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

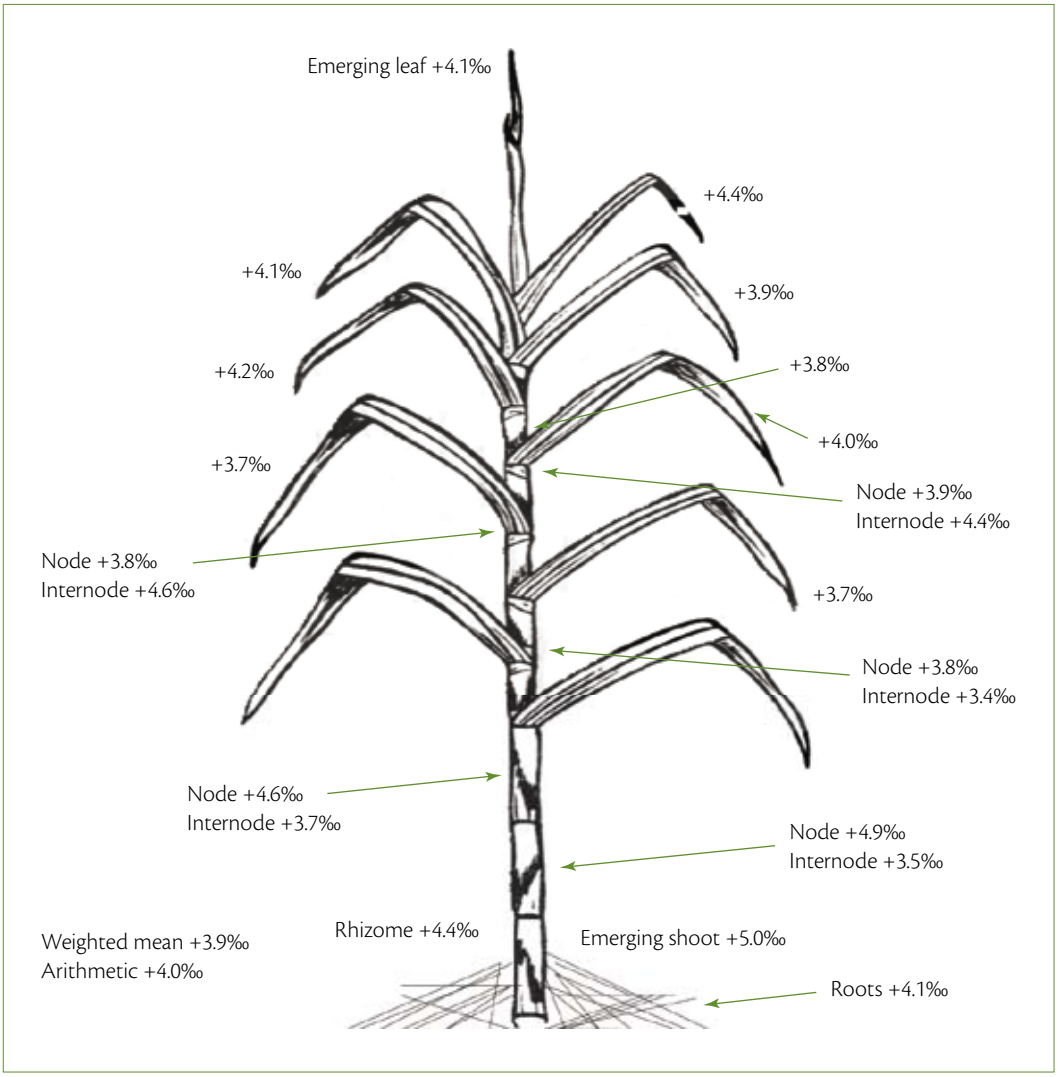

Figure 40. $\delta^{15}N$ natural abundance in leaves, stem (nodes and internodes), roots and emerging shoots of a sugarcane plant (cv. SP 70-1143) growing in the field in a sandy soil. Data are from Boddey et al. (2001).

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.

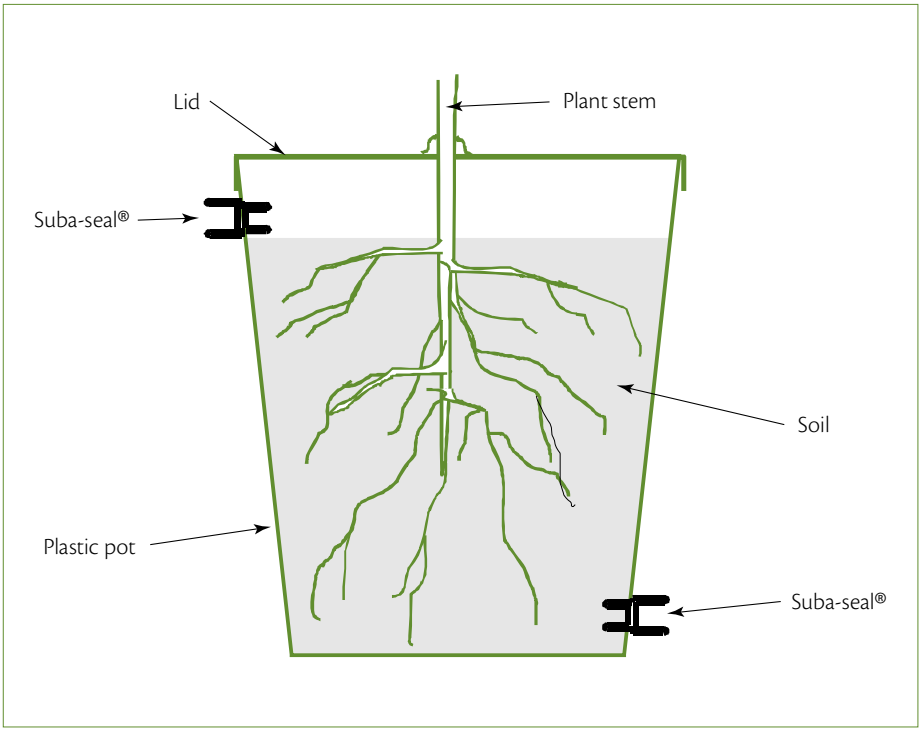

Figure 41. Incubation system for undisturbed acetylene-reduction assays of root systems of intact plants. A porous sand medium that allows easy diffusion of gases is preferred to loam or clay soils that present significant diffusion problems. Note the rubber ports for injection and withdrawal of acetylene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

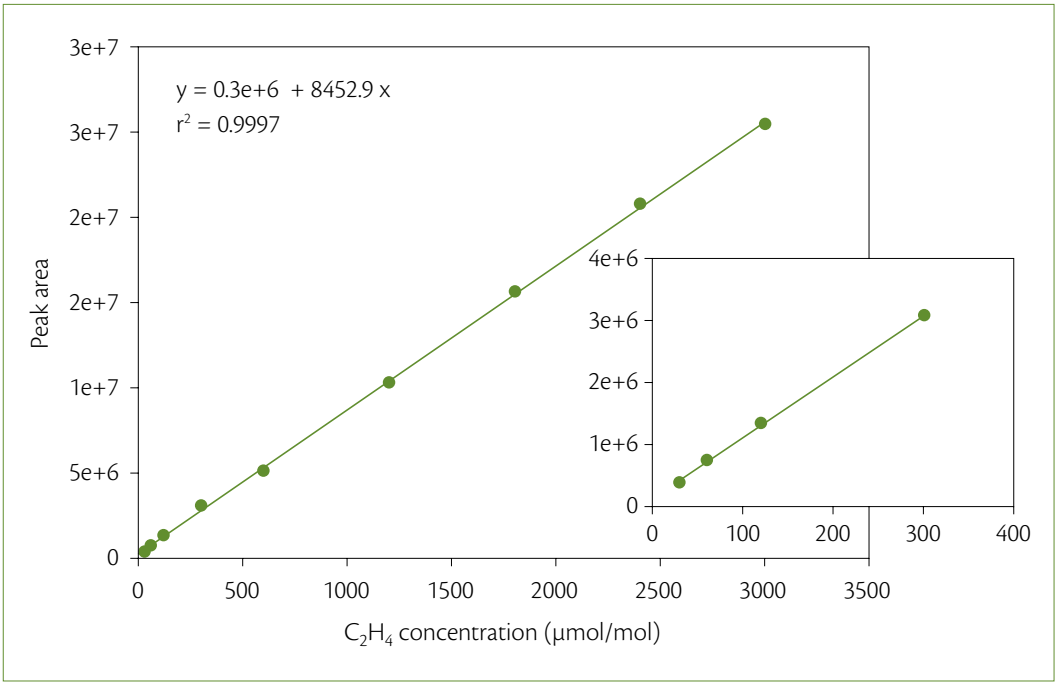

Figure 42. Relationship between peak area and ethylene standards (30–3,000 µmol/mol) from a Perkin Elmer chromatograph equipped with a flame ionisation detector and a 1.0 m Porapak N column. Temperatures were 100 °C for the injector, 95 °C for the oven and 230 °C for the detector. The inset graph is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

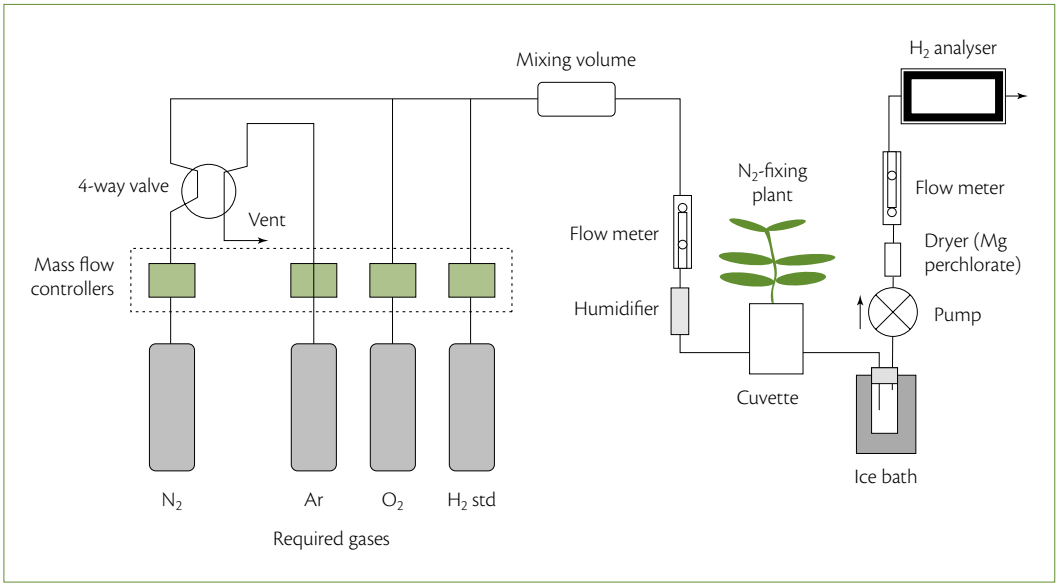

Figure 43. A flow-through system described in Hunt et al. (1989) to measure H_2 efflux from the root nodules of a single plant

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.

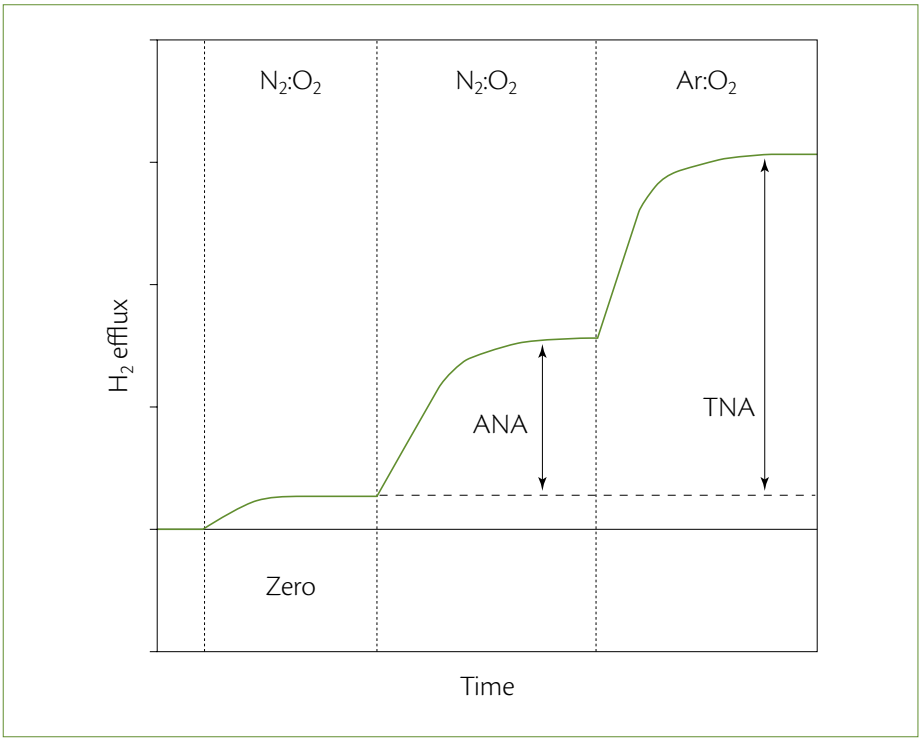

Figure 44. Hypothetical changes in H_2 efflux following sequential measurements of a N_2-fixing system under an atmosphere of N_2 and argon, both containing ambient O_2 concentration

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.

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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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