plant (cv. SP 70-1143) growing in the field in a sandy soil. Data are from Boddey et al. (2001).

tend to underestimate, rather than overestimate, the true values. A further point from the data in Table 34 is that weeds sampled at 6-monthly intervals showed essentially the same $\delta^{15}N$, suggesting that $\delta^{15}N$ of the plant-available N can be constant with time.

Table 34. $\delta^{15}N$ (‰) of five preselected elephant grass (*Pennisetum purpureum*) genotypes and three weed species in a field in Brazil. Values are the means of four replicate blocks to which no fertiliser N had been added. Values followed by the same letter within a column are not significantly different by analysis of variance ($P<0.05$).

	$\delta^{15}N$ (‰)	
	Dec 2005	June 2006
Elephant grass genotypes		
Bag	4.94 b	3.88 b
F 06-3	3.89 b	3.31 b
F 79-2	4.62 b	3.22 b
Gramafante	5.04 b	4.74 b
Cameroon	4.87 b	4.08 b
Weed species		
Colonião	7.52 a	6.78 a
Poaia Branca	7.32 a	7.15 a
Vassourinha	7.27 a	7.45 a

Source: Fiusa, Pacheco, Urquiaga, Boddey and Alves, unpublished data

The $\delta^{15}N$ strategy of Boddey et al. (2001) is arguably the best way of studying N_2 fixation of large associative N_2-fixing plants such as sugarcane and elephant grass in an open field environment. If the improvements on this technique to assess possible changes in $\delta^{15}N$ of plant-available N with soil depth and with time (see also section 8.4.) are integrated into the strategy, there is a strong possibility that N_2 fixation by these species, and others, can be quantified in the field. It does depend, however, on growing the plants in a soil with reasonably high and stable ^{15}N abundance. Parallel studies that provide additional evidence of N_2 fixation may also be required to reduce uncertainties that surround reference plant $\delta^{15}N$.

Table 35. $\delta^{15}N$ (‰) of three weed species planted in soil samples taken from different depths from a field soil in Brazil planted to five genotypes of elephant grass (*Pennisetum purpureum*). Values followed by the same letter within a column are not significantly different by analysis of variance ($P<0.05$).

Depth (cm)	Alpiste	Sorghum	Pé de Galinha
0–10	8.47 c	7.88 c	8.12 b
10–20	11.01 b	11.16 b	12.84 a
20–30	11.63 b	11.43 b	13.25 a
30–50	13.67 a	11.73 b	13.64 a
50–70	13.83 a	14.21 a	13.75 a

Source: Fiusa, Pacheco, Urquiaga, Boddey and Alves, unpublished data

Hand weeding the rice field, Myanmar

10.3 Final word

None of the methods for measuring N_2 fixation is completely adequate for associative N_2-fixing systems, which often make only modest contributions to the total N accumulated by the plants. Because these methods have particular limitations at low rates of N_2 fixation, they cannot always provide surety that N_2 fixation occurs, even if low rates are indicated. Techniques such as acetylene reduction (section 11.1) and $^{15}N_2$ feeding provide the most direct proof of N_2 fixation under these circumstances, and coupling these with ^{15}N isotope dilution or $\delta^{15}N$ techniques provides the most reliable approach for quantification. Where plants are known to be able to fix a substantial portion (>20%) of their N from associative N_2 fixation, then ^{15}N natural abundance or ^{15}N isotope dilution alone should provide robust quantification. It is very important to collect supporting information, use a variety of approaches (field and glasshouse) and take extra care with sampling and analysis, to be able to ensure that unambiguous results are obtained.

In some situations, experiments on N_2 fixation of associative plants produce slow-growing N-starved material. Under these circumstances contributions of N from contaminant sources (irrigation water, rainfall, atmospheric NH_3) may be equally important sources of N for plant growth, and will need to be measured or at least be very carefully considered when interpreting results.

11

Assays of nitrogenase activity

Biological N_2 fixation, the conversion of atmospheric N_2 to NH_3, is catalysed by the enzyme nitrogenase (equation (17)):

$$N_2 + 6H^+ + 6e^- \rightarrow 2NH_3 \qquad (17)$$

Our understanding of the physiology of N_2 fixation has been facilitated by studies of activity of the nitrogenase enzyme, assessed in three principal ways: $^{15}N_2$ feeding in short-term laboratory studies (e.g. Schulze et al. 2006); the substitution of N_2 with acetylene, which is reduced by nitrogenase to ethylene (Dilworth 1966); and evolution of H_2 by N_2-fixing systems (e.g. van Kessel et al. 1983). Of these three techniques, only $^{15}N_2$ is quantitative (see section 7.3). However, owing to their sensitivity and ease of use, acetylene reduction and H_2 evolution are valuable additional tools in the study of plant-associated N_2 fixation.

11.1 The acetylene reduction method

11.1.1 How it works

When acetylene (C_2H_2) gas is exposed to nitrogenase, it is converted to ethylene (C_2H_4) gas (equation (18)). Ethylene can be easily detected by gas chromatography with high sensitivity, using a flame ionisation detector.

$$\underset{\text{acetylene}}{HC{\equiv}CH} + 2H^+ + 2e^- \rightarrow \underset{\text{ethylene}}{H_2C{=}CH_2} \quad \text{(acetylene reduction)} \qquad (18)$$

Measurement of the rate of ethylene production after exposing N_2-fixing systems to acetylene is a simple method for indicating relative rates of nitrogenase activity.

However, in the process of N_2 fixation, not all the electrons flowing to nitrogenase are used for N_2 reduction; at least 25% go to H_2 production (equation (19) and see Hunt and Layzell 1993).

$$N{\equiv}N + 8H^+ + 8e^- \rightarrow 2NH_3 + H_2 \quad (N_2 \text{ fixation}) \qquad (19)$$

Theoretically, six electrons are required for N_2 reduction and two for the reduction of protons. However, if N_2 is replaced by C_2H_2, all eight electrons are needed to reduce C_2H_2 to C_2H_4, and thus four moles of acetylene should theoretically be reduced for each mole of N_2 fixed, with no protons reduced to H_2.

In practice the ratio of H_2 reduced for each mole of N_2 fixed varies considerably with organisms and environmental conditions. In the absence of acetylene, nitrogenase always reduces some protons to H_2, but at concentrations of acetylene above 100 mL/L, H_2 production is essentially zero.

The advantage of the acetylene reduction technique is very high sensitivity and rapid sample analysis. Using equipment that is easily and cheaply obtained, the technique is relatively simple and a large number of analyses can be performed per day.

11.1.2 Problems with acetylene reduction assays with nodulated legumes

Traditionally, acetylene reduction assays were conducted on decapitated root systems, groups of detached (or even single) nodules or entire plants. Assays were undertaken in closed vessels containing approximately 0.1 atmosphere acetylene (e.g. Mague and Burris 1972; Hardy et al. 1973; Dart 1986). The recommended incubation times were from 30 to 60 minutes, and ethylene evolution appeared to be constant with time.

However, it was subsequently found that physical disturbance of the nodule–soil and root–soil interfaces can influence nitrogenase activity in both nodulated legume and associative N_2-fixing systems. In the case of nodulated legumes, this is principally due to changes in nodule gas exchange (Minchin et al. 1986; Hunt and Layzell 1993). Both result in substantial reductions in nitrogenase activity. Virtually all diazotrophs are micro-aerophilic, requiring low O_2 concentration for survival and/or N_2 fixation.

Assaying intact legumes in pots with flow-through gas systems was used to overcome this disturbance problem. Even in the absence of physical plant–soil disturbance, nitrogenase activity in nodulated legumes was shown to decline in the presence of acetylene due to the interruption to NH_3 production within the nodule (Minchin et al. 1983a, 1986). The true rate of acetylene reduction in legumes, indicative of nitrogenase activity, probably only lasts a few minutes and can be detected only by using a continuous flow system (Minchin et al. 1983b). Details of design and construction of flow-through systems can

be found in Minchin et al. (1983a), Drevon et al. (1988) and McNeill et al. (1989) and are not discussed further here. Thus, static systems are likely to be more practical and convenient for most applications.

The use of the acetylene reduction technique as a quantitative assay of N_2 fixation has been generally discouraged since the 1980s. Yet, despite its limitations, the simple, rapid detection of ethylene evolution in the presence of acetylene gas is a powerful technique for verifying whether or not nodulated legume roots are actively fixing N_2.

11.1.3 Assay procedures and problems with acetylene reduction assays for associative N_2-fixing systems

Incubation systems for field assays

Almost any type of non-porous container can be used for static assays, with size usually determined by the size of the plant or root being assayed. Glass tubes with rubber septa, preserving jars, plastic syringes, plastic bags and plant pots have all been used for acetylene reduction assays. The lower the dead volume, the greater will be the concentration of ethylene produced and the sensitivity of the assay. Regardless of what is used, the incubation vessel must be gastight. Whole root systems are preferable for assay, such as illustrated in Figure 41, and with porous sandy soils to permit gas diffusion. Alternatively, soil cores with decapitated plants, or intact sections of root systems gently removed from soils, can be enclosed for incubation.

Plant disturbance is a serious source of error in estimating nitrogenase activity and concerted efforts to reduce this are warranted. For rainfed crops we recommend that plants be seeded directly into the centre of a steel or PVC cylinder, or the cylinder driven into the soil around the plant at least a week before assay. The cylinders should be of sufficient dimensions to encompass most of the root system. Cylinders of 18–20 cm diameter and 18 cm height (depth) are usually sufficient; smaller cylinders will cause considerable root disturbance and further disrupt the soil-pore structure (see Wani et al. 1983). In many sandy soils it is impossible to remove soil cores from the field without serious soil loss from the bottom of the core, but in loamy or high-clay soils these assays may be performed successfully if great care is taken. Twenty-four hours before the assay a layer of 2–3 cm of dry sand is placed on top of the soil surface within the cylinder to inhibit potential N_2-fixing activity by cyanobacteria.

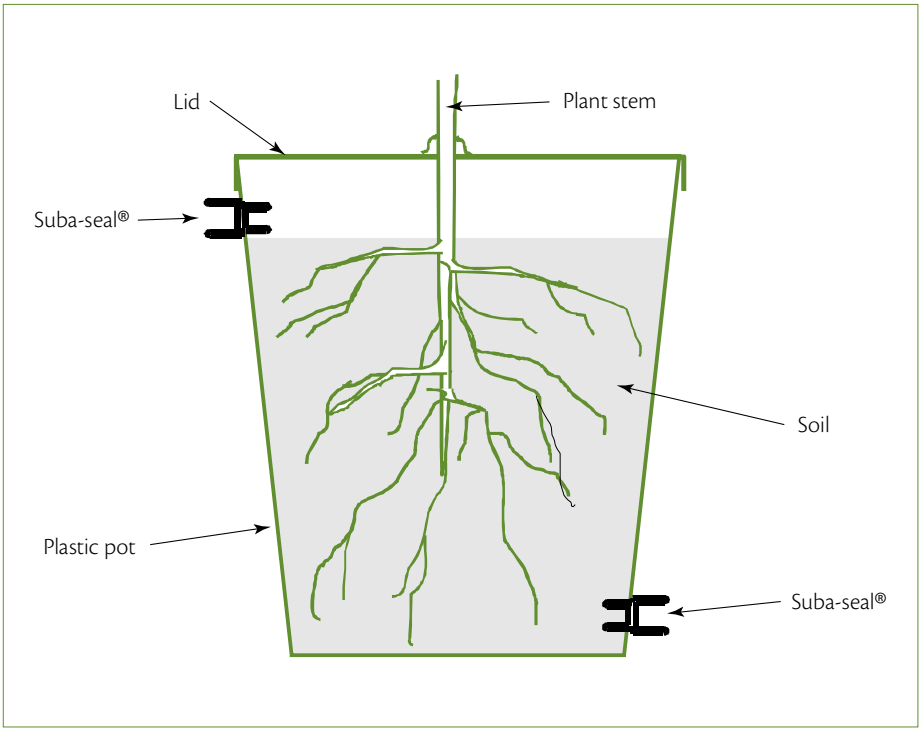


Figure 41. Incubation system for undisturbed acetylene-reduction assays of root systems of intact plants. A porous sand medium that allows easy diffusion of gases is preferred to loam or clay soils that present significant diffusion problems. Note the rubber ports for injection and withdrawal of acetylene and for assisting with mixing of gases within the pot.

For assay, the cylinder is dug out with great care, the plant decapitated (cut) a few centimetres above the soil level, and the whole root–soil system contained in the cylinder immediately transferred to an airtight plastic container (e.g. plastic food storage boxes) fitted with a needle-puncture rubber stopper to allow the gas space to be sampled. Because decapitation of the plant may reduce acetylene reduction activity, intact plants are preferred, but containers large enough to hold whole plants are not always easily obtained.

In waterlogged or flooded soils such as in rice systems, there are serious problems of gas diffusion, especially the release of ethylene from the rhizosphere to the atmosphere of the incubation container (Lee et al. 1977; Boddey and Ahmad 1981). Cyanobacteria in irrigation floodwater may also contribute to high apparent acetylene reduction rates, independent of any associations with plants. This can be controlled by using either an antibiotic or the herbicide

Propanil (3', 4'-dichloropropionanilide), which inhibits photosynthesis and kills most weeds but does not affect rice plants (Boddey et al. 1978; Habte and Alexander 1980).

Intact plants with single stems can be assayed in the field without decapitation if grown in cylinders. Instead of removing the soil core, a plastic sleeve with the same diameter can be pulled over the plant and sealed around the cylinder and the plant stem with string and agar, silicon, 'blue-tak' or Terostat® putty (Teroson GmbH, Heidelberg, FRG). We find polypropylene bags are less permeable to gases than polyethylene. The incubations are performed as described above for soil cores.

This method can easily be adapted to estimate acetylene reduction activity in pots, which may have sealable lids that close when the assay is performed (Figure 41). With plants grown in pots, especially in the greenhouse, great care must be taken to inhibit the activity of cyanobacteria, which often thrive under humid conditions.

Saturation of nitrogenase with acetylene

NOTE: Acetylene is a dangerous, explosive gas, and great care must be taken with its use. It should not be mixed with oxygen and never be used in confined (unventilated) rooms or near sources of ignition.

Acetylene is readily obtainable as a compressed gas in steel cylinders from commercial suppliers, or it can be produced by adding water to calcium carbide. Industrial grade acetylene is usually adequate (see Tough and Crush 1979). An incubation atmosphere of 10% acetylene (100 mL/L) is sufficient to assess maximum nitrogenase activity. It can be added to the incubation vessel using a plastic syringe and, after mixing, an equivalent volume of gas removed by the same syringe to maintain atmospheric pressure. Note that this concentration may not be sufficient for bacteria such as *Beijerinckia* or *Derxia* growth on Petri dishes. These bacteria were reported to require a very high partial pressure of C_2H_2 to overcome diffusion resistance through a viscous gum barrier produced under aerobic conditions (see Spiff and Odu 1973).

If the effective volume of the container when filled with the soil core is not known, a small known volume (e.g. 10 mL) of propane is injected through the seal (see Figure 41) after the injection of acetylene (100–150 mL/L airspace). The propane is used to estimate the volume of the enclosed gas space.

Diffusion of ethylene and other gases

The acetylene injected must reach the site of N_2 fixation in the soil, and the ethylene produced from nitrogenase must also be able to diffuse back to the atmosphere of the incubation vessel. This can be a major problem in intact soil–plant systems (van Berkum and Day 1980), and especially for rice grown in waterlogged soil (Watanabe et al. 1978). Be aware in these circumstances that underestimation of nitrogenase activity may occur.

Incubation period

The period of incubation should be the minimum necessary to precisely detect the ethylene evolved. Hourly samples up until 4 hours after introducing acetylene should be sufficient. Incubations of >4 hours are not recommended due to changes in the concentration of O_2 in the soil (Wani et al. 1983). Further, bacteria may multiply on the dead roots within the incubation chamber so that long incubations may not represent rates of N_2 fixation associated with living plants. Ensure that you record the time when you first disturb your plants, the time of acetylene injection and the time each gas sample is withdrawn from the incubation chamber for analysis. Ideally, the gas samples are analysed immediately but they can be stored in airtight syringes inserted into a rubber bung for up to 1 hour or in Vacutainers® for longer periods.

Environmental conditions

Environmental conditions during the assay should be similar to those of the undisturbed system before assay. Temperature, concentrations of CO_2 and O_2, humidity / soil water content, and light intensity in the case of intact systems, all have an impact on nitrogenase activity and should be controlled.

Endogenous production of ethylene in soils

Intact systems with roots in soil need to be corrected for background ethylene production. Ethylene is frequently produced and simultaneously oxidised in soils, but acetylene inhibits this natural ethylene oxidation. Injection of acetylene means that the 'endogenous' soil-produced ethylene will add to the nitrogenase-produced ethylene, giving apparently higher values. Control incubations can be run with low concentrations of acetylene (e.g. 500 µL/L) that still inhibit ethylene oxidation but are too low to register anything other than trace levels of acetylene reduction (Nohrstedt 1983). An alternative control uses the normal concentration of acetylene and 20–40 mL/L of

carbon monoxide (CO), a powerful inhibitor of nitrogenase activity, in the incubation. However, great caution should be used when handling CO as it is odourless and can be lethal if inhaled in modest quantities. It must never be used in confined spaces.

While such corrections for endogenous ethylene production can be used, the natural production of ethylene by soils and soil–plant systems remains a substantial, some would argue insurmountable (Giller and Merckx 2003), problem for application of the acetylene reduction assay to soils. Both the implementation of the assay and the interpretation of results should therefore be conducted with great care.

11.1.4 Gas chromatography

The chromatographic columns most commonly used to separate acetylene, ethylene and propane are generally micro-beads of polypropylene (Porapak N or Porapak T) packed into 0.32 cm (1/8 inch) external diameter stainless steel columns of approximately 2 m length passing through an oven at 100–120 °C (e.g. Hardy et al. 1968; Burris 1975). Shorter columns (40–50 cm) at lower temperatures (e.g. 40 °C) may enable faster separation of these gases (Boddey 1987). Chromatographs should be fitted with a flame ionisation detector. For most studies 0.5 mL of gas is extracted from the incubation chamber headspace and injected into the gas chromatograph. If possible, inject the same volume of sample, standard and gas to make the calculations simpler. Variable gas volumes can be accommodated as well, so make sure you record everything carefully.

With older gas chromatographs equipped with pen recorders, the areas under the peaks recorded on paper charts are proportional to the quantities of each substance, with the relationship of area to component concentration computed for standards of each gas. The peak height can often be used instead of areas under the peaks, with only small losses in accuracy.

With more modern gas chromatographs, the output is typically fed into an integrator that automatically gives the area under the curve of each peak and this area can be related to the concentration of each component.

The relationship of ethylene standard concentrations and area is linear for a wide concentration range (Figure 42), which makes it possible to use only one standard to calculate ethylene concentration in the sample without incurring significant error.

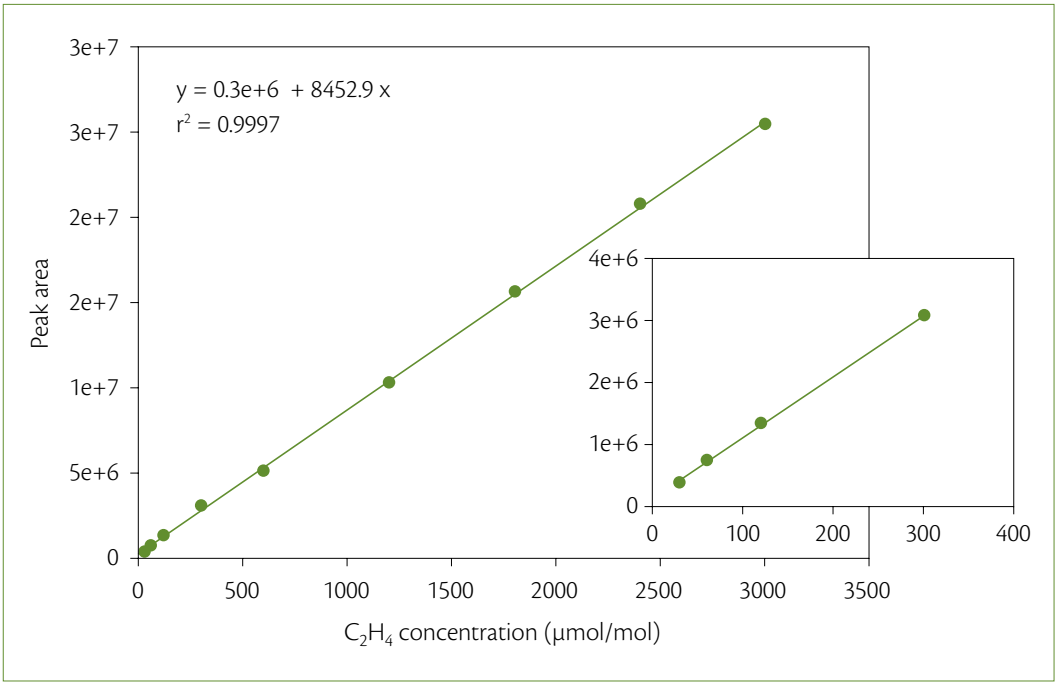


Figure 42. Relationship between peak area and ethylene standards (30–3,000 µmol/mol) from a Perkin Elmer chromatograph equipped with a flame ionisation detector and a 1.0 m Porapak N column. Temperatures were 100 °C for the injector, 95 °C for the oven and 230 °C for the detector. The inset graph is an enlarged view of the lower ethylene concentrations.

11.1.5 Calculations for acetylene reduction assays

The first assumption of the calculations below is that same volume of gas is injected into the chromatograph, usually 0.5 mL, for both the samples and standards. In this case, the areas under the peaks are proportional to the quantities of each component analysed. For ethylene concentration (equation (20):

$$\frac{C_E}{E} = \frac{K_E}{S_E} \qquad (20)$$

where C_E and K_E are the ethylene concentration in the sample and standard, respectively, (µL/L or parts per million by volume), and E (sample) and S_E (standard) are the respective areas obtained after chromatographic analyses.

To express the results in molar units it is assumed that samples and standards are injected into the chromatograph at 25 °C (298 K). Hence, as the volume of one mole of any gas at 298 K and 10 kPa is 24.45 L, the ethylene concentration in the sample, in µmol/mL, is (equation (21)):

$$C_E = \frac{E \times K_E}{S_E \times 24{,}450} \quad (21)$$

If two gas samples are taken from a flask of volume F (mL) after a time interval t (hours) between samplings, the ethylene production rate (X), in µmol/hour, during the time t is (equation (22)):

$$X = \frac{(C_{E2} - C_{E1}) \times F}{t} \quad (22)$$

where C_{E1} and C_{E2} are the initial and final ethylene concentrations in the flask.

Incorporating equations (20)–(22), the rate of ethylene production, in µmol C_2H_4/hour, is (equation (23)):

$$X = \frac{(E_2 - E_1) \times F \times K_E}{24{,}450 \times S_E \times t} \quad (23)$$

where E_1 and E_2 are the initial and final sample peak areas.

The X value can be considered the acetylene reduction rate by organisms in the flask of volume F, assuming that:

- the only ethylene source is from acetylene reduction
- there are no gas leaks during incubation or in the injection of gas samples and standards in the chromatograph
- acetylene reduction is constant with time.

Discussion of the use of external standards and the corrections for possible leaks can be found in Patriquin and Keddy (1978) and Turner and Gibson (1980). Examples of use of the equations are provided in Balandreau and Dommergues (1973) and Boddey et al. (1978).

11.1.6 Final word

The attraction of the acetylene reduction assay is its simplicity and sensitivity in indicating nitrogenase activity. Because of this, it is useful in identifying systems that are active in N_2 fixation. In the context of this book, it is not sufficiently reliable for field quantification. It is valuable, however, for identifying systems or conditions for further study using more robust quantitative techniques. It is likely to be most beneficial for studies of associative N_2-fixing systems where it is often difficult to provide unequivocal evidence of N_2 fixation. Under no circumstances should results from acetylene reduction assays be used to calculate amounts of N_2 fixed. Disturbance of the O_2 concentration in N_2-fixing systems, the direct influence of acetylene on nitrogenase activity, the short-term nature of the assays, and endogenous ethylene production from soils during assay are all factors that confound any extrapolation beyond the incubation chamber and incubation conditions.

Studying N_2 fixation using H_2 efflux

11.2

During N_2 fixation, part of the electron flux to nitrogenase is diverted to H_2 production (Bulen et al. 1965). The fact that H_2 is produced in parallel with N_2 allows H_2 efflux to be used as an indirect measure of nitrogenase activity (Layzell et al. 1984). However, some bacteria also possess an uptake hydrogenase enzyme that oxidises H_2, greatly reducing its liberation during N_2 fixation. These so called Hup^+ bacteria are thus capable of redirecting some of the energy generated by H_2 oxidation back to the nitrogenase complex to fix N_2. Thus, little or no H_2 efflux may be observed from nodules or colonies of Hup^+ bacteria.

As shown in equation (16), eight electrons are involved in the reduction of N_2 during N_2 fixation, but at least 25% of this electron flux is diverted to H_2 production. Thus, H_2 measurement under normal atmospheres can give only an approximate measure of nitrogenase activity as the majority of electrons are used to reduce N_2 to NH_3. However, if N_2 is replaced by an inert gas such as argon, all the electrons are diverted to H_2 production, provided that the proportion of O_2 in the mixture is maintained the same as in air (Layzell et al. 1984). H_2 efflux will then provide a direct measure of nitrogenase activity under assay conditions. The H_2 assay has advantages of requiring only low-cost apparatus such as that equipped with a solid-state detector (Qubit Systems Inc., Kingston, Canada <www.qubitsystems.com>) or by chromatographic analysis using a thermal conductivity detector (Bertelsen 1985).

However, the argon-induced inhibition of nitrogenase occurs for only about 2 hours, which limits the usefulness of the technique for N_2 fixation studies (King and Layzell 1991).

11.2.1 How it works

The H_2-efflux technique can be used in static incubation systems (Dong et al. 1995), flow-through systems using a simple portable detector (Rainbird et al. 1983) and more complex computer-monitored systems equipped with valves and sensors (Hunt et al. 1989). The rate of N_2 fixation (X) is calculated by subtracting the partial or apparent nitrogenase activity (ANA) measured in air from that measured in the absence of N_2 (i.e. presence of argon), which is termed total nitrogenase activity (TNA). The result is divided by three, which represents the number of electron pairs (e) required to reduce N_2 to NH_3, as follows (equation (24)):

$$X = \left(\frac{TNA - ANA}{e} \right) \tag{24}$$

The relative allocation of electrons by nitrogenase involved in the production of H_2 and N_2 reduction is calculated as (equation (25)):

$$\text{electron allocation coefficient } (EAC) = 1 - \left(\frac{ANA}{TNA} \right) \tag{25}$$

The theoretical maximum EAC is 0.75, but experimental values range from 0.59 to 0.70 (Hunt and Layzell 1993).

Simple assays can be performed on vials or flasks containing culture media (e.g. diazotrophic bacteria), from which air samples are withdrawn periodically until the maximum H_2 efflux rate is obtained (Bertelsen 1985). The procedure used by Dong et al. (1995) is described below.

When working with more heterogeneous systems including plants, it is recommended that measurements be made under normal atmospheres, alternated with an Ar:O_2 atmosphere, with the latter condition for as short a time as possible. Continuous flow-through systems equipped with H_2 detectors allow apparent and total nitrogenase activity to be measured for short periods.

In this case, plants must be grown in a medium that allows gas to flow freely through the root system. Grade 16 silica sand or industrial quartz of 3-mesh size (0.65 mm sand) provides appropriate porosity and contains no N that could inhibit nitrogenase. However, several experiments have been carried out with vermiculite (Witty and Minchin 1998; Gordon et al. 1999), where plants were grown in a N-free medium, and no problems were reported. Soil is unsuitable for use in flow-through systems unless it is sterilised, since the presence of H_2 induces changes in the soil biology population towards organisms capable of oxidising H_2. Within a short period all the H_2 produced will be consumed by the microflora and will not be measurable with H_2 detectors (Dong and Layzell 2001).

The advantages of the H_2 efflux technique to monitor nitrogenase activity can be summarised as follows (after Hunt and Layzell 1993):

- The method does not carry a significant health and safety hazard (unlike acetylene/O_2 mixtures which are potentially explosive).
- The H_2 analyser is less expensive than a gas chromatograph, which is required for acetylene reduction activity.
- Measurements of apparent nitrogenase activity and short-term measurements of total nitrogenase activity do not inhibit nitrogenase, and therefore the technique can be applied to the same plant continuously or intermittently.
- Using flow-through systems it is possible to continuously monitor nitrogenase activity with high sensitivity, allowing for measurements of ANA, TNA, EAC and N_2 fixation rate.

Among the disadvantages are the following:

- The method is not useful for Hup+ micro-organisms, which recycle most of the H_2 produced by nitrogenase.
- It is unsuitable for use with soil-based systems.
- The impact of plant disturbance on nitrogenase activity and H_2 evolution may be similar to those described for acetylene reduction assays.
- H_2 analysers are sensitive to water vapour and temperature changes, and analysis can be affected by O_2 and carrier gases, thus requiring careful calibration.
- Extended exposure to Ar:O_2 causes a decline in TNA.

An apparatus for H_2 measurement in plant systems is available from QubitSystems of Canada <http://www.qubitsystems.com/>.

11.2.2 Static H_2 procedure (after Dong et al. 1995)

Paired flasks with culture media are sealed with gastight serum stoppers to allow for H_2 accumulation. The atmosphere of one of them has its air content replaced by a mixture of 80% Ar : 20% O_2 by flushing it for 5 minutes. To avoid changes in bacterial behaviour and H_2 evolution in response to changes in temperature etc. (e.g. Bertelsen 1985), flasks must be kept under exactly the same conditions prior to the H_2 assay. Gas samples of 1 mL are withdrawn with plastic syringes at 10-minute intervals and analysed in a gas chromatograph equipped with a thermal conductivity or semiconductor sensor. The maximum H_2 evolution rate is usually reached after 1–2 hours. The N_2 fixation rate can be calculated using equation (24).

11.2.3 Flow-through H_2 systems

A scheme for use with intact nodulated legumes, based on that of Hunt et al. (1989), is shown in Figure 43.

The open-flow gas analysis system is used to monitor rates of H_2 exchange from nodulated roots in atmospheres of N_2:O_2 or Ar:O_2. The system is composed of mass flow controllers, one each of N_2, Ar and O_2 in the mixture. A H_2 standard in Ar is also connected to a flow controller to

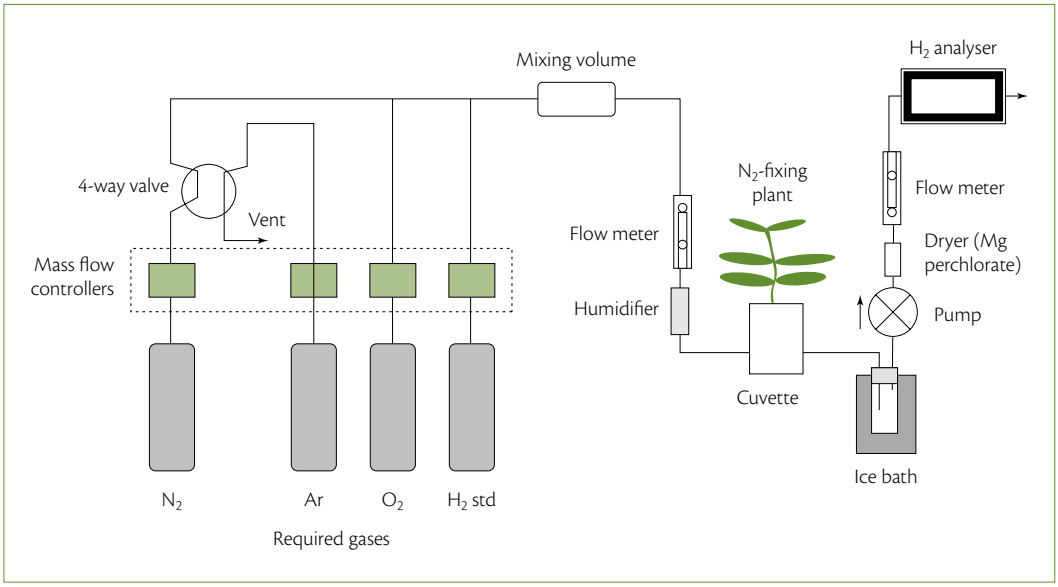


Figure 43. A flow-through system described in Hunt et al. (1989) to measure H_2 efflux from the root nodules of a single plant

calibrate the H_2 analyser in both N_2:O_2 and Ar:O_2. The analytical gas stream is humidified by bubbling through water and is then passed through the growth pot (cuvette) enclosing the root system of a plant. To maintain a low back-pressure and minimise leaks, most of the effluent gas stream is vented to the atmosphere. A portion of the gas stream is sampled by a pump and dehumidified by initial passage through an ice-water bath and then a more rigorous water trap such as magnesium perchlorate, before H_2 analysis.

H_2 evolution in a N_2:O_2 atmosphere provides a continuous monitor of relative nitrogenase activity in an atmosphere that is similar to air. After replacement with an Ar:O_2 atmosphere, all the electron flow is diverted to H_2 production, and the rate of H_2 evolution is taken as a measure of total nitrogenase activity (TNA). Since TNA declines with continuous exposure to Ar, the Ar:O_2 treatment is imposed only until maximal rates of H_2 evolution are measured (about 5 minutes). Figure 44 shows a time course of H_2 efflux under atmospheres of N_2 and Ar, both with ambient O_2 concentrations.

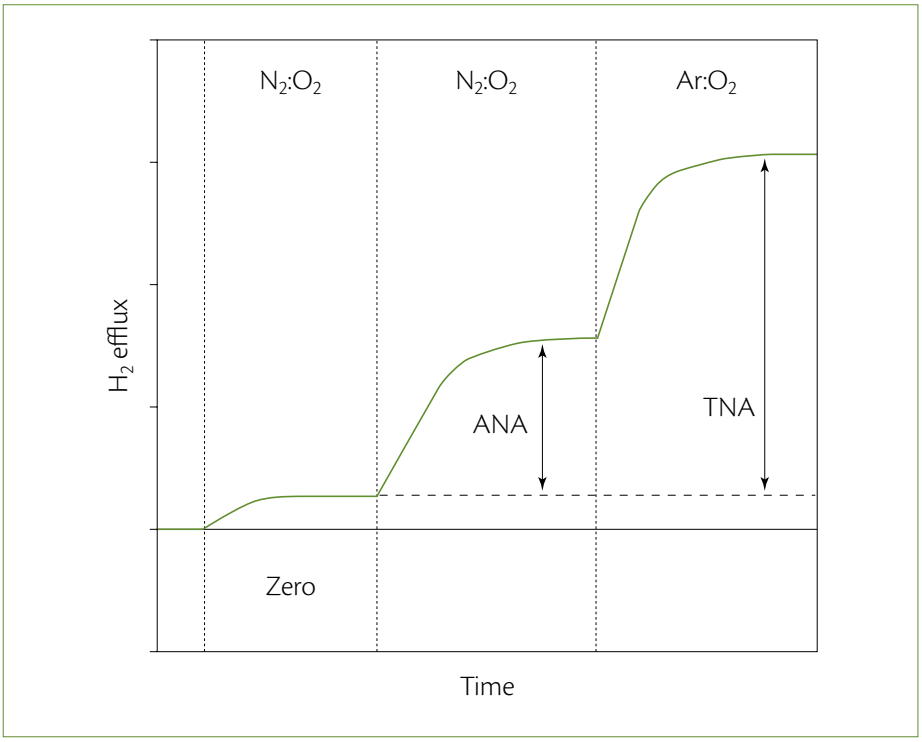


Figure 44. Hypothetical changes in H_2 efflux following sequential measurements of a N_2-fixing system under an atmosphere of N_2 and argon, both containing ambient O_2 concentration

11.2.4 Final word

The analysis of H_2 evolution is a sensitive procedure that can be used to provide evidence of nitrogenase activity, but only in systems that lack the hydrogenase enzyme. It suffers from a number of the same limitations described for the acetylene reduction assay, and is not well suited for use under field conditions for other than short-term monitoring of N_2-fixing activity by excavated roots. Results cannot be extrapolated beyond the incubation vessel or in time, and thus it cannot provide quantitative field estimates of N_2 fixation.

References

Addiscott T., Whitmore A. and Powlson D. 1991. Farming, fertilisers and the nitrate problem. CAB International: Wallingford, UK.

Alves B., Resende A., Urquiaga S. and Boddey R. 2000a. Biological nitrogen fixation by two tropical forage legumes assessed from the relative ureide abundance of stem solutes: ^{15}N calibration of the technique in sand culture. Nutrient Cycling in Agroecosystems 56, 165–176.

Alves B., Zotarelli L., Resende A., Polidoro J., Urquiaga S. and Boddey R. 2000b. Rapid and sensitive determination of nitrate in plant tissue using flow injection analysis. Communications in Soil Science and Plant Analysis 31, 2739–2750.

Alves B.J.R., Resende C.de P., A S, Macedo R., Tarre R.M., Urquiaga S. and Boddey R.M. 2000c. Estimation of N_2 fixation of *Desmodium ovalifolium* from the relative ureide abundance of stem solutes: comparison with the ^{15}N–dilution and in situ soil core technique. Nutrient Cycling in Agroecosystems 56, 177–193.

Anderson G., Fillery I.R.P., Dunin F.X., Dolling P.J. and Asseng S. 1998. Nitrogen and water flows under pasture–wheat and lupin–wheat rotations in deep sands in Western Australia. 2. Drainage and nitrate leaching. Australian Journal of Agricultural Research 49, 345–362.

Anderson J. and Ingram J. (eds) 1993. Tropical soil biology and fertility: a handbook of methods. CAB International: Wallingford, UK.

Arnold S.L. and Schepers J.S. 2004. A simple roller-mill grinding procedure for plant and soil samples. Communications in Soil Science and Plant Analysis 35, 537–545.

Baker D., Fried M. and Parotta J. 1995. Theoretical implications for the estimation of dinitrogen fixation by large perennial plant species using isotope dilution. Pp. 225–236 in 'Nuclear techniques in soil–plant studies for sustainable agriculture and environmental preservation'. IAEA: Vienna.

Balandreau J. and Dommergues Y. 1973. Assaying nitrogenase (C_2H_2) activity in the field. Pp. 247–254 in 'Modern methods in the study of microbial ecology', ed. by T. Rosswall. Bulletin of the Ecological Research Committee (Stockholm) 17: Stockholm.

Barraclough D. 1991. The use of mean pool abundances to interpret ^{15}N tracer experiments. I. Theory. Plant and Soil 131, 89–96.

Barley K.P. 1970. The configuration of the root system in relation to nutrient uptake. Advances in Agronomy 22, 159–201.

Barrie A., Brookes S.T., Prosser S.J. and Debney S. 1995. High productivity analysis of ^{15}N and ^{13}C in soil/plant research. Fertilizer Research 42, 43–59.

Batten G.D., Blakeney A.B., Glennie Holmes M.G., Henry R.J., McCaffery A.C., Bacon P.E. and Heenan D.P. 1991. Rapid determination of shoot nitrogen status in rice using near infrared reflectance spectroscopy. Journal of the Science of Food and Agriculture 54, 191–197.

Bergersen F.J. 1980a. Measurement of nitrogen fixation by direct means. Pp. 111–138 in 'Methods for evaluating biological nitrogen fixation', ed. by F.J. Bergersen. John Wiley & Sons: Chichester, UK.

Bergersen F.J. (ed.) 1980b. 'Methods for evaluating biological nitrogen fixation.' John Wiley & Sons: Chichester, UK.

Bergersen F.J., Brockwell J., Gault R.R., Morthorpe L., Peoples M.B. and Turner G.L. 1989. Effects of available soil nitrogen and rates of inoculation on nitrogen fixation by irrigated soybeans and evaluation of d^{15}N methods for measurement. Australian Journal of Agricultural Research 40, 763–780.

Bergersen F.J., Turner G.L., Amarger N., Mariotti F. and Mariotti A. 1986. Strain of *Rhizobium lupini* determines natural abundance of ^{15}N in root nodules of *Lupinus* species. Soil Biology and Biochemistry 18, 97–101.

Bertelsen H. 1985. Effect of temperature on H_2 evolution and acetylene reduction in pea nodules and in isolated bacteroids. Plant Physiology 77, 335–338.

Bethlenfalvay G.J., Reyes-Solis M.G., Camel S.B. and Ferrera-Cerrato R. 1991. Nutrient transfer between root zones of soybean and maize plants connected by a common mycorrhizal mycelium. Physiologia Plantarum 82, 423–432.

Blakeney A.B., Batten G.D. and Welsh L.A. 1995. Choosing materials as NIR standards. Pp. 36–39 in 'Leaping ahead with near infrared spectroscopy', ed. by G.D. Batten, P.C. Flinn, L.A. Welsh and A.B. Blakeney. Near Infrared Spectroscopy Group, RACI: North Melbourne.

Blakeney A.B., Dunn B., Batten G.D., Ciavarella S. and Beecher H.G. 2008. Choosing materials as standards for the Bruker FT-NIR. In 'Proceedings of the 13th International Conference on Near Infrared Spectroscopy'. Umeå-Vasa, Sweden and Finland. IM Publications: Chichester, UK.

Boddey R.M. 1987. Methods for quantification of nitrogen fixation associated with Gramineae. CRC Critical Reviews in Plant Science 6, 209–266.

Boddey R.M. and Ahmad N. 1981. Seasonal variations in nitrogenase activity of various rice varieties measured with an in situ acetylene reduction technique. Pp. 219–229 in 'Associative N_2 fixation', ed. by P. Vose and A. Ruschel. CRC Press: Boca Raton, FL.

Boddey R.M., Baldani V., Baldani J. and Döbereiner J. 1986. Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field-grown wheat. Plant and Soil 95, 109–121.

Boddey R.M., de Oliveira O., Alves B. and Urquiaga S. 1995a. Field application of the ^{15}N isotope dilution technique for the reliable quantification of plant-associated biological nitrogen fixation. Fertilizer Research 42, 77–87.

Boddey R.M., de Oliveira O., Urquiaga S., Reis V., Olivares F., Baldani V. and Dobereiner J. 1995b. Biological nitrogen fixation associated with sugarcane and rice: contributions and prospects for improvement. Plant and Soil 174, 195–209.

Boddey R.M., Peoples M.B., Palmer B. and Dart P. 2000. Use of the ^{15}N natural abundance technique to quantify biological nitrogen fixation by woody plants. Nutrient Cycling in Agroecosystems 57, 235–270.

Boddey R.M., Polidoro J., Carlos, Resende A.S., Alves B.J.R. and Urquiaga S. 2001. Use of the^{15}N natural abundance technique for the quantification of the contribution of N_2 fixation to sugar cane and other grasses. Functional Plant Biology 28, 889–895.

Boddey R.M., Quilt P. and Ahmad P. 1978. Acetylene reduction in the rhizosphere of rice: methods of assay. Plant and Soil 50, 567–574.

Boddey R.M., Reis V.M., Alves B.J.R. and Urquiaga S. 2006. Biological nitrogen fixation in agroecosystems. Pp. 177–189 in 'Biological approaches to sustainable soil Systems', ed. by N. Uphoff et al. CRC Press: Boca Raton, FL.

Boddey R.M. and Victoria R.L. 1986. Estimation of biological nitrogen fixation associated with *Brachiaria* and *Paspalum* grasses using ^{15}N-labelled organic matter and fertilizer. Plant and Soil 90, 265–292.

Bolan N., Saggar S., Luo J., Bhandral R. and Singh J. 2004. Gaseous emissions of nitrogen from grazed pastures: Processes, measurements and modelling, environmental implications, and mitigation Advances in Agronomy 84, 37–119.

Bonilla C.A., Kroll D.G., Norman J.M., Yoder D.C., Molling C.C., Miller P.S., Panuska J.C., Topel J.B., Wakeman P.L. and Karthikeyan K.G. 2006. Instrumentation for measuring runoff, sediment, and chemical losses from agricultural fields. Journal of Environmental Quality 35, 216–223.

Bremer E. and van Kessel C. 1990. Appraisal of the nitrogen-15 natural abundance method for quantifying dinitrogen fixation. Soil Science Society of America, Journal 54, 404–411.

Bremner J.M. and Mulvaney C.S. 1982. Nitrogen—total. Pp. 595–624 in 'Methods of soil analysis', ed. by A.L. Page. American Society of Agronomy and Soil Science Society of America: Madison, WI.

Brockwell J., Bottomley P.J. and Thies J.E. 1995. Manipulation of rhizobia microflora for improving legume productivity and soil fertility: a critical assessment. Plant and Soil 174, 143–180.

Bulen W.A., Burns R.C. and Lecomte J.R. 1965. Nitrogen fixation: hydrosulphite as electron donor with cell- free preparations of *Azotobacter vinelandii* and *Rhodospirillum rubrum*. Proceedings of the National Academy of Science, USA 53, 532–539.

Burris R.H. 1975. The acetylene reduction technique. Pp. 249–257 in 'Nitrogen fixation by free-living microorganisms', ed. by W.D.P. Stewart. Cambridge: Cambridge University Press.

Burris R.H., Eppling F.J., Wahlin H.B. and Wilson P.W. 1942. Studies of biological nitrogen fixation with isotopic nitrogen. Soil Science Society America Proceedings 7, 258–262.

Busse M. 2000. Suitability and use of the^{15}N-isotope dilution method to estimate nitrogen fixation by actinorhizal shrubs. Forest Ecology and Management 136, 85–95.

Cadisch G., Hairiah K. and Giller K.E. 2000. Applicability of the natural ^{15}N abundance technique to measure N_2 fixation in *Arachis hypogaea* grown on an Ultisol. Netherlands Journal of Agricultural Science 48, 31–45.

Cadisch G., Sylvester-Bradley R. and Nosberger J. 1989. ^{15}N-based estimation of nitrogen fixation by eight tropical forage-legumes at two levels of P:K supply. Field Crops Research 22, 181–195.

Carlsson G., Palmborg C. and Huss-Danell K. 2006. Discrimination against ^{15}N in three N_2-fixing *Trifolium* species as influenced by *Rhizobium* strain and plant age. Acta Agriculturae Scandinavica B56, 31–38.

Cataldo D.A., Haroon M., Schrader L.E. and Youngs V.L. 1975. Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Communications in Soil Science and Plant Analysis 6, 71–80.

Chalk P.M. 1985. Estimation of N_2 fixation by isotope dilution: an appraisal of techniques involving ^{15}N enrichment and their application. Soil Biology and Biochemistry 17, 389–410.

— 1996a. Nitrogen transfer from legumes to cereals in intercropping. Pp. 351–374 in 'Dynamics of roots and nitrogen in intercropping systems of the semi-arid tropics', ed. by O. Ito, C. Johansen, J. Adu-Gyamfi, K. Katayama, J. Kumar Rao and T. Rego. Japanese International Research Centre for Agricultural Sciences: Tsukuba.

— 1996b. Estimation of N_2 fixation by ^{15}N isotope dilution—the A-value approach. Soil Biology and Biochemistry 28, 1123–1130.

— 1998. Dynamics of biologically fixed N in legume–cereal rotations: a review. Australian Journal of Agricultural Research 49, 303-316.

— 2000. Integrated effects of mineral nutrition on legume performance. Soil Biology & Biochemistry 32, 577–579.

Chalk P.M. and Ladha J.K. 1999. Estimation of legume symbiotic dependence: an evaluation of techniques based on ^{15}N dilution. Soil Biology and Biochemistry 31, 1901-1917.

Chalk P.M., Smith C.J., Hopmans P. and Hamilton S.D. 1996. A yield–independent, ^{15}N–isotope dilution method to estimate legume symbiotic dependence without a non–N_2–fixing reference plant. Biology and Fertility of Soils 23, 196–199.

Chen D., Chalk P.M. and Freney, J.R. 1991. External-source contamination during extraction–distillation in isotope-ratio analysis of soil inorganic nitrogen. Analytica Chimica Acta 245, 49–55.

Ciavarella S., Batten G.D., Blakeney A.B. and Marr K.M. 1995. Modified NIR cells and packing techniques. Pp. 40-43 in 'Leaping ahead with near infrared spectroscopy', ed. by G.D. Batten, P.C. Flinn, L.A. Welsh and A.B. Blakeney. Near Infrared Spectroscopy Group, RACI: North Melbourne.

Cleveland C.C., Townsend A.R., Schimel D.S., Fisher H., Howarth R., Hedin L., Perakis S., Latty E., von Fischer J., Elsroad A. and Wasson M. 1999. Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. Global Biogeochemical Cycles 13, 623–645.

Clifton K. and Clifton L. 1991. A field method for the determination of total nitrogen in plant tissue. Communications in Soil Science and Plant Analysis 22, 851–860.

Corbin E.J., Brockwell J. and Gault R.R. 1977. Nodulation studies on chickpea (*Cicer arietinum*). Australian Journal of Experimental Agriculture and Animal Husbandry 17, 126–134.

Craswell E.T. and Eskew D.L. 1991. Nitrogen and nitrogen-15 analysis using automated mass and emission spectrometers. Soil Science Society of America, Journal 55, 750–756.

Crews T., Brockwell J. and Peoples M. 2005. Host–rhizobia interaction for effective inoculation: evaluation of the potential use of the ureide assay to monitor the symbiotic performance of tepary bean (*Phaseolus acutifolius* A. Gray). Soil Biology and Biochemistry 36, 1223–1228.

Dagliesh N. and Foale M. 2000. Soil matters: monitoring soil water and nutrients in dryland farming. Agricultural Production Systems Research Unit, CSIRO: Brisbane.

Danso S.K.A. and Kumarishinge K.S. 1990. Assessment of potential sources of error in nitrogen fixation measurements by the nitrogen-15 isotope dilution technique. Plant and Soil 125, 87–93.

Dart P.J. 1986. Nitrogen fixation associated with non-legumes in agriculture. Plant and Soil 90, 303–334.

Dart P.J. and Day J.M. 1975. Non-symbiotic nitrogen fixation in soils. Pp. 225–252 in 'Soil microbiology—a critical review', ed. by N. Walker. Butterworths: London.

Dawson T. and Brooks P.D. 2001. Fundamentals of stable isotope chemistry and measurement. Pp. 1–18 in 'Application of stable isotope techniques to study biological processes and functioning of ecosystems', ed. by M.J. Unkovich, J.S. Pate, A.M. McNeill and D.J. Gibbs. Kluwer Academic: Dordrecht.

Dawson T., Mambelli S., Plamboeck A., Templer P. and Tu K. 2002. Stable isotopes in plant ecology. Annual Review Ecology and Systematics 33, 507–559.

De Polli H., Matsui E., Dobereiner J. and Salati E. 1977. Confirmation of nitrogen fixation in two tropical grasses by $^{15}N_2$ incorporation. Soil Biology and Biochemistry 9, 119–123.

Dilworth M.J. 1966. Acetylene reduction by nitrogen fixing preparations from *Clostridium pasteurianum*. Biochimica et Biophyica Acta 127, 285–294.

Dobereiner J. and Day J.M. 1975. Associative symbioses in tropical grasses: characterization of microorganisms and nitrogen-fixing sites. Pp. 518–538 in 'Proceedings of the 1st International Symposium on Nitrogen Fixation, Pullman', ed. by W. Newton and C. Nyman. Washington State University Press.

Dong Y., Iniguez A.L. and Triplett E.W. 2003. Quantitative assessments of the host range and strain specificity of endophytic colonization by *Klebsiella pneumoniae*. Plant and Soil 257, 49–59.

Dong Z., Heydrich M., Bernard K. and McCully M.E. 1995. Further evidence that the N_2-fixing endophytic bacterium from the intercellular spaces of sugarcane stems is *Acetobacter diazotrophicus*. Applied and Environmental Microbiology 61, 1843–1846.

Dong Z. and Layzell D.B. 2001. H_2 oxidation, O_2 uptake and CO_2 fixation in hydrogen treated soils. Plant and Soil 229, 1–12.

Do Rosario M., Oliveira G., van Noordwijk M., Gaze S., Brouwer G., Bona S., Mosca G. and Hairiah K. 2000. Augur sampling, ingrowth cores and pinboard methods. Pp. 175–210 in 'Root methods: a handbook', ed. by A. Smit, A.G. Bengough, C. Engels, M. van Noordwijk, S. Pelerin, S. van de Geijn. Springer Press: Berlin and Heidelberg.

Doughton J.A., Vallis I. and Saffigna P.G. 1992. An indirect method for estimating N-15 isotope fractionation during nitrogen fixation by a legume under field conditions. Plant and Soil 144, 23–29.

Drevon J., Kalia V., Heckman M. and Pedalahore P. 1988. In situ open-flow assay of acetylene reduction activity by soybean root nodules: influence of acetylene and oxygen. Plant Physiology and Biochemistry 26, 73–78.

Eriksen J. and Hogh-Jensen H. 1998. Variations in the natural abundance of ^{15}N in ryegrass/white clover shoot material as influenced by cattle grazing. Plant and Soil 205, 67–76.

Eskew D.L., Eaglesham A.R.J. and App A.A. 1981. Heterotrophic $^{15}N_2$ fixation and distribution of newly fixed nitrogen in a rice–flooded soil system. Plant Physiology 68, 48–52.

Evans J. and Taylor A. 1987. Estimating dinitrogen (N_2) fixation and soil accretion of nitrogen by grain legumes. Journal of the Australian Institute of Agricultural Science 53, 78–82.

Evans J., Turner G.L., O'Connor G.E. and Bergersen F.J. 1987. Nitrogen fixation and accretion of soil nitrogen by field grown lupins. Field Crops Research 16, 309–322.

Farnworth J. 1997. Agri Info: guidelines for world crop and livestock production. John Wiley & Sons: Chichester, UK.

Fehr W.R., Caviness C.E., Burmood D.T. and Pennington J.S. 1971. Stage of development descriptions for soybeans, *Glycine max* (L.) Merrill. Crop Science 11, 929–931.

Fiedler R. 1984. The measurement of ^{15}N. Pp. 233–282 in 'Soil–plant–water relationships', ed. by M.F. L'Annunziata and J.O. Legg. Academic Press: London.

Fillery I. 2001. The fate of biologically fixed nitrogen in legume-based dryland farming systems. Australian Journal of Experimental Agriculture 41, 361–381.

Firth P., Thitipoca H., Suthipradit S., Wetselaar R. and Beech D.F. 1973. Nitrogen balance studies in the central plain of Thailand. Soil Biology and Biochemistry 5, 41–46.

Focht D. and Poth M. 1987. Measurement of biological nitrogen fixation by ^{15}N techniques. Pp. 257–288 in 'Symbiotic nitrogen fixation technology', ed. by G. Elkan. Marcel Dekker: New York.

Fried M. and Broeshart H. 1975. An independent measurement of the amount of nitrogen fixed by a legume crop. Plant and Soil 43, 707– 711.

Gathumbi S., Cadisch G. and Giller K.E. 2002. ^{15}N natural abundance as a tool for assessing N_2-fixation of herbaceous, shrub and tree legumes in improved fallows. Soil Biology and Biochemistry 34, 1059–1071.

Gault R.R., Peoples M.B., Turner G.L., Lilley D., Brockwell J. and Bergersen F.J. 1995. Nitrogen fixation by irrigated lucerne during the first three years after establishment. Australian Journal of Agricultural Research 46, 1401–1425.

Gibson A. 1980. Methods for legumes in glasshouses and controlled environment cabinets. Pp. 139–184 in 'Methods for evaluating biological nitrogen fixation', ed. by F.J. Bergersen. John Wiley & Sons: Chichester, UK.

Giller K.E. 2001. Nitrogen fixation in tropical cropping systems. CAB Publishing: Wallingford, UK.

Giller K.E., Cadisch G., Ehaliotis C., Adams E., Sakala W. and Mafongoya P. 1997. Building soil nitrogen capital in Africa. Pp. 151–192 in 'Replenishing soil fertility in Africa', ed. by R. Buresh, P. Sanchez and F. Calhoun. American Society for Agronomy, Crop Science Society of America, Soil Science Society of America: Madison, WI.

Giller K.E. and Day J.M. 1985. Nitrogen fixation in the rhizosphere: significance in natural and agricultural systems. Pp. 127–147 in 'Ecological interactions in soil: plants, microbes and animals', ed. by A.H. Fitter, D. Atkinson, D.J. Read and M.B. Usher. Blackwell Scientific: Oxford, UK.

Giller K.E. and Merckx R. 2003. Exploring the boundaries of N_2 fixation in cereals and grasses: an hypothetical framework. Symbiosis 35, 3–17.

Giller K.E., Wani S. and Day J. 1986. Use of isotope dilution to measure nitrogen fixation associated with the roots of sorghum and millet genotypes. Plant and Soil 90, 255–263.

Glenister R.A. and LaRue T.A. 1986. A spot test for estimating ureides in soybean petioles. Experimental Agriculture 22, 405–416.

Gordon A.J., Minchin F.R., James C.L. and Komina O. 1999. Sucrose synthase in legume nodules is essential for nitrogen fixation. Plant Physiology and Biochemistry 120, 867–877.

Goulding K.W.T., Bailey N.J., Bradbury N.J., Hargreaves P., Howe M., Murphy D.V., Poulton P.R. and Willison T.W. 1998. Nitrogen deposition and its contribution to nitrogen cycling and associated soil processes. New Phytologist 139, 49–58.

Gregory P. 2006. Plant roots; growth, activity and interaction with soils. Blackwell Publishing: Oxford, UK.

Guimarães A., de Morais R., Urquiaga S., Boddey R. and Alves B. 2008. *Bradyrhizobium* strain influences the ^{15}N natural abundance quantification of biological N_2 fixation in soybean. Scientia Agricola, in press.

Guthrie J.A., Liebenberg C.J. and Walsh K.B. 2006. NIR model development and robustness in prediction of melon fruit total soluble solids. Australian Journal of Agricultural Research 57, 1–8.

Guthrie J.A., Reid D.J. and Walsh K.B. 2005. Assessment of internal quality attributes of mandarin fruit. 2. NIR calibration model robustness. Australian Journal of Agricultural Research 56, 417–426.

Habte M. and Alexander M. 1980. Nitrogen fixation by photosynthetic bacteria in lowland rice culture. Applied and Environmental Microbiology 39, 342–347.

Hamilton S.D., Smith C.J., Chalk P.M. and Hopmans P. 1992. A model based on measurement of soil and plant ^{15}N enrichment to estimate N_2 fixation by soybean (*Glycine max* L. Merrill) grown in pots. Soil Biology and Biochemistry 24, 71–78.

Hansen A.P. and Pate J.S. 1987. Evaluation of the ^{15}N natural abundance method and xylem sap analysis for assessing N_2 fixation of understorey legumes in jarrah (*Eucalyptus marginata* Donn ex Sm.) forest in S.W. Australia. Journal of Experimental Botany 38, 1446–1458.

Hansen A.P., Rerkasem B., Lordkaew S. and Martin P. 1993. Xylem-solute technique to measure N_2 fixation by *Phaseolus vulgaris* L.: calibration and sources of error. Plant and Soil 150, 223–231.

Hardy R.W.F., Burns R.C. and Holsten R.D. 1973. Application of the acetylene–ethylene assay for measurement of nitrogen fixation. Soil Biology and Biochemistry 5, 47–81.

Hardy R.W.F., Holsten R.D., Jackson E.K. and Burns R.C. 1968. The acetylene–ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Physiology 43, 1185–1207.

Harris G. and Hesterman O.B. 1990. Quantifying the nitrogen contribution from alfalfa to soils and two succeeding crops using nitrogen-15. Agronomy Journal 82, 129–134.

Herridge D.F. 1982a. A whole-system approach to quantifying biological nitrogen fixation by legumes and associated gains and losses of nitrogen in agricultural systems. Pp. 593–608 in 'Biological nitrogen fixation technology for tropical agriculture', ed. by P.H. Graham and S.C. Harris. CIAT: Cali, Colombia.

— 1982b. Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. Plant Physiology 70, 1–6.

— 1984. Effects of nitrate and plant development on the abundance of nitrogenous solutes in root-bleeding and vacuum extracted exudates of soybean. Crop Science 25, 173–179.

— 1988. The narrow leaved lupin (*Lupinus angustifolius*) as a nitrogen-fixing rotation crop for cereal production. I. Indices of nitrogen fixation. Australian Journal of Agricultural Research 39, 1003–10015.

Herridge D.F., Atkins C.A., Pate J.S. and Rainbird R.M. 1978. Allantoin and allantoic acid in the nitrogen economy of the cowpea (*Vigna unguiculata* [L.] Walp.). Plant Physiology 62, 495–498.

Herridge D.F., Bergersen F.J. and Peoples M.B. 1990. Measurement of nitrogen fixation by soybean in the field using the ureide and natural ^{15}N abundance methods. Plant Physiology 93, 708–716.

Herridge D.F. and Doyle A.D. 1988. The narrow leaved lupin (*Lupinus angustifolius*) as a nitrogen-fixing rotation crop for cereal production. II. Estimates of nitrogen fixation by field-grown crops. Australian Journal of Agricultural Research 39, 1017–10028.

Herridge D.F., Marcellos H., Felton W., Turner G. and Peoples M. 1998. Chickpea in wheat based cropping systems of northern New South Wales III. Prediction of N_2 fixation and N balance using soil nitrate at sowing and chickpea yield. Australian Journal of Agricultural Research 49, 409–418.

Herridge D.F., Marcellos H., Felton W.L., Turner G.L. and Peoples M.B. 1995. Chickpea increases soil N fertility in cereal systems through nitrate sparing and N_2 fixation. Soil Biology and Biochemistry 27, 545–551.

Herridge D.F., O'Connell P. and Donnelly K. 1988. The xylem ureide assay of nitrogen fixation: Sampling procedures and sources of error. Journal of Experimental Botany 39, 12–22.

Herridge D.F., Palmer B., Nurhayati D.P. and Peoples M.B. 1996. Evaluation of the xylem ureide method for measuring N_2 fixation in six tree legume species. Soil Biology and Biochemistry 28, 281–289.

Herridge D.F. and Peoples M.B. 1990. The ureide assay for measuring nitrogen fixation by nodulated soybean calibrated by ^{15}N methods. Plant Physiology 93, 495–503.

— 2002a. Timing of xylem sampling for ureide analysis of nitrogen fixation. Plant and Soil 238, 57–67.

— 2002b. Calibrating the xylem-solute method for nitrogen fixation measurement of ureide-producing legumes: cowpea, mungbean and black gram. Communications in Soil Science and Plant Analysis 33, 425–437.

Herridge D.F., Peoples M. and Boddey R.M. 2008. Global inputs of biological nitrogen fixation in agricultural systems. Plant and Soil 311, 1–18.

Herridge D.F. and Rose I.A. 2000. Breeding for enhanced nitrogen fixation in crop legumes. Field Crops Research 65, 229–248.

Herridge D.F., Roughley R.J. and Brockwell J. 1984. Effects of rhizobia and soil nitrate on the establishment and functioning of the soybean symbiosis in the field. Australian Journal of Agricultural Research 35, 149–161.

Hogberg P. 1997. ^{15}N natural abundance in soil–plant systems: Tansley Review No. 95. New Phytologist 137, 179–203.

Holdensen L., Hauggaard-Nielsen H. and Jensen E.S. 2007. Short-range spatial variability of soil $\delta^{15}N$ natural abundance—effects on symbiotic N_2-fixation estimates in pea. Plant and Soil 298, 265–272.

Hughes R.M. and Herridge D.F. 1989. Effect of tillage on yield, nodulation and N_2 fixation of soybean in far north-coastal New South Wales. Australian Journal of Experimental Agriculture and Animal Husbandry 29, 671–677.

Hungria M., Boddey L., Santos M. and Vargas M. 1998. Nitrogen fixation capacity and nodule occupancy by *Bradyrhizobium japonicum* and *B. elkanii* strains. Biology and Fertility of Soils 27, 393–399.

Hungria M., Franchini J., Campo R., Crispino C., Moraes J., Sibaldelli R., Mendes L. and Arihara J. 2006. Nitrogen nutrition of soybean in Brazil: contributions of biological N_2 fixation and N fertilizer to grain yield. Canadian Journal of Plant Science 86, 927–939.

Hungria M. and Neves M. 1987. Cultivar and *Rhizobium* strain effect on nitrogen fixation and transport in *Phaseolus vulgaris* L. Plant and Soil 103, 111–121.

Hunt P., Burnham K. and Matheny T. 1987. Precision and bias of various soybean dry matter sampling techniques. Agronomy Journal 79, 425–428.

Hunt S., King B.J. and Layzell D.B. 1989. Effects of gradual increases in O_2 concentration on nodule activity in soybean. Plant Physiology 91, 315–321.

Hunt S. and Layzell D. 1993. Gas exchange of legume nodules and the regulation of nitrogenase activity. Annual Reviews of Plant Physiology and Plant Molecular Biology 44, 483–511.

Iniguez A.L., Dong Y. and Triplett E.W. 2004. Nitrogen fixation in wheat provided by *Klebsiella pneumoniae*. Molecular Plant–Microbe Interactions 17, 1078–1085.

IAEA (International Atomic Energy Agency) 2001. Use of isotope and radiation methods in soil and water management and crop nutrition. FAO/IAEA: Vienna.

Kamphake L.J., Hannah S.A. and Cohen J.M. 1967. Automated analysis for nitrate by hydrazine reduction. Water Research 1, 205–216.

Khan D.F., Peoples M., Chalk P.M. and Herridge D. 2002. Quantifying below ground nitrogen of legumes. 2. A comparison of ^{15}N and non-isotopic methods. Plant and Soil 239, 277–289.

Khan D.F., Peoples M., Schwenke G., Felton W.L., Chen D. and Herridge D.F. 2003. Effects of below-ground nitrogen on N balances of field-grown fababean, chickpea and barley. Australian Journal of Agricultural Research 54, 333–340.

King B.J. and Layzell D.B. 1991. Effect of increases in O_2 concentration during the argon-induced decline in nitrogenase activity in root nodules of soybean. Plant Physiology 96, 376–381.

King C. and Purcell L. 2005. Inhibition of N fixation in soybean is associated with elevated ureides and amino acids. Plant Physiology 137, 389–1396.

Kowalenko C.G. 2001. Assessment of Leco CNS-2000 analyser for simultaneously measuring total carbon, nitrogen and sulfur in soil. Communications in Soil Science and Plant Analysis 32, 2065–2078.

Kumar Rao J., Thompson J., Sastry P., Giller K.E. and Day J. 1987. Measurement of N_2-fixation in field-grown pigeonpea [*Cajanus cajan* L.] using ^{15}N-labelled fertilizer. Plant and Soil 101, 107–113.

Kyei-Boahen S., Slankard A. and Walley F. 2002. Isotopic fractionation during N_2 fixation by chickpea. Soil Biology and Biochemistry 34, 417–420.

Ladha J.K., Peoples M.B., Barrity D.P., Capuno V.T. and Dart P.J. 1993. Estimating dinitrogen fixation of hedgerow vegetation using the ^{15}N natural abundance method. Soil Science Society of America, Journal 57, 732–737.

Layzell D.B., Weagle G.E. and Canvin D.T. 1984. A highly sensitive flow through H_2 analyser for use in nitrogen fixation studies. Plant Physiology 75, 582–585.

Ledgard S.F., Freney J.R. and Simpson J.R. 1984. Variations in natural enrichment of ^{15}N in the profiles of some Australian pasture soils. Australian Journal of Soil Research 22, 155–164.

Ledgard S.F. and Giller K.E. 1995. Atmospheric N_2 fixation as an alternative N source. Pp. 443–486 in 'Nitrogen Fertilization and the Environment', ed. by P.E. Bacon. Marcel Dekker Inc.: New York.

Lee, K, Alimango B. and Yoshida T. 1977. Field technique using the acetylene reduction method to assay nitrogenase activity and its association with the rice rhizosphere. Plant and Soil 47, 519–526.

Lethbridge G. and Davidson M.S. 1983. Root-associated nitrogen-fixing bacteria and their role in the nitrogen nutrition of wheat estimated by ^{15}N isotope dilution. Soil Biology and Biochemistry 15, 365.

Letolle R. 1980. Nitrogen-15 in the natural environment. Pp. 407–433 in 'Handbook of environmental isotope geochemistry', ed. by P. Fritz and J.C. Fontes. Elsevier: Amsterdam.

Lima E., Boddey R. and Döbereiner J. 1987. Quantification of biological nitrogen fixation associated with sugar cane using a ^{15}N aided nitrogen balance. Soil Biology and Biochemistry 19, 165–170.

McAuliffe C., Chamblee D., Uribe-Arango H. and Woodhouse W. 1958. Influence of inorganic nitrogen on nitrogen fixation of legumes as revealed by ^{15}N. Agronomy Journal 50, 334–337.

McClure P.R., Israel D.W. and Volk R.J. 1980. Evaluation of the relative ureide content of xylem sap as an indicator of N_2 fixation in soybeans. Plant Physiology 60, 720–725.

McNeill A. and Fillery I. 2008. Field measurement of lupin belowground nitrogen accumulation and recovery in the subsequent cereal–soil system in a semi-arid Mediterranean-type climate. Plant and Soil 302, 297–316.

McNeill A.M., Pilbeam C.J., Harris H.C. and Swift R.S. 1996. Seasonal variation in the suitability of different methods for estimating biological nitrogen fixation by grain legumes under rainfed conditions. Australian Journal of Agricultural Research 47, 1061–1073.

— 1998. Use of residual fertiliser ^{15}N in soil for isotope dilution estimates of N_2 fixation by grain legumes. Australian Journal of Agricultural Research 49, 821–828.

McNeill A.M., Sheehy J.E. and Drennan D.S.H. 1989. The development and use of a flow-through apparatus for measuring nitrogenase activity and photosynthesis in field crops. Journal of Experimental Botany 40, 187–194.

McNeill A.M. and Unkovich M.J. 2007. The nitrogen cycle in terrestrial ecosystems. Pp. 37–64 in 'Nutrient cycling in terrestrial ecosystems', ed. by P. Marschner and Z. Rengel. Springer Verlag: Amsterdam.

McNeill A.M., Wood M. and Gates R.P. 1993. The use of a closed system flow-through enclosure apparatus for studying the effects of partial pressure of dinitrogen in the atmosphere on growth of *Trifolium repens* L and *Lolium perenne* L. Journal of Experimental Botany 44, 1021–1028.

McNeill A.M., Zhu C. and Fillery I. 1997. Use of *in situ* ^{15}N-labelling to estimate the total below-ground nitrogen of pasture legumes in intact plant–soil systems. Australian Journal of Agricultural Research 48, 295–304.

Mague T.M. and Burris R.H. 1972. Reduction of acetylene and nitrogen by field grown soybeans. New Phytologist 71, 275–286.

Mariotti A. 1983. Atmospheric nitrogen is a reliable standard for ^{15}N natural abundance measurements. Nature 303, 685–687.

Mariotti A., Mariotti F., Amarger N., Pizelle G., Ngambi J.-M., Champigny M.-L. and Moyse A. 1980. Fractionnements isotopiques de l'azote lors des processus d'absorption des nitrates et de fixation de l'azote atmosphérique par les plantes. Physiologie Végetale, 18, 163–181.

Matsumoto T., Yatazawa M. and Yamamoto Y. 1977. Incorporation of 15N into allantoin in nodulated soybean plants supplied with 15N2. Plant Cell Physiology 18, 859–862.

Meeks J.C. 1993. 13N Techniques. Pp. 273–304 in 'Nitrogen isotope techniques', ed. by R. Knowles and T.H. Blackburn. Academic Press: San Diego.

Minchin F.R., Sheehy J.E. and Witty J.F. 1986. Further errors in the acetylene reduction assay: effects of plant disturbance. Journal of Experimental Botany 37, 1581–1591.

Minchin F.R., Witty J. and Sheehy J.E. 1983a. A new technique for the measurement of respiratory costs of symbiotic nitrogen fixation. Pp. 201–217 in 'Temperate legumes: physiology, genetics and nodulation', ed. by P.G. Jones and D.R. Davies. Pitman Books: London.

— 1983b. A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. Journal of Experimental Botany 34, 641–649.

Morgan R. 1986. Soil erosion and conservation. Longman Scientific and Technical: London.

Mulvaney R.L. 1993. Mass spectrometry. Pp. 11–58 in 'Nitrogen isotope techniques', ed. by R. Knowles and T.H. Blackburn. Academic Press: San Diego.

Murphy D., Fillery I. and Sparling G. 1997. Method to label soil cores with $^{15}NH_3$ gas as a prerequisite for ^{15}N isotopic dilution and measurement of gross N mineralization. Soil Biology and Biochemistry 29, 1731–1741.

Neves M., Didonet A., Duque F. and Dobereiner J. 1985. Rhizobium strain effects on nitrogen transport and distribution in soybeans. Journal of Experimental Botany 36, 1179–1192.

Nguluu S., Probert M., McCown R., Myers R. and Waring S. 2001. Isotopic discrimination associated with symbiotic nitrogen fixation in stylo (*Stylosanthes hamata* L.) and cowpea (*Vigna unguiculata* L.). Nutrient Cycling in Agroecosystems 62, 11–14.

Nohara T., Nakayama N., Takahashi M., Maruyama S., Shimada S. and Arihara J. 2005. Cultivar differences in dependence on nitrogen fixation of soybeans in the field with a high soil nitrate level determined by the relative ureide abundance method. Japanese Journal of Crop Science 74, 316–324.

Nohrstedt H. 1983. Natural formation of ethylene in forest soils and methods to correct results given by the acetylene reduction assay. Soil Biology and Biochemistry 15, 281–286.

Norhayati M., Mohd Noor S., Chong K., Faizah A.W., Herridge D.F., Peoples M.B. and Bergersen F.J. 1988. Adaptation of methods for evaluating N_2 fixation in food legumes and legume cover crops. Plant and Soil 108, 143–150.

Ohtake N., Nishiwaki T., Mizukoshi K., Takahashi Y., Chinushi T. and Ohyama T. 1995. Amino acid composition in xylem sap of soybean related to the evaluation of N_2 fixation by the relative ureide method. Soil Science and Plant Nutrition 41, 95–102.

Ohyama T. and Kumazawa K. 1981. A simple method for the preparation, purification and storage of $^{15}N_2$ gas for biological nitrogen fixation studies. Soil Science and Plant Nutrition 27, 263–265.

Ojiem J., Vanlauwe B., de Ridder N. and Giller K.E. 2007. Niche-based assessment of contributions of legumes to the nitrogen economy of Western Kenya smallholder farms. Plant and Soil 292, 119–135.

Okito A., Alves B., Urquiaga S. and R B. 2004. Isotope fractionation during N_2 fixation by four tropical legumes. Soil Biology and Biochemistry 36, 1179–1190.

Oliveira E. 1992. Estudo da associa¢io entre bacterias diazotroficas e arroz. MSc Thesis, Univ. Fed. Rural Rio de Janeiro.

Osborne B.G., Fearn T. and Hindle P.H. 1993. Practical NIR spectroscopy with applications in food and beverage analysis. Longman Scientific and Technical: Harlow, UK.

Osborne S. and Riedell W. 2006. Soybean growth response to low rates of nitrogen at planting in the northern great plains. Journal of Plant Nutrition and Soil Science 29, 985–1002.

Ovalle C., Longeri L., Aronson J., Herrera A. and Avendano J. 1996. N_2-fixation, nodule efficiency and biomass accumulation after two years in three Chilean legume trees and tagasaste *Chamaecytisus proliferus* subsp. *palmensis*. Plant and Soil 179, 131–140.

Pate J.S. 1980. Transport and partitioning of nitrogenous solutes. Annual Review of Plant Physiology 31, 312–340.

Pate J.S., Atkins C., White S., Rainbird R. and Woo K. 1980. Nitrogen nutrition and xylem transport of nitrogen in ureide-producing grain legumes. Plant Physiology 65, 961–965.

Pate J.S. and Unkovich M.J. 1999. Measuring symbiotic nitrogen fixation—case studies of natural and agricultural ecosystems in a Western Australian setting. Pp. 153–173 in 'Advances in physiological plant ecology', ed. by M.C. Press, J.D. Scholes and M.G. Barker. Blackwell Scientific: Oxford.

Pate J.S., Unkovich M.J., Armstrong E.L. and Sanford P. 1994. Selection of reference plants for ^{15}N natural abundance assessment of N_2 fixation by crop and pasture legumes in southwest Australia. Australian Journal of Agricultural Research 45, 133–147.

Patriquin D.G. and Keddy C. 1978. Nitrogenase activity (acetylene reduction) in a Nova Scotian salt marsh: its association with angiosperms and the influence of some edaphic factors. Aquatic Botany 4, 227–244.

Patterson T.G. and LaRue T.A. 1983. N_2 fixation (C_2H_2) and ureide contents of soybean: ureides as an index of fixation. Crop Science 23, 825–831.

Peoples M.B., Bell M. and Bushby V. 1992. Effect of rotation and inoculation with *Bradyrhizobium* on nitrogen fixation and yield of peanut (*Arachis hypogaea* L. cv Virginia Bunch). Australian Journal of Agricultural Research 43, 595–607.

Peoples M.B., Bergersen F.J., Turner G.L., Sampet C., Rerkasem B., Bhromsiri A., Nurhayati D.P., Faizah A.W., Sudin M.N., Norhayati M. and Herridge D.F. 1991. Use of the natural enrichment of ^{15}N in plant available soil N for the measurement of symbiotic N_2 fixation. Pp. 117–128 in 'Stable isotopes in plant nutrition, soil fertility and environmental studies'. International Atomic Energy Agency: Vienna.

Peoples M.B., Boddey R.M. and Herridge D.F. 2002. Quantification of nitrogen fixation. Pp. 357–389 in 'Nitrogen fixation at the millenium', ed. by G.J. Leigh. Elsevier Science: Amsterdam.

Peoples M.B., Bowman A., Gault R., Herridge D., McCallum M., McCormick K., Norton R., Rochester I., Scammell G. and Schwenke G. 2001. Factors regulating the contributions of fixed nitrogen by pasture and crop legumes to different farming systems of eastern Australia. Plant and Soil 228, 29–41.

Peoples M.B., Faizah A.W., Rerkasem B. and Herridge D.F. 1989b. Methods for evaluating biological nitrogen fixation by nodulated legumes in the field. Australian Centre for International Agricultural Research: Canberra.

Peoples M.B., Hebb D.M., Gibson A.H. and Herridge D.F. 1989a. Development of the xylem ureide assay for the measurement of nitrogen fixation by pigeonpea (*Cajanus cajan* [L.] Millsp.). Journal of Experimental Botany 40, 535–542.

Peoples M.B., Herridge D.F. and Ladha J.K. 1995b. Biological nitrogen fixation: an efficient source of nitrogen for sustainable agricultural production? Plant and Soil 174, 3–28.

Peoples M.B., Ladha J.K. and Herridge D.F. 1995a. Enhancing legume N_2 fixation through plant and soil management. Plant and Soil 174, 83–101.

Peoples M.B., Palmer B., Lilley D., Duc L. and Herridge D. 1996. Application of ^{15}N and xylem ureide methods for assessing N_2 fixation of three shrub legumes periodically pruned for forage. Plant and Soil 182, 125–137.

Peoples M.B., Pate J.S., Atkins C.A. and Bergersen F.J. 1986. Nitrogen nutrition and xylem sap composition of peanut (*Arachis hypogaea* L. cv Virginia Bunch). Plant Physiology 82, 946–951.

Peoples M.B., Sudin M.N. and Herridge D.F. 1987. Translocation of nitrogenous compounds in symbiotic and nitrate-fed amide-exporting legumes. Journal of Experimental Botany 38, 567–579.

Peoples M.B., Turner G.L., Shah Z., Aslam M., Ali S., Maskey S., Bhattari S., Afandi F., Schwenke G. and Herridge D. 1997. Evaluation of the ^{15}N natural abundance technique for measuring N_2 fixation in experimental plots and farmers' fields. Pp. 57–75 in 'Extending nitrogen fixation research to farmers fields', ed. by O.P. Rupella, C. Johansen and D.F. Herridge. ICRISAT: Patancheru, India.

Preston C. 1993. Optical emission spectrometry. Pp. 59–87 in 'Nitrogen isotope techniques', ed. by R. Knowles and T.H. Blackburn. Academic Press: San Diego, California.

Purcell L., Serraj R., Sinclair T. and De A. 2004. Soybean N_2 fixation estimates, ureide concentrations, and yield responses to drought. Crop Science 44, 484–492.

Rainbird R.M., Atkins C.A. and Pate J.S. 1983. Effect of temperature on nitrogenase functioning in cowpea nodules. Plant Physiology 73, 392–394.

Ramos M., Parsons R. and Sprent J. 2005. Differences in ureide and amino acid content of water stressed soybean inoculated with *Bradyrhizobium japonicum* and *B. elkanii*. Pesquisa Agropecuaria Brasileira 40, 453–458.

Reis V.M., dos Reis Jr F., B., Quesada D.M., de Oliveira O.C.A., Alves B.J.R., Urquiaga S. and Boddey R.M. 2001. Biological nitrogen fixation associated with tropical pasture grasses. Functional Plant Biology 28, 837–844.

Reiter K., Schmidtke K. and Rauber R. 2002. Estimation of symbiotic N_2 fixation by a low-level, large scale ^{15}N application technique. Soil Biology and Biochemistry 34, 303–314.

Rerkasem B., Rekasem K., Peoples M., Herridge D. and Bergersen F. 1988. Measurement of N_2 fixation in maize (*Zea mays* L.)–ricebean (*Vigna umbellate* [Thumb.] Ohwi and Ohashi) intercrops. Plant and Soil 108, 125–135.

Riffkin P., Cameron F., Kearney G., Quigley P., Gault R., Peoples M. and Thies J. 1999. Factors associated with biological nitrogen fixation in dairy pastures in south-western Victoria. Australian Journal of Agricultural Research 50, 261–272.

Rochester I., Peoples M., Constable G.A. and Gault R. 1998. Faba beans and other legumes add nitrogen to irrigated cotton cropping systems. Australian Journal of Experimental Agriculture 38, 253–260.

Rowe E. and Cadisch G. 2002. Implications on heterogeneity on procedures for estimating plant ^{15}N recovery in hedgerow intercrop systems. Agroforestry Systems 54, 61–70.

Ruschel A.P., Henis Y. and Salati E. 1975. Nitrogen-15 tracing of N-fixation with soil-grown sugar cane seedlings. Soil Biology and Biochemistry 7, 181–182.

Ruschel A., Vose P., Victoria R. and Salati E. 1979. Comparison of isotope techniques and non-nodulating isolines to study the effect of ammonium fertilization on dinitrogen fixation in soybean, *Glycine max*. Plant and Soil 53, 513–525.

Russell C.A. and Fillery I.R.P. 1996. Estimates of lupin below-ground biomass N, dry matter and N turnover to wheat. Australian Journal of Agricultural Research 47, 1047–1059.

Sanchez C., Blackmer A., Horton R. and Timmons D. 1987. Assessment of errors associated with plot size and lateral movement of nitrogen-15 when studying fertilizer recovery under field conditions. Soil Science 144, 344–351.

Santos V., Neves M. and Rumjanek N. 1997. Differential symbiotic efficiency by shading of soybean nodulated by *B. japonicum* and *B. elkanii* strains. Soil Biology and Biochemistry 29, 1015–1018.

Schjoerring J.K. 1998. Atmospheric ammonia and impacts of nitrogen deposition: uncertainties and challenges. New Phytologist 139, 59–60.

Schulze J., Temple G., Temple S.J., Beschow H. and Vance C.P. 2006. Nitrogen fixation by white lupin under phosphorus deficiency. Annals of Botany 98, 731–740.

Schwenke G., Peoples G., Turner G. and Herridge D. 1998. Does nitrogen fixation of commercial dryland chickpea and faba bean crops in north-west New South Wales maintain or enhance soil nitrogen? Australian Journal of Experimental Agriculture 38, 61–70.

Shearer G. and Kohl D.H. 1986. N_2 fixation in field settings: estimates based on natural ^{15}N abundance. Australian Journal of Plant Physiology 13, 699–756.

Shearer G., Kohl D. and Harper J.E. 1980. Distribution of ^{15}N among plant parts of nodulating and non-nodulating isolines of soybeans. Plant Physiology 66, 57–60.

Shearer G., Kohl D.H., Virginia R.A., Bryan B.A., Skeeters J.L., Nilsen E.T., Sharifi M.R. and Rundel P.W. 1983. Estimates of N_2-fixation from variation in the natural abundance of ^{15}N in Sonoran desert ecosystem. Oecologia 56, 365–373.

Shrestha R.K. and Ladha J.K. 1996. Genotypic variation in promotion of rice dinitrogen fixation as determined by nitrogen-15 dilution. Soil Science Society of America, Journal 60, 1815–1821.

Shutsrirung A., Sutigoolabud P., Santasups C., Senoo K., Tajima S., Hisamatsu M. and Bhromsiri A. 2002. Symbiotic efficiency and compatibility of native rhizobia in northern Thailand with different soybean cultivars. Soil Science and Plant Nutrition 48, 491–499.

Sinclair T., Purcell L., King C., Sneller C., Chen P. and Vadez V. 2006. Drought tolerance and yield increase of soybean resulting from improved symbiotic N_2 fixation. Field Crops Research 101, 68–71.

Smil V. 1999. Nitrogen in crop production: an account of global flows. Global Biogeochemical Cycles 13, 647–662.

Smith C.J., Chalk P.M., Hamilton S.D. and Hopmans P. 1992. Estimating N_2 fixation by field-grown lupins (*Lupinus angustifolius* L.) using soil and plant ^{15}N enrichment. Biology and Fertility of Soils 13, 235–241.

Smith C.J., Chalk P.M., Noble C.L., Prendergast J.B. and Robertson F. 1993. Nitrogen fixation in a white clover-grass pasture irrigated with saline groundwater. Irrigation Science 13, 189–194.

Smith C.J., Whitfield D. and Gyles O. 1989. Estimation of available N status of soil by wheat and barley: A-values. Soil Biology and Biochemistry 21, 699–756.

Song L., Carroll B.J., Gresshoff P.M. and Herridge D.F. 1995. Field assessment of supernodulating genotypes of soybean for yield, N_2 fixation and benefit to subsequent crops. Soil Biology and Biochemistry 27, 563–569.

Spiff E. and Odu C. 1973. Acetylene reduction by *Beijerinkia* under various partial pressures of oxygen and acetylene. Journal of General Microbiology 78, 207–209.

Sprent J. 2001. Nodulation in legumes. Royal Botanic Gardens: Kew, UK.

Steele K.W., Bonish P.M., Daniel R.M. and O'Hara G.W. 1983. Effect of Rhizobial strains and host plant on nitrogen isotopic fractionation in legumes. Plant Physiology 7, 11001–11004.

Stevenson F., Knight J. and van Kessel C. 1995. Dinitrogen fixation in pea: controls at the landscape- and micro-scale. Soil Science Society of America, Journal 59, 1603–1611.

Stewart G.R. 2001. What do $d^{15}N$ signatures tell us about nitrogen relations in natural ecosystems. Pp. 91–101 in 'Application of stable isotope techniques to study biological processes and functioning of ecosystems', ed. by M.J. Unkovich, J.S. Pate, A.M. McNeill and D.J. Gibbs. Kluwer Academic: Dordrecht.

Swan A., Peoples M., Gault R., Crews T., Khan D.F., Urquiaga S., Alves B., Boddey R.M. and Forrester R. 2003. Evaluating sources of error when quantifying N_2 fixation in commercial legume crops. Pp. 1–4 in '11th Australian Agronomy Conference, Geelong'. Australian Society of Agronomy.

Sylvester-Bradley R., Ayarza M., Mendez J. and Moriones R. 1983. Use of undisturbed soil cores for evaluation of Rhizobium strains and methods for inoculation of tropical forage legumes in a Colombian Oxisol. Plant and Soil 74, 237–247.

Tajima S. and Yamamoto Y. 1975. Enzymes of purine catabolism in soybean plants. Plant Cell Physiology 16, 271–282.

Takahashi Y., Chinushi T., Nakano T. and Ohyama T. 1992. Evaluation of N_2 fixation and N absorption activity by relative ureide method in field-grown soybean plants with deep placement of coated urea. Soil Science and Plant Nutrition 30, 699–708.

Tarrand J.J., Krieg N.R. and Dobereiner J. 1978. A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense* sp. nov. Canadian Journal of Microbiology 24, 967–980.

Tewari K., Suganuma T., Fujikake H., Ohtake N., Sueyoshi K., Takahashi Y. and Ohyama T. 2004. Effect of deep placement of N fertilisers and different inoculation methods of Bradyrhizobia on growth, N_2 fixation activity and N absorption rate of field grown soybean plants. Journal of Agronomy and Crop Science 190, 46–58.

Tough H. and Crush J. 1979. Effect of grade of acetylene on ethylene production by white clover (*Trifolium repens* L), during acetylene assays on nitrogen fixation. New Zealand Journal of Agricultural Research 22, 581–583.

Turner G.L., Bergersen F.J. and Tantala H. 1983. Natural enrichment of ^{15}N during decomposition of plant material in soil. Soil Biology and Biochemistry 15, 495–497.

Turner G.L., Gault R.R., Morthorpe L., Chase D.L. and Bergersen G.L. 1987. Differences in the natural abundance of ^{15}N in the extractable mineral N of croppped and fallowed surface soils. Australian Journal of Agricultural Research 38, 15–26.

Turner G.L. and Gibson A.H. 1980. Measurement of nitrogen fixation by indirect means. Pp. 111–138 in 'Methods for evaluating biological nitrogen fixation', ed. by F.J. Bergersen. John Wiley & Sons Ltd: Chichester, UK.

Turpin J.E., Herridge D.F. and M.J. R. 2002. Nitrogen fixation and soil nitrate interactions in field-grown chickpea and fababean. Australian Journal of Soil Research 53, 599–608.

Unkovich M.J. and Pate J. 2000. An appraisal of recent field measurements of symbiotic N_2 fixation by annual legumes. Field Crops Research 211, 211–228.

Unkovich M.J. and Pate J.S. 2001. Assessing N_2 fixation in annual legumes using ^{15}N natural abundance. Pp. 103–118 in 'Application of stable isotope techniques to study biological processes and functioning of ecosystems', ed. by M.J. Unkovich, J.S. Pate, A.M. McNeill and D.J. Gibbs. Kluwer Academic: Dordrecht.

Unkovich M.J., Pate J.S. and Hamblin M.J. 1994a. The nitrogen economy of broadacre lupin in southwest Australia. Australian Journal of Agricultural Research 45, 149–164.

Unkovich M.J., Pate J., Lefroy E. and Arthur D. 2000. Nitrogen isotope fractionation in the fodder tree legume tagasaste (*Chamecytisus proliferus*) and assessment of N_2 fixation inputs in deep sandy soils of Western Australia. Australian Journal of Plant Physiology 27, 921–929.

Unkovich M.J., Pate J.S. and Sanford P. 1993. Preparation of plant samples for high precision nitrogen isotope ratio analysis. Communications in Soil Science and Plant Analysis 24, 2093–2106.

Unkovich M.J., Pate J.S., Sanford P. and Armstrong E.L. 1994b. Potential precision of the $d^{15}N$ natural abundance method in field estimates of nitrogen fixation by crop and pasture legumes in S.W. Australia. Australian Journal of Agricultural Research 45, 119–132.

Unkovich M.J., Sanford P., Pate J.S. and Hyder M. 1998. Effects of grazing on plant and soil nitrogen relations of pasture–crop rotations. Australian Journal of Agricultural Research 49, 475–485.

Urquiaga S., Cruz K.H.S. and Boddey R.M. 1992. Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen balance estimates. Soil Science Society of America, Journal 56, 105–114.

Vadez V. and Sinclair T. 2001. Leaf ureide degradation and N_2 fixation tolerance to water deficit in soybean. Journal of Experimental Botany 52, 153–159.

Vallis I. 1973. Sampling for soil nitrogen changes in large areas of grazed pasture. Communications in Soil Science and Plant Analysis 4, 163–170.

van Berkum P. and Day J. 1980. Nitrogenase activity associated with soil cores of grasses in Brazil. Soil Biology and Biochemistry 12, 137–140.

van Kessel C., Farrell R., Roskoski J. and Keane K. 1994. Recycling of the naturally-occurring ^{15}N in an established stand of *Leucaena leucocephala*. Soil Biology and Biochemistry 26, 757–762.

van Kessel C., Roskoski J.P., Wood T. and Montano J. 1983. $^{15}N_2$ fixation and H_2 evolution by six species of tropical leguminous trees. Plant Physiology 72, 909–910.

Vessey J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255, 571.

Viera-Vargas M., De Oliveira O., Souto C., Cadisch G., Urquiaga S. and Boddey R. 1995b. Use of different ^{15}N labelling techniques to quantify the contribution of biological nitrogen fixation to legumes. Soil Biology and Biochemistry 27, 1185–1192.

Viera-Vargas M., Souto C., Urquiaga S. and Boddey R. 1995a. Quantification of the contribution of N_2 fixation to tropical forage legumes and transfer to associated grass. Soil Biology and Biochemistry 27, 1193–1200.

Vikman P. and Vessey J.K. 1992. The decline in N_2 fixation rate in common bean with the onset of pod-filling: fact or artefact. Plant and Soil 147, 95–105.

Vincent J.M. 1970. A manual for the practical study of root-nodule bacteria. Blackwell Scientific: Oxford, UK.

Vose P., Ruschel A., Victoria R., Saito S. and Matsui E. 1982. ^{15}N as a tool in biological nitrogen fixation research. Pp. 575–592 in 'Biological nitrogen fixation technology for tropical agriculture', ed. by P.H. Graham and S.C. Harris. CIAT: Cali, Colombia.

Wang G., Peoples M.B., Herridge D.F. and Rerkasem B. 1993. Nitrogen fixation, growth and yield of soybean grown under saturated soil culture and conventional irrigation. Field Crops Research 32, 257–268.

Wani S., Dart P. and Upadhyaya M. 1983. Factors affecting nitrogenase activity (C_2H_2 reduction) associated with sorghum and millet estimated using the soil core assay. Canadian Journal of Microbiology 29, 1063–1069.

Wani S.P. 1986. Cereal nitrogen fixation. Proceedings of a working group meeting 9–12 October 1984, ICRISAT, Patancheru, India.

Warembourg F.R. 1993. Nitrogen fixation in soil and plant systems. Pp. 127–156 in 'Nitrogen isotope techniques', ed. by R. Knowles and T.H. Blackburn. Academic Press Inc.: San Diego.

Warembourg F.R., Montange D. and Bardin R. 1982. The simultaneous use of $^{14}CO_2$ and $^{15}N_2$ labelling techniques to study the carbon and nitrogen economy of legumes grown under natural conditions. Physiologia Plantarum 56, 46–55.

Watanabe I., Chiu C. and Yoshida T. 1990. Estimation of N_2 fixation in soybean and cowpea by using soil residual ^{15}N. Soil Science and Plant Nutrition 36, 375–381.

Watanabe I., Lee K. and Alimango B. 1978. Seasonal change of N_2-fixing rate in rice field assayed by in situ acetylene reduction assay technique I. Experiments in long term fertility plots. Soil Science and Plant Nutrition 24, 1–13.

Watanabe I., Yoneyama T., Padre B. and Ladha J.K. 1987. Difference in natural abundance of ^{15}N in several rice (*Oryza sativa* L.) varieties: application for evaluating N_2 fixation. Soil Science and Plant Nutrition 33, 407–415.

Westfall D., Henson M. and Evans E. 1978. The effect of soil sample handling between collection and drying on nitrate concentration. Communications in Soil Science and Plant Analysis 9, 169–185.

Wetselaar R., Jakobsen P. and Chaplin G. 1973. Nitrogen balance in crop systems in tropical Australia. Soil Biology and Biochemistry 5, 35–40.

Williams P.C., Norris K.H., Gehrke C.W. and Bernstein K. 1983. Comparison of near-infrared methods for measuring protein and moisture in wheat. Cereal Foods World 150, 149–152.

Witty J. 1979. Algal nitrogen fixation on temperate arable fields; the broadbalk experiments. Plant and Soil 52, 151–164.

Witty J.F. 1983. Estimating N_2-fixation in the field using ^{15}N-labelled fertilizer: some problems and solutions. Soil Biology and Biochemistry 15, 631–639.

Witty J.F. and Day J.M. 1979. Use of $^{15}N_2$ in evaluating asymbiotic N_2 fixation. Pp. 135 in 'Isotopes in biological nitrogen fixation'. International Atomic Energy Agency: Vienna.

Witty J.F. and Minchin F.R. 1988. Measurement of nitrogen fixation by the acetylene reduction assay; myths and mysteries. Pp. 331–343 in 'Nitrogen fixation by legumes in Mediterranean agriculture', ed. by D.P. Beck and L.A. Materon. Martinus Nijhoff: Dordrecht.

— 1998. Hydrogen measurements provide direct evidence for a variable physical barrier to gas diffusion in legume nodules. Journal of Experimental Botany 49, 1015–1020.

Yamakawa T. and Ishizuka J. 2002. Effect of nodulation with *Bradyrhizobium japonicum* and *Shinorhizobium fredii* on xylem sap composition of Peking (*Glycine max* L. Merr.). Soil Science and Plant Nutrition 48, 521–527.

Yemm E.W. and Cocking E.C. 1955. The determination of amino acids with ninhydrin. Analyst 80, 209–213.

Ying J., Herridge D.F., Peoples M.B. and Rerkasem B. 1992. Effects of N fertilization on N_2 fixation and N balances of soybean grown after lowland rice. Plant and Soil 147, 235–242.

Yoneyama T., Fujita K., Toshida T., Matsumoto T., Kambayashi I. and Yazaki J. 1986. Variation in natural abundance of ^{15}N among plant parts and in $^{15}N/^{14}N$ fractionation during N_2 fixation in the legume–rhizobia symbiotic system. Plant Cell Physiology 27, 791–799.

Yoneyama T., Muraoka T., Kim T.H., Dacanay E.V. and Nakanishi Y. 1997. The natural ^{15}N abundance of sugarcane and neighbouring plants in Brazil, the Philippines and Miyako (Japan). Plant and Soil 189, 239–244.

Yoshida T. and Yoneyama T. 1980. Atmospheric dinitrogen fixation in the flooded rice rhizosphere as determined by the N-15 isotope technique. Soil Science and Plant Nutrition 26, 551–559.

Young E.G. and Conway C.F. 1942. On the estimation of allantoin by the Rimini-Schryver reaction. Journal of Biological Chemistry 142, 839–853.

Appendixes

Appendix 1. Nutrient solutions for glasshouse culture of legumes

McKnight's solution (N-free complete nutrients)		
Preparation of individual stock solutions (each in a 1 L flask)		
1.	Calcium chloride ($CaCl_2$)	add 20 g/L distilled H_2O
2.	Magnesium sulfate ($MgSO_4.7H_2O$)	add 400 g/L distilled H_2O
3.	Potassium dihydrogen orthophosphate (KH_2PO_4)	add 100 g/L distilled H_2O
4.	Potassium chloride (KCl)	add 150 g/L distilled H_2O
5.	Trace elements stock solution: add to a 1 L flask:	
	boric acid (H_3BO_3)	2.86 g
	manganese sulfate ($MnSO_4.4H_2O$)	2.03 g
	zinc sulfate ($ZnSO_4.7H_2O$)	0.222 g
	copper sulfate ($CuSO_4.5H_2O$)	0.079 g
	molybdic acid ($H_2MoO_4.H_2O$)	0.09 g
	Then add 1 L of distilled H_2O and mix.	
6.	EDTA stock solution: add to a 1 L flask:	
	ethylene diamine tetra-acetic, sodium salt (EDTA)	2 g
	60% wt/vol ferric chloride solution ($FeCl_3$)	16.8 mL
	Make up to 1 L with distilled water.	
7.	1N sodium hydroxide. (NaOH): add to a 1L flask:	
	sodium hydroxide (NaOH)	40 g
	Make up to 1L with distilled water.	

Preparation of McKnight's solution for glasshouse plants		
Add the following aliquots of each of the stock solutions prepared above into a large (150–200 L) container:		
1.	Calcium chloride	25 mL
2.	Magnesium sulfate	25 mL
3.	Potassium dihydrogen orthophosphate	100 mL
4.	Potassium chloride	100 mL

Appendix 1. (continued)

Preparation of McKnight's solution for glasshouse plants		
5.	Trace elements stock solution	50 mL
6.	EDTA–$FeCl_3$ stock solution	75 mL
7.	Sodium hydroxide	25 mL
Make up to 100 L with tap water (final pH should be 6.6).		
The nutrient solution is now ready to be supplied to glasshouse-grown plants.		

Appendix 2. Examples of 'B' values for shoots of temperate crop legumes

Legume	Shoot $\delta^{15}N$ (‰)		Location and source of information
	Range[a]	Mean[b]	
Chickpea	−1.65 to −1.50	−1.58	Australia – Figure 27, Herridge et al. (1995)
	−1.34	−1.34	Australia – Unkovich and Pate (2000)
	−2.10	−2.10	Australia – Doughton et al. (1992)
	−2.82 to −1.31	−2.00	Canada – Kyei-Boahen et al. (2002)
		−1.75	
Faba bean	−0.32 to −0.40	−0.36	Australia – Figure 27, Peoples (unpublished data)
	−0.63	−0.63	France – Mariotti et al. (1980)
		−0.50	
Field pea	−0.74 to −0.41	−0.60	Australia – Peoples (unpublished data)
	−0.68 to −0.20	−0.33	Australia – Unkovich et al. (1994b, unpublished data)
	−1.00	−1.00	France – Mariotti et al. (1980)
	−0.7	−0.7	Denmark – Holdensen et al. (2007)
		−0.66	
Grasspea (lathyrus)	−0.11	**−0.11**	Australia – Unkovich (unpublished data)
Lentil	−0.60	−0.60	Australia – Peoples (unpublished data)
	−0.51	−0.51	Australia – Unkovich and Pate (2000)
		−0.56	
Lupin	−0.70 to −0.20	−0.44	Australia – Unkovich et al. (1994b, unpublished data)
	−1.16 to +0.10	−0.53	Australia/France – Bergersen et al. (1986)
	−0.55	−0.55	Australia – Evans et al. (1987)
	−0.77	−0.77	France – Mariotti et al. (1980)
		−0.57	
Narbon bean	−0.25	**−0.25**	Australia – Unkovich (unpublished data)

[a] Further explanation of treatments imposed to generate the range of shoot 'B' values for some of the data summarised here is provided in Table 16.

[b] These values represent the mean where more than one rhizobial strain or variety was tested, or where the data are derived from several experiments undertaken by the same laboratory. Mean values in bold are the means for the particular species.

Appendix 3. Examples of 'B' values for shoots of subtropical and tropical crop legumes

Legume	Shoot $\delta^{15}N$ (‰)		Location and source of information
	Range[a]	Mean[b]	
Adzuki bean	−0.91	**−0.91**	Australia – Peoples (unpublished data)
Black gram	−1.82 to −1.67	**−1.75**	Australia – Peoples (unpublished data)
Common bean	−2.00	−2.00	Australia – Peoples (unpublished data)
	−3.2 to −1.8	−2.50	Japan – Yoneyama et al. (1986)
	−1.97	−1.97	France – Mariotti et al. (1980)
		−2.16	
Cowpea	−1.66	−1.66	Australia – Peoples (unpublished data)
	−1.50	−1.50	Australia – Nguluu et al. (2001)
		−1.61	
Green gram	−2.20 to −1.14	−1.60	Australia – Peoples (unpublished data)
	−2.5	−2.50	Pakistan/Nepal – Peoples et al. (1997)
		−2.05	
Groundnut (peanut)	−2.50 to −1.40	−1.95	Indonesia – Cadisch et al. (2000)
	0.65 to 0.70	+0.67	Australia – Peoples et al. (1992, unpublished data)
	−1.70 to −1.00	−1.35	Brazil – Okito et al. (2004)
		−0.88	
Pigeon pea	−1.45 to −0.90	−1.18	Australia – Peoples (unpublished data)
	−1.06	−1.06	Kenya – Gathumbi et al. (2002)
		−1.12	
Rice bean	−0.91	**−0.91**	Thailand – Rerkasem et al. (1988)

Appendix 3. (continued)

Legume	Shoot $\delta^{15}N$ (‰)		Location and source of information
	Range[a]	Mean[b]	
Soybean	−1.40 to −1.15	−1.30	Australia – Bergersen et al. (1989)
	−1.70 to −1.00	−1.40	Australia – Peoples (unpublished data)
	−2.06 to −1.00	−2.00	New Zealand – Steele et al. (1983)
	−1.50 to −0.90	−1.20	Thailand – Peoples (unpublished data)
	−1.54	−1.54	Japan – Yoneyama et al. (1986)
Soybean (continued)	−2.39 to −1.31	−1.83	Brazil – Okito et al. (2004); Guimarães et al. (2008)
	−4.54 to −3.49	−3.61	Brazil – Okito et al. (2004); Guimarães et al. (2008)
	−2.24 to −1.60	−1.99	Brazil – Guimarães et al. (2008)
	−2.50	−2.50	Pakistan/Nepal – Peoples et al. (1997)
	−1.2 to −1.9	−1.83	France – Mariotti et al (1980)
	−0.90	−0.90	USA – Shearer et al. (1980)
		−1.83	
Winged bean	−1.97 to −1.04	**−1.54**	Japan – Yoneyama et al. (1986)

[a] Further explanation of treatments imposed to generate the range of shoot 'B' values for some of the data summarised here is provided in Table 16.

[b] These values represent the mean where more than one rhizobial strain or variety was tested, or where the data are derived from several experiments undertaken by the same laboratory. Mean values in bold are the means for the particular species.

Appendix 4. Examples of 'B' values for shoots of temperate forage legumes

Legume species	Shoot ^{15}N (‰)		Location and source of information
	Range[a]	Mean[b]	
Annuals			
Balansa clover	−0.55	**−0.55**	Australia – Peoples (unpublished data)
Berseem clover	−0.64	**−0.64**	Australia – Peoples (unpublished data)
Lotus	−1.20 to +0.8	**−0.12**	New Zealand – Steele et al. (1983)
Medic	−0.56 to −0.20	−0.38	Australia – Peoples (unpublished data)
	−0.38 to +0.02	−0.21	Australia – Unkovich and Pate (2001, unpublished data)
		−0.30	
Persian clover	−0.57 to −0.71	**−0.64**	Australia – Peoples (unpublished data)
Subterranean clover	−0.40 to −0.90	−0.67	Australia – Unkovich et al. (1994)
	−1.37 to +0.06	−0.58	Australia – Unkovich and Pate (2001)
	−0.88 to −0.60	−0.78	Australia – Peoples (unpublished data)
		−0.68	
Vetch	−0.59 to −0.94	−0.76	Australia – Unkovich (unpublished data)
	−0.47 to −0.94	−0.70	Australia – Peoples (unpublished data)
	−1.17 to −0.56	−0.90	Japan – Yoneyama et al. (1986)
		−0.79	
Perennials			
Alsike clover	−2.5 to −0.6	−1.20	Sweden – Carlsson et al. (2006)
	−1.0 to −0.67	−0.83	Japan – Yoneyama et al. (1986)
		−1.00	
Lucerne (alfalfa)	−1.24 to −0.44	−0.84	Australia – Peoples (unpublished data)
	0	0	New Zealand – Steele et al. (1983)
	−0.92	−0.92	France – Mariotti et al. (1980)
	−1.1 to −0.7	−0.95	Japan – Yoneyama et al. (1986)
		−0.68	

Appendix 4. (continued)

Legume species	Shoot ^{15}N (‰)		Location and source of information
	Range[a]	Mean[b]	
Red clover	−0.78	−0.78	Australia – Peoples (unpublished data)
	−0.88	−0.88	France – Mariotti et al. (1980)
	−2.6 to 0	−1.30	Sweden – Carlsson et al. (2006)
	−1.26 to −0.37	−0.82	Japan – Yoneyama et al. (1986)
		−0.94	
White clover	−3.93 to −1.48	−2.48	Australia – Riffkin et al. (1999)
	−0.92 to −0.64	−0.78	Australia – Peoples (unpublished data)
	−1.4	−1.40	Denmark – Eriksen and Høgh-Jensen (1998)
	−4.5 to −0.6	−1.70	Sweden – Carlsson et al. (2006)
	−2.6 to −1.4	−1.80	New Zealand – Steele et al. (1983)
	−1.21 to −0.22	−0.71	Japan – Yoneyama et al. (1986)
		−1.48	

[a] Further explanation of treatments imposed to generate the range of shoot 'B' values for some of the data summarised here is provided in Table 16.

[b] These values represent the mean where more than one rhizobial strain or variety was tested, or where the data are derived from several experiments undertaken by the same laboratory. Mean values in bold are the means for the particular species.

Appendix 5. Examples of 'B' values for shoots or foliage of tropical legume species used as forages or covercrops

Legume species	Shoot/foliage ^{15}N (‰)		Location and source of information
	Range	Mean[a]	
Forages			
Centrosema	−1.08	−1.08	Australia – Peoples (unpublished data)
	−2.23	−2.23	Japan – Yoneyama et al. (1986)
		−1.65	
Desmodium	−0.77	−0.77	Australia – Peoples (unpublished data)
	−1.19	−1.19	Kenya – Mwangi et al. (unpublished data)
	−1.45	−1.45	Japan – Yoneyama et al. (1986)
		−1.14	
Macrotyloma axillare	−1.83	**−1.83**	Kenya – Mwangi et al. (unpublished data)
Neonotonia wightii	−1.75	**−1.75**	Kenya – Mwangi et al. (unpublished data)
Siratro	−4.6 to −2.7	−3.43	New Zealand – Steele et al. (1983)
	−1.16	−1.16	Australia – Peoples (unpublished data)
	−2.47	−2.47	Japan – Yoneyama et al. (1986)
		−2.35	
Stylo	−0.74	−0.74	Australia – Peoples (unpublished data)
	−0.81	−0.81	Japan – Yoneyama et al. (1986)
	−1.86	−1.86	Australia – Nguluu et al. (2001)
		−1.14	

Appendix 5. (continued)

Legume species	Shoot/foliage ^{15}N (‰)		Location and source of information
	Range	Mean[a]	
Covercrops			
Astragalus (milk vetch)	−0.82	**−0.82**	Japan – Yoneyama et al. (1986)
Calopogonium	−0.95	**−0.95**	Malaysia – Peoples (unpublished data)
Crotalaria (sunnhemp)	−1.73 to −1.02	−1.37	Brazil – Okito et al. (2004)
	−0.57	−0.57	Kenya – Gathumbi et al. (2002)
	−1.31	−1.31	Philippines – Peoples (unpublished data)
		−1.08	
Lablab	−1.36	−1.36	Australia – Peoples (unpublished data)
	−0.82	−0.82	Kenya – Gathumbi et al. (2002)
		−1.09	
Velvet bean (Mucuna)	−2.04 to −1.48	**−1.82**	Brazil – Okito et al. (2004)
Pueraria	−1.22	**−1.22**	Malaysia – Peoples (unpublished data)

[a] These values represent the mean where more than one rhizobial strain or variety was tested, or where the data are derived from several experiments undertaken by the same laboratory. Mean values in bold are the means for the particular species.

Appendix 6. Examples of 'B' values for shoots or foliage of temperate or tropical legume shrubs or trees

Legume species	Shoot/foliage ^{15}N (‰)	Location and source of information
	Mean[a]	
Temperate		
Tagasaste	**–0.47**	Australia – Unkovich et al. (2000)
Tropical		
Acacia mangium	**–0.89**	Philippines – Mercado et al. (unpublished data)
Calliandra	–1.29	Australia – Peoples (unpublished data)
	–0.50	Kenya – Cadisch (unpublished data)
	–0.90	
Leucaena	**–0.34**	Australia – Peoples (unpublished data)
Flemingia congesta	**–1.32**	Indonesia – Hairiah et al. (unpublished data)
Gliricidia	–1.45	Australia – Peoples (unpublished data)
	–1.11	Indonesia – Hairiah et al. (unpublished data)
	–1.28	
Sesbania grandiflora	**–2.89**	Australia – Peoples (unpublished data)
Sesbania sesban	**–1.76**	Kenya – Gathumbi et al. (2002)

[a] Values in bold are the means for the particular species. No data are presented to indicate a range of values as for the most part only a single legume line – rhizobial association was examined in each study.

Appendix 7. Scientific and common names of plants referred to in the text

Legumes	
Acacia mangium	Black wattle, hickory wattle
Arachis hypogaea	Groundnut (peanut)
Astragalus spp.	Astragalus (milk vetch)
Cajanus cajan	Pigeon pea
Calliandra calothyrsus	Calliandra
Calopogonium caeruleum	Calopogonium
Centrosema pubescens	Centro
Centrosema spp.	Centrosema (butterfly pea)
Chamaecytisus palmensis	Tagasaste
Cicer arietinum	Chickpea
Codariocalyx gyroides	Codarrio
Crotalaria juncea	Crotalaria (sunnhemp)
Cyamopsis tetragonoloba	Guar
Desmodium ovalifolium	Desmodium
Desmodium rensonii	Tick trefoil
Flemingia congesta	Flemingia
Flemingia macrophylla	Apa apa
Galactia striata	Galactia
Gliricidia sepium	Gliricidia
Glycine max	Soybean
Lablab purpureus	Hyacinth bean (lablab)
Lathyrus sativus	Grasspea
Lens culinaris	Lentil
Leucaena spp.	Leucaena
Lotus spp.	Lotus
Lupinus angustifolius	Lupin

Appendix 7. (continued)

Macroptilium atropurpureum	Siratro
Macrotyloma axillare	Horse gram
Macrotyloma geocarpum	Kersting's groundnut
Medicago sativa	Alfalfa (lucerne)
Mucuna pruriens	Velvet bean (mucuna)
Neonotonia wightii	Soya perenne
Phaseolus acutifolius	Tepary bean
Phaseolus coccineus	Runner bean
Phaseolus lunatus	Lima bean
Phaseolus vulgaris	Common bean
Pisum sativum	Field pea
Prosopis juliflora	Mesquite
Psophocarpus tetragonolobus	Winged bean
Pueraria phaseoloides	Kudzu
Senna spectabilis	Cassia
Sesbania grandiflora	Sesban
Sesbania sesban	Egyptian pea
Stylosanthes guianensis	Stylo
Trifolium alexandrinum	Berseem clover
Trifolium balansa	Balansa clover
Trifolium hybridum	Alsike clover
Trifolium incarnatum	Crimson clover
Trifolium pratense	Red clover
Trifolium repens	White clover
Trifolium resputinatum	Persian clover
Trifolium subterraneum	Subterraneum clover
Vicia faba	Faba bean
Vicia sativa	Vetch

Appendix 7. (continued)

Vigna aconitifolia	Moth bean
Vigna angularis	Adzuki bean
Vigna mungo	Black gram
Vigna radiata	Mung bean (green gram)
Vigna subterranea	Bambara groundnut
Vigna umbellata	Rice bean
Vigna unguiculata	Cowpea
Non legumes	
Avena sativa	Oats
Brachiaria brizantha	Beard grass
Brachiaria arrecta	Tanner grass
Brassica napus	Canola
Euphorbia hirta	Asthma weed
Hordeum vulgare	Barley
Oryza sativa	Rice
Oxalis corniculata	Yellow wood sorrel
Panicum maximum	Guinea grass
Poa chinensis	Red sprangletop
Pennisetum purpureum	Elephant grass
Saccharum officinarum	Sugarcane
Senecio vulgaris	Common groundsel
Sorghum bicolor	Sorghum
Sorghum sudanense	Sudan grass
Triticum aestivum	Wheat
Zea mays	Maize

Appendix 8. Some suppliers of ^{15}N-labelled materials

There are many suppliers of stable isotope materials throughout the world and these can be found through an internet search. A few well-known suppliers are listed below.

China Nuclear Energy Industry Corporation (CNEIC)
Building No. 9, Huayuan Street
Xi Cheng District, Beijing 100032, China
Fax: Intl+ 86 10 66297165
Phone: Intl+ 86 10 66297 170
<www.cneic.com.cn>

Chemotrade
Brahestr. 27, D-04347 Leipzig, Germany
Fax: Intl+ 49 3412444922
Phone: Intl+ 49 341 24449 28
Email: <asp@chemotrade-leipzig.de>
<www.chemotrade-leipzig.de/>

Rashtriya Chemicals & Fertilizers Limited
Eastern Express Highway
Sion, Mumbai 400-022, India
Fax: Intl+ 91 22 4045111
Phone: Intl+ 91 22 4078175 or 22 8078176
<www.rcfltd.com/>

National Institute of Research & Development for Isotopic & Molecular Technology (INCDTIM)
PO Box 700, R-3400 Cluj-Napoca, Romania
Fax: Intl+ 40 2644 20042
Phone: Intl+ 40 264 584037
<www.itim-cj.ro/>

Isotec, a Division of Sigma-Aldrich
3858 Benner Rd, Miamisburg, OH 45342-4304
United States of America
Fax: Intl+ 1 9378594878
Phone: Intl+ 1 937 859 1808
<www.sigmaaldrich.com/Area_of_Interest/Chemistry/Stable_Isotopes__ISOTEC_.html>

Appendix 9. Some laboratories providing ^{15}N analyses

There are many laboratories providing stable isotope analysis throughout the world and these can be found through an internet search. A few well-known ones are listed below.

Iso-Analytical Limited
Millbuck Way
Sandbach, CW11 3HT, UK
Email: info@iso-analytical.com
Tel: Intl +44 (0)1270 766771
Fax: Intl +44 (0)1270 766709
<www.iso-analytical.co.uk/>

Isotech Laboratories, Inc.
1308 Parkland Court
Champaign, Illinois 61821 USA
Tel: Intl +1 877 362 4190
Fax: Intl +1 217 398 3493
<http://www.isotechlabs.com/index.html>

University of California Davis
Stable Isotope Facility
Department of Plant Sciences
One Shields Avenue, Mail Stop #1
Davis, California, 95616, USA
David Harris
Email: <dharris@ucdavis.edu>
Tel: Intl +1 530 754 7517
Fax: Intl +1 530 752 4361

Center for Stable Isotope Biogeochemistry (CSIB)
Integrative Biology
University of California, Berkeley
3060 VLSB,
Berkeley CA-94720 USA
Email: <stableisotopes@berkeley.edu>
Phone: Intl +1 510 643 1748
<http://ib.berkeley.edu/groups/biogeochemistry/>

Appendix 9. (continued)

Stable Isotope Research Facility for Environmental Research
Department of Biology
University of Utah
257 S 1400 E
Salt Lake City, Utah 84112 USA
Email: <sirfer@bioscience.utah.edu>
Tel: Intl +1 801 581 4654
Tel: Intl +1 801 581 4665
<http://ecophys.biology.utah.edu/sirfer.html>

Environmental Isotopes Pty Ltd
Riverside Corporate Park
11 Julius Ave, North Ryde NSW 2113, Australia
Email: <anita.andrew@isotopic.com.au>
Tel: Intl +61 2 9490 8743
Fax: Intl +61 2 9490 8740
<www.isotopic.com.au>

CSIRO Land and Water Isotope Analysis Service
Waite Road
Urrbrae SA 5064, Australia
Postal Address:
Private Bag No. 2
Glen Osmond SA 5064 Australia
Email: <Fred.Leaney@csiro.au>
Ph: Intl +61 8 8303 8728
Fax:Intl +61 8 8303 8750
<http://www.clw.csiro.au/services/isotope/>

Melbourne School of Land and Environment
University of Melbourne
Victoria, 3010 Australia
Email: <ron.teo@unimelb.edu.au>
Ph: Intl +61 3 8344 5028
Fax: Intl +61 3 8344 4665

Appendix 9. (continued)

Western Australian Biogeochemistry Centre
School of Plant Biology
Faculty of Natural & Agricultural Sciences
The University of Western Australia
35 Stirling Highway
Crawley WA 6907 Australia
Email: <pfgblue@plants.uwa.edu.au>
Ph: Intl +61 8 6488 7926

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