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).

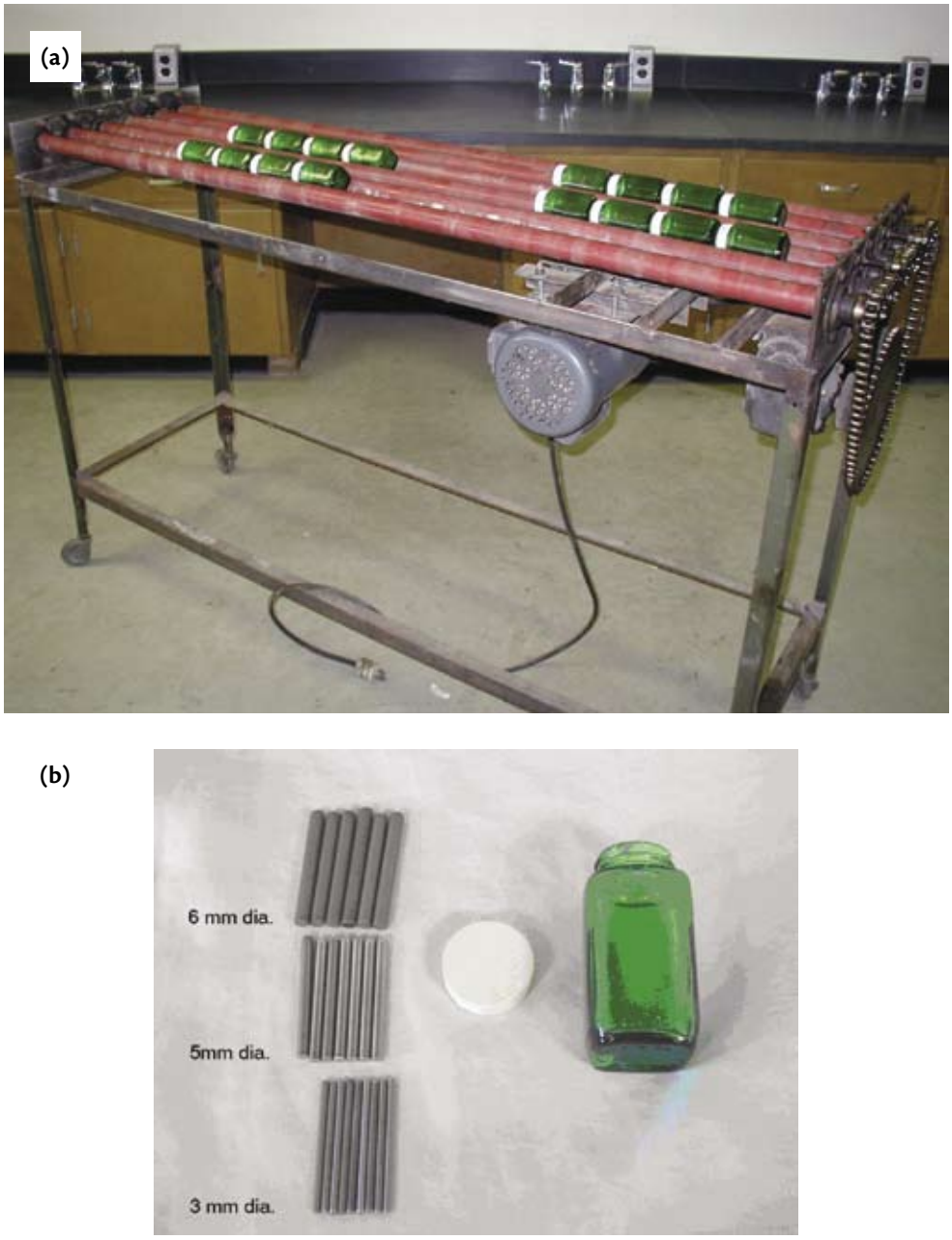

Figure 5. A simple (a) roller grinder that can be made from readily available materials, including (b) steel rods and square glass bottles (see Arnold and Schepers 2004)

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.

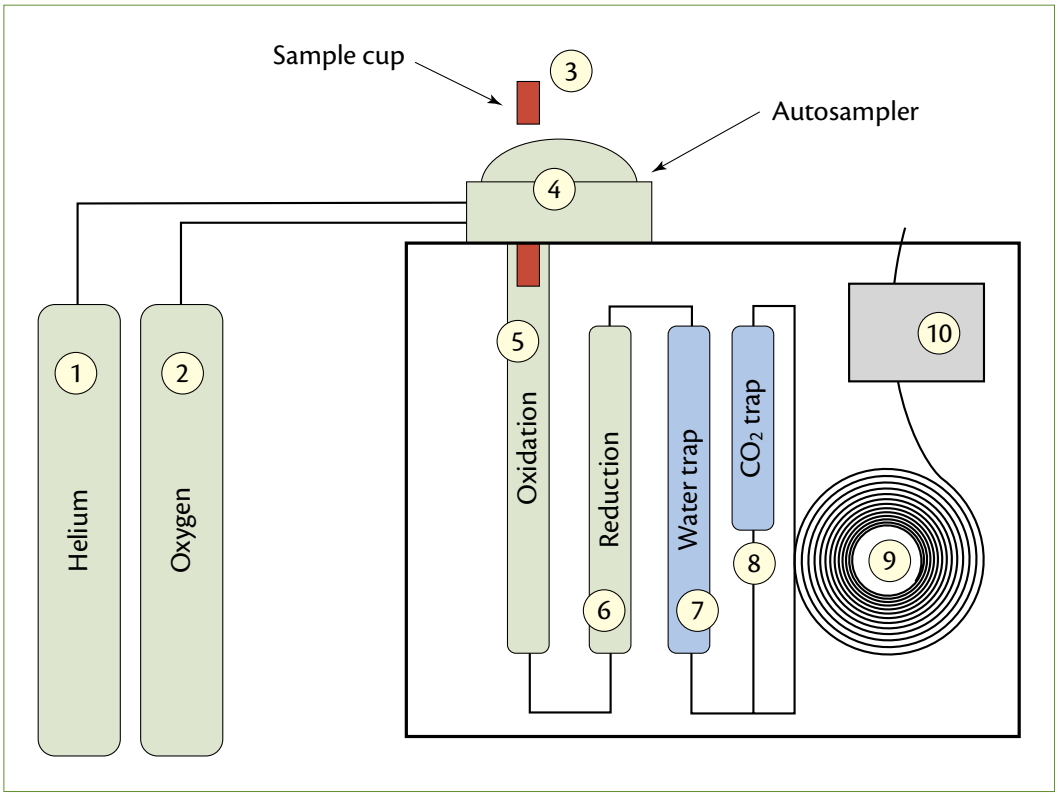

Figure 6. Schematic diagram of an automated combustion system for analysis of total carbon, nitrogen or sulfur in plants or soils. A description of the components and operation is given in the text.

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

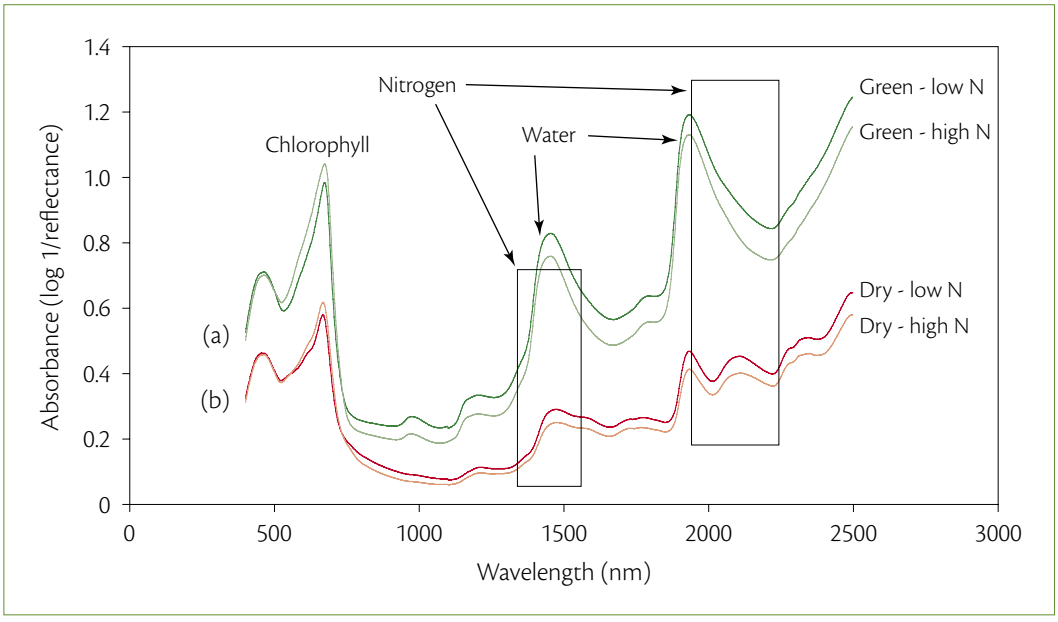

Figure 7. Typical spectra of (a) fresh and (b) dry, ground rice tissue, showing the location of N, water and chlorophyll absorption bands

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).

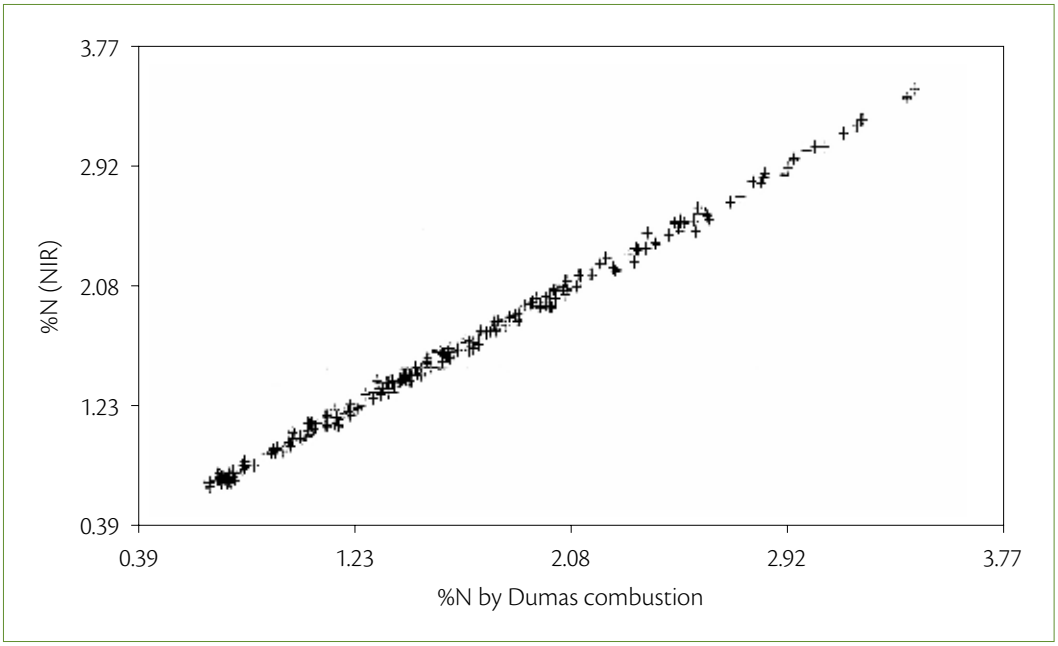

Figure 8. Example of correlation between %N of rice shoots determined by Dumas combustion and near infra-red reflectance (NIR). Such NIR calibrations need to be done for each instrument and plant material type and species.

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).

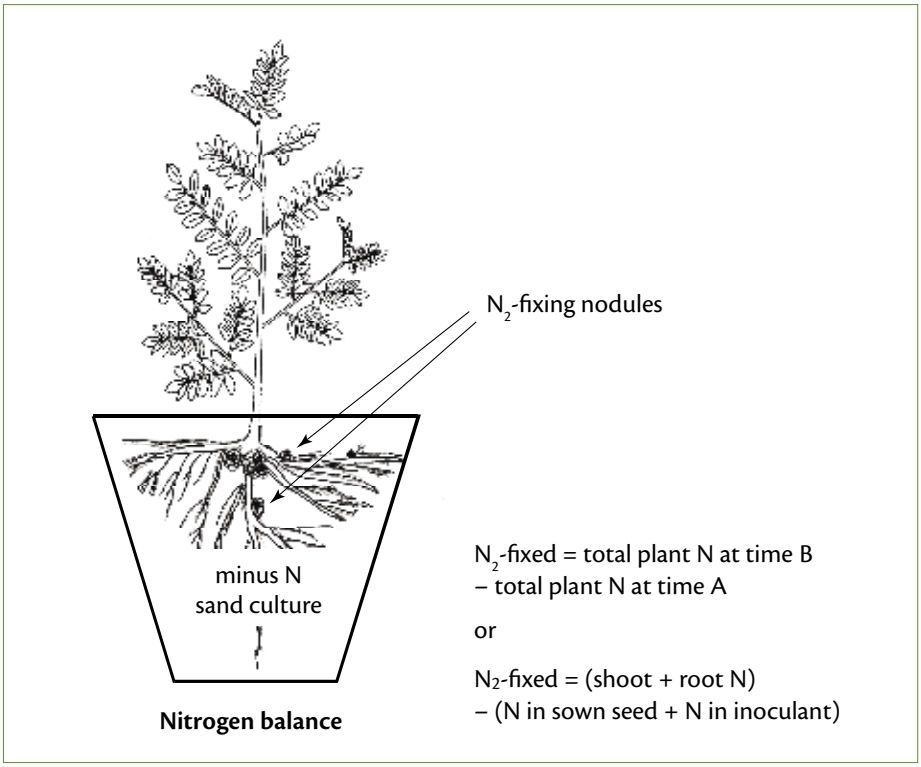

Figure 9. Estimating N_2 fixation by N balance. For a N_2-fixing plant grown in minus N culture, total plant N minus sown seed N equates to N_2 fixation. In this case it is assumed that any N remaining in the pot soil after plant harvest is negligible compared with total plant N.

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

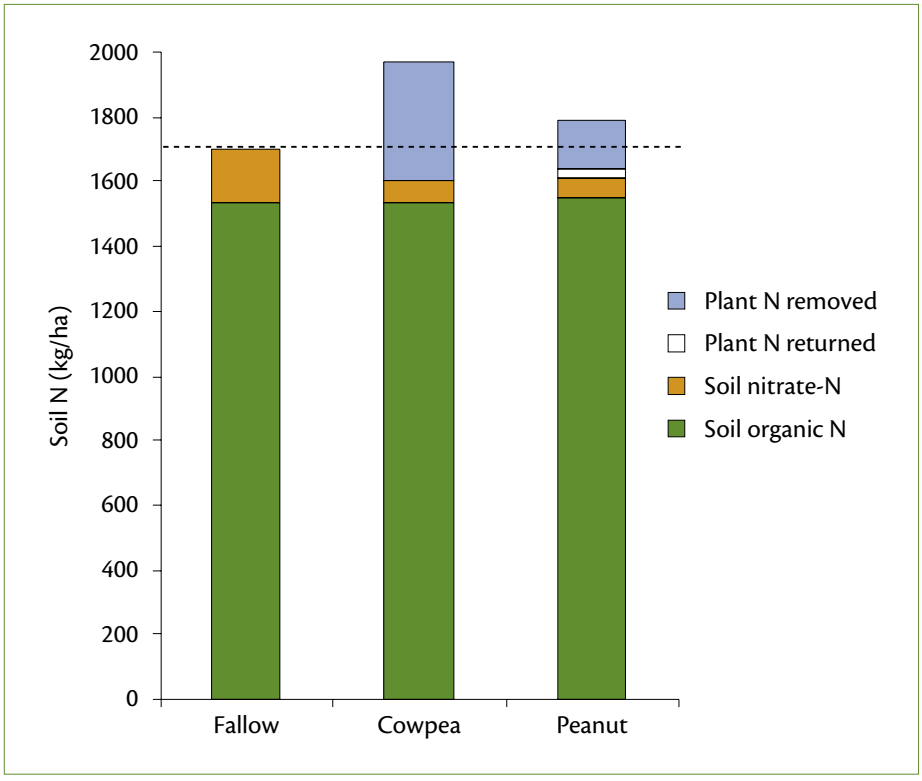

Figure 10. Field-based N balances for three cropping systems. Crops were grown for three consecutive years, with cowpea cut for forage and peanut grown as a grain crop (from Wetselaar et al. 1973). Compared with the fallow, both systems with legumes returned a positive N balance.

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.

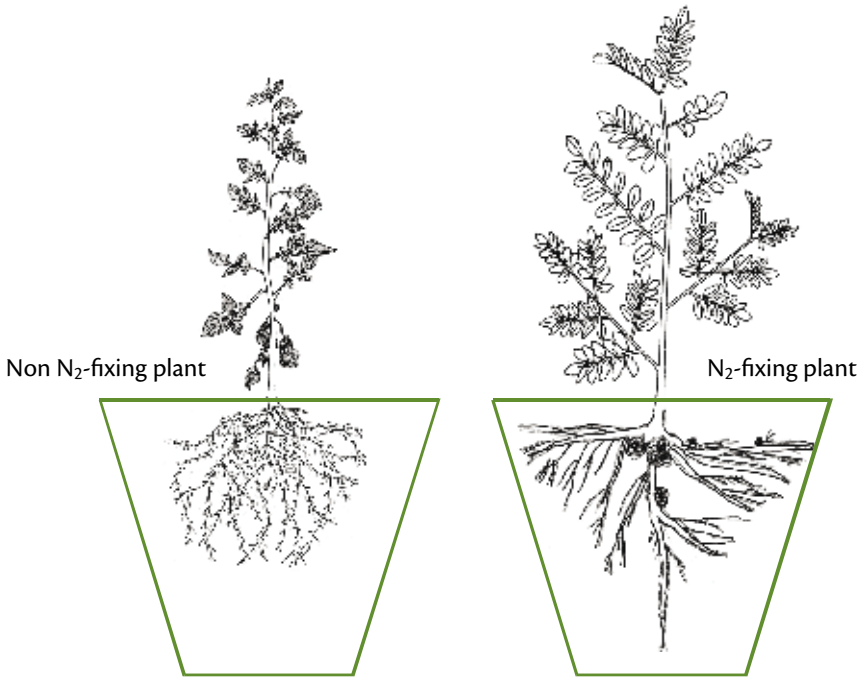

Figure 11. The N difference method for estimating N_2 fixation in a pot study. The difference in total N accumulation between the N_2-fixing and non N_2-fixing plants grown in the same soil is attributed to N_2 fixation. The assumption here is that the non N_2-fixing and N_2-fixing plants extract the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

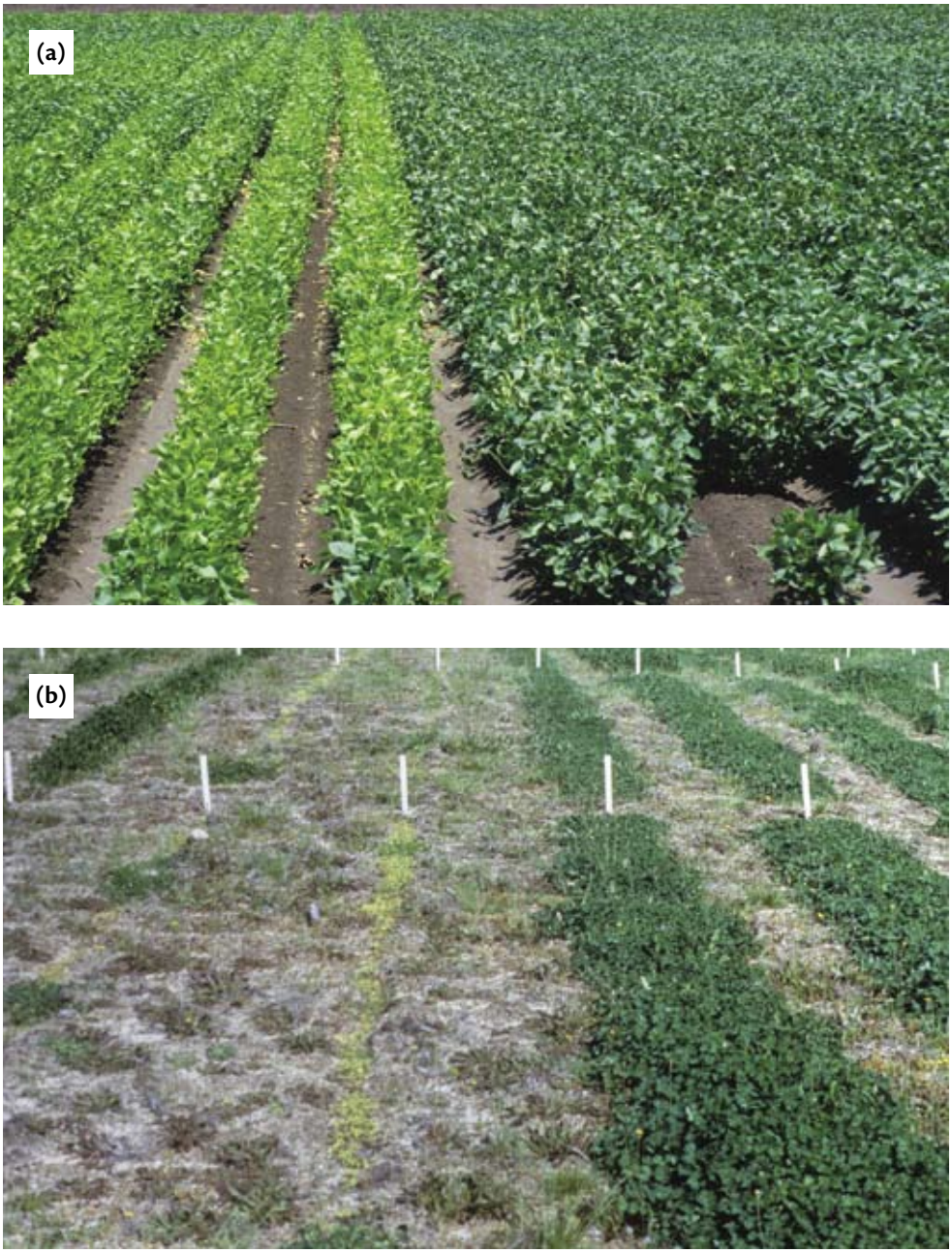

Figure 12. Using N difference to estimate N_2 fixation by field-grown (a) soybean and (b) subterranean clover. In both cases, the difference in total N accumulation between the inoculated and uninoculated plants was attributed to N_2 fixation.

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.

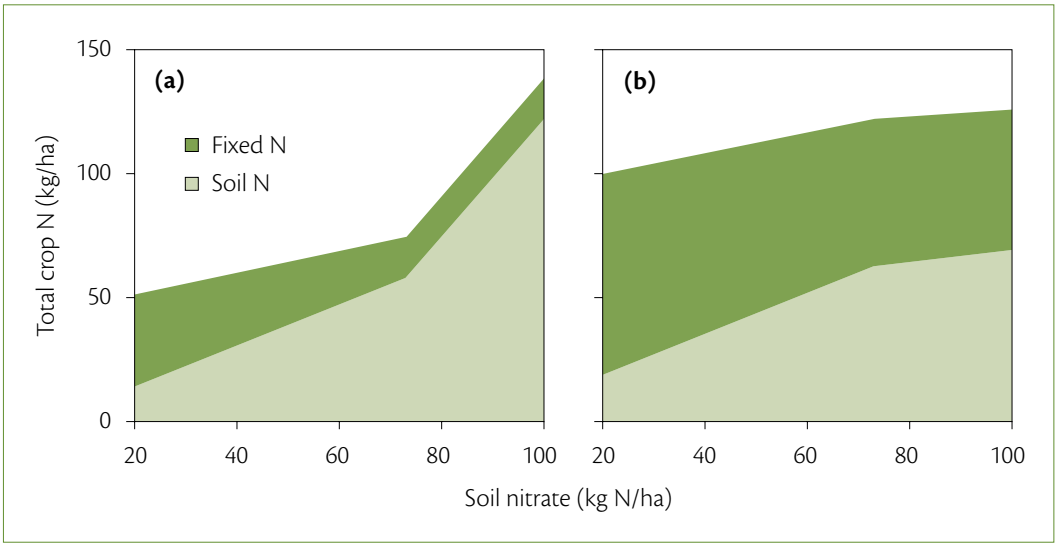

Figure 13. N_2 fixation and uptake of mineral N by field grown (a) chickpea and (b) faba bean at different levels of soil nitrate (from Turpin et al. 2002). Note that at the same levels of soil nitrate, substantially more N was fixed by faba bean than by chickpea.

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).