8

^{15}N natural abundance method

8.1 How it works

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

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

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

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

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

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

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

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

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

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

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

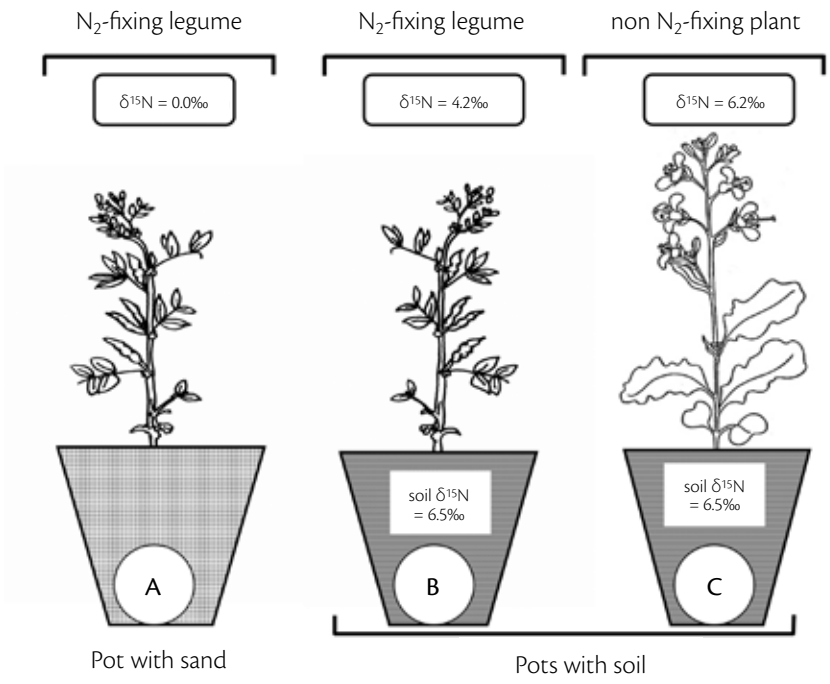

Figure 25. Illustration of the $\delta^{15}N$ natural abundance method for estimating N_2 fixation, highlighting that the $\delta^{15}N$ of a legume completely dependent on atmospheric N_2 (A) should have a $\delta^{15}N$ in shoots very close to that of its N source, atmospheric N_2 (0‰); that a plant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

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

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

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

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

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

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

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

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

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

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

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

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

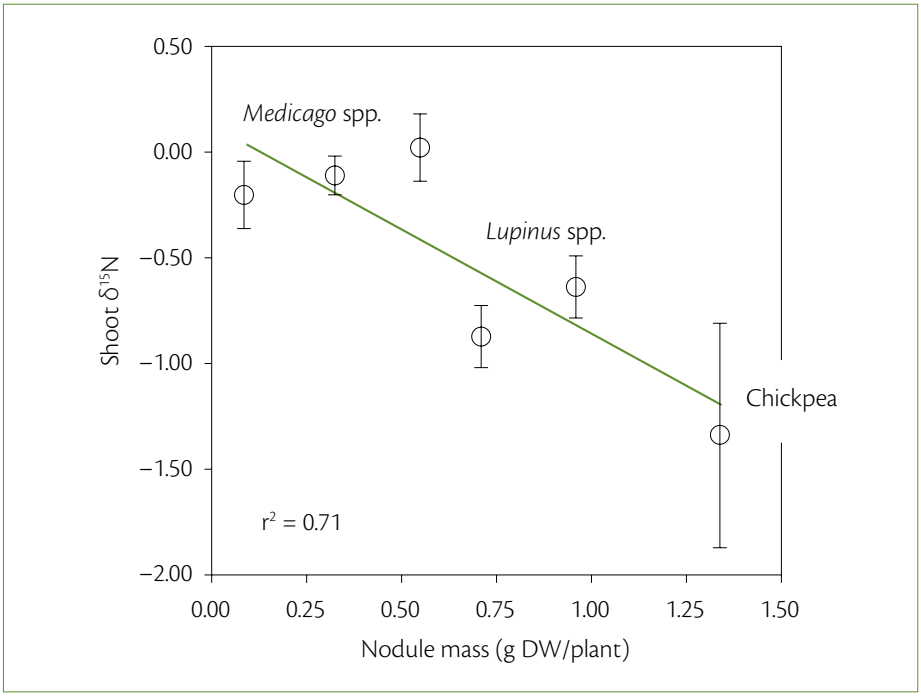

Figure 26. The relationship between legume shoot $\delta^{15}N$ and nodule mass for glasshouse-grown plants relying solely upon N_2 fixation for growth (DW, dry weight). Source: M. Unkovich, unpublished data

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

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

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

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

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

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

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

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

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

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

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

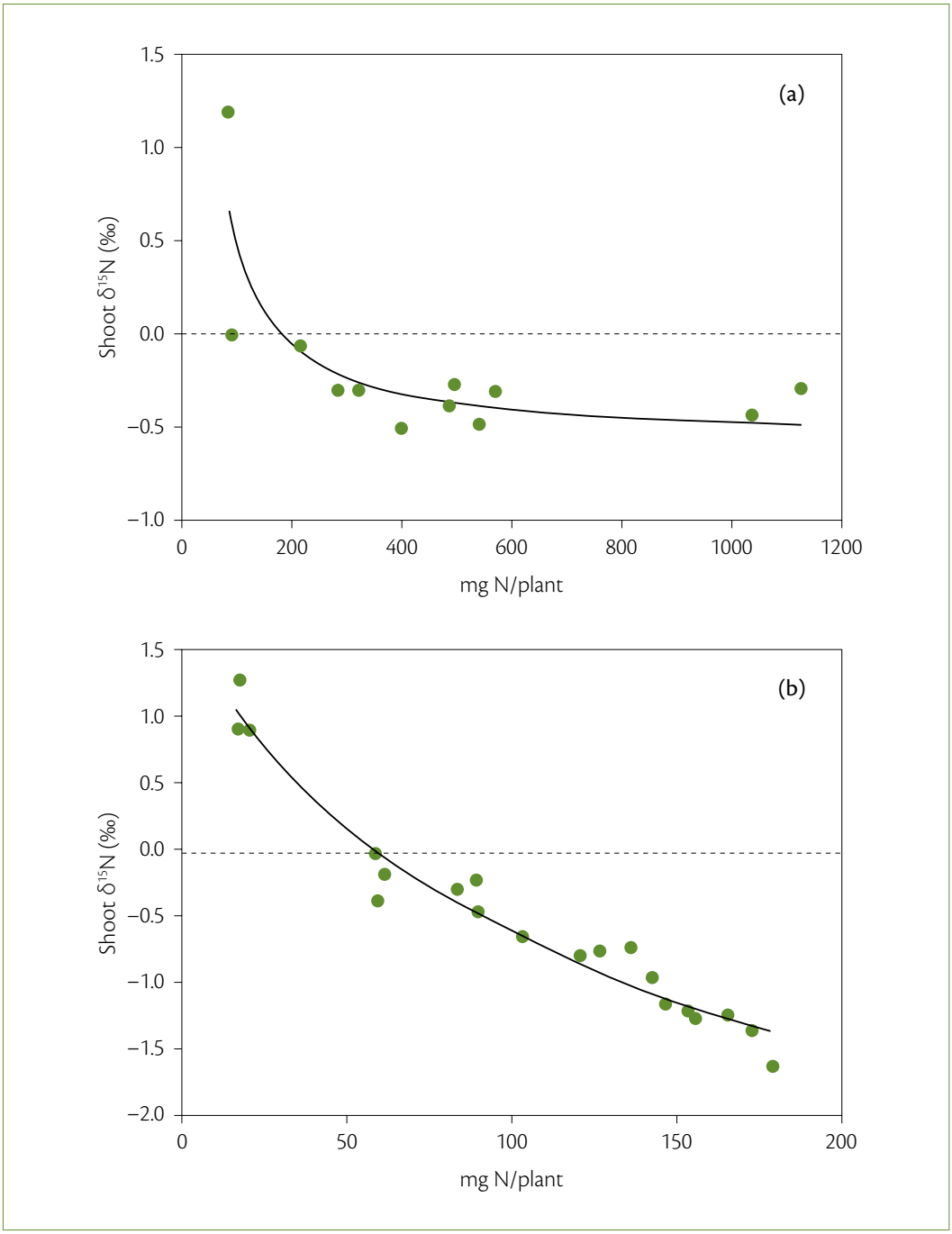

Figure 27. Changes during development in the $\delta^{15}N$ composition of shoots of effectively nodulated (a) faba bean and (b) chickpea growing in sand culture in the absence of combined N in a glasshouse and totally dependent on N_2 fixation

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

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

8.2.3 Recommendations on what 'B' value to use

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

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

8.3 'B' value determination

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

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

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

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

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

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

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

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

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

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

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

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

8.4 Non N_2-fixing reference plants

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

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

Reference plants used in natural abundance studies have included:

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

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

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

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

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

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

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

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

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

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

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

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

The key points from Table 20 are that:

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

Magnitude and variability of soil ^{15}N abundance

8.5

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

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

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

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

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

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

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

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

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

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

[b] Distance between sampling points

Glasshouse application of ^{15}N natural abundance

8.6

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

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

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

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

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

8.7.1 Pre-experiment information

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

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

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

8.7.2 Experimental design

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

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

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

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

(a) Classical randomisation

legume 2
reference
legume 5
legume 1
legume 3
legume 4

legume 4
legume 1
legume 5
legume 3
legume 2
reference

(b) Reference plant strips

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

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

(c) Reference plants interspersed

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

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

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

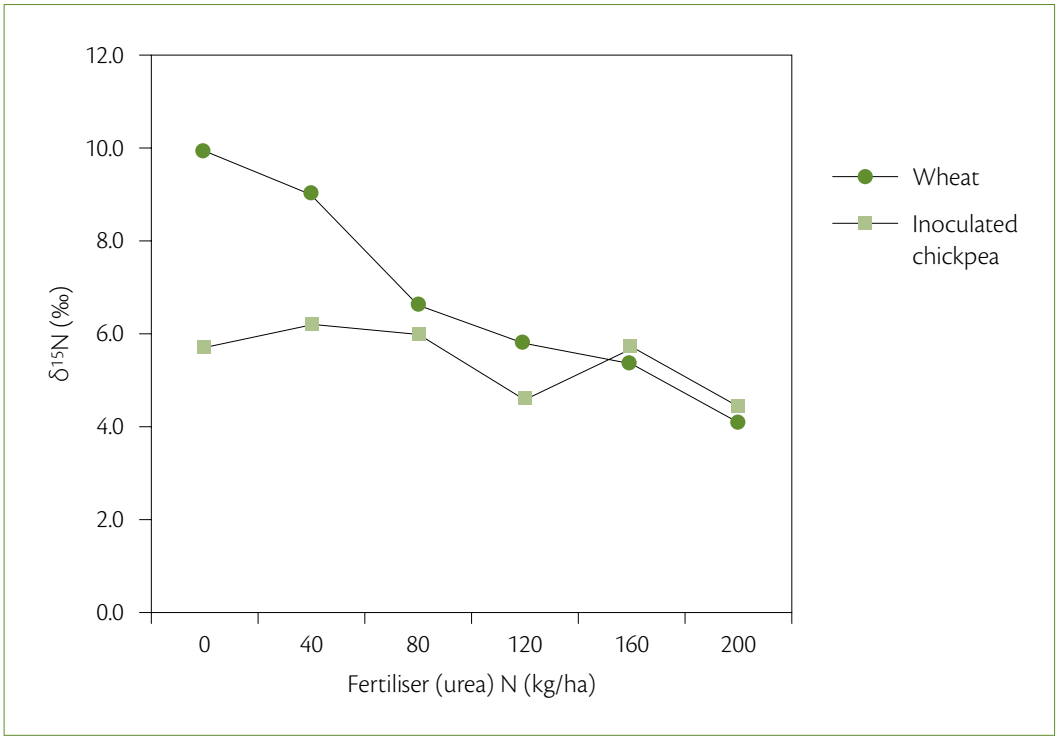

Figure 29. Effects of applied fertiliser (urea) N on the $\delta^{15}N$ of soil mineral N, assessed as the $\delta^{15}N$ of the non N_2-fixing wheat, and on $\delta^{15}N$ and calculated %Ndfa of chickpea, grown in the field in Australia. Source: Herridge et al. (1998)

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

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

Sampling farmers' fields

8.8

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

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

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

8.8.1 Sampling procedures—annual legumes

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

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

8.8.2 Sampling procedures—perennial legumes

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

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

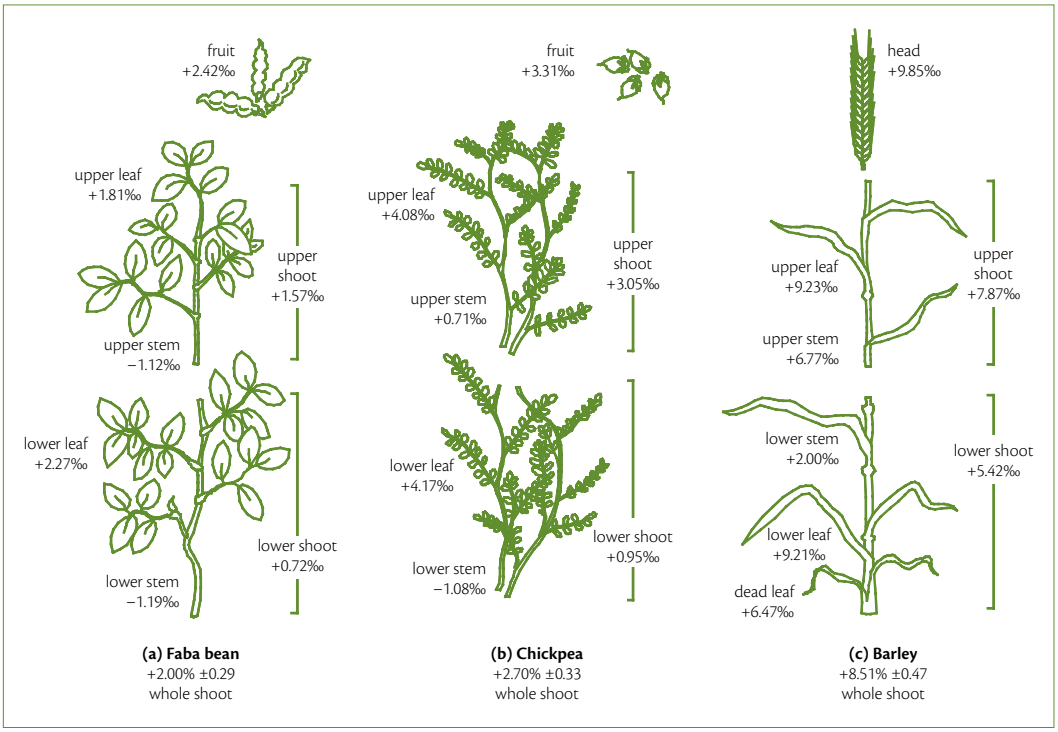

Figure 30. $\delta^{15}N$ in different leaf and stem strata, shoot fractions, reproductive parts and whole shoots of field-grown (a) faba bean, (b) chickpea and (c) barley. Data are from Peoples et al. (2002).

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

8.10 Final word

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

9

^{15}N isotope dilution method

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

9.1 How it works

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

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

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

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

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

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

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

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

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

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

Source: McNeill et al. (1996)

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

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

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

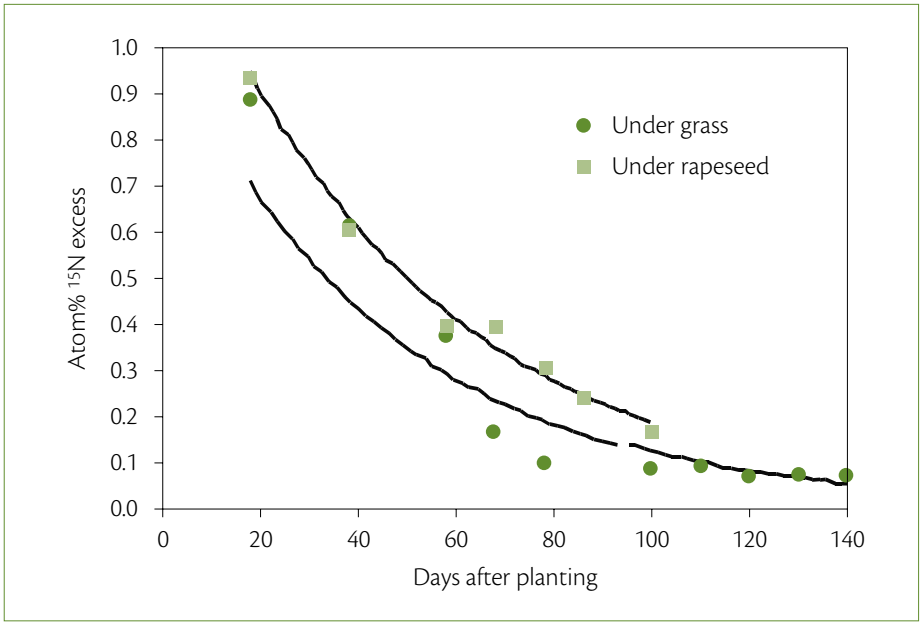

Figure 31. Changes in the ^{15}N enrichment of soil mineral N under grass or rapeseed following application to soil of 3.85 atom% ^{15}N excess KNO_3 at 30 kg N/ha. Data are from Witty (1983).

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

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

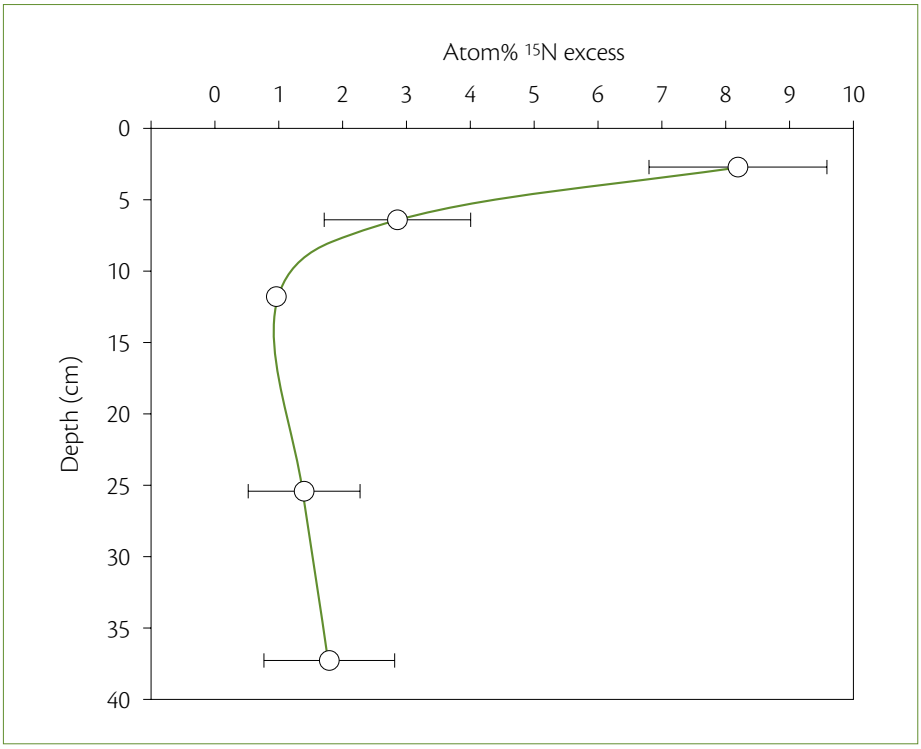

Figure 32. Variation in ^{15}N enrichment of total soil N with depth after labelling with 10 atom% ^{15}N excess $(^{15}NH_4)_2SO_4$. Error bars are s.d. of the means. Data are from Witty (1983).

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

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

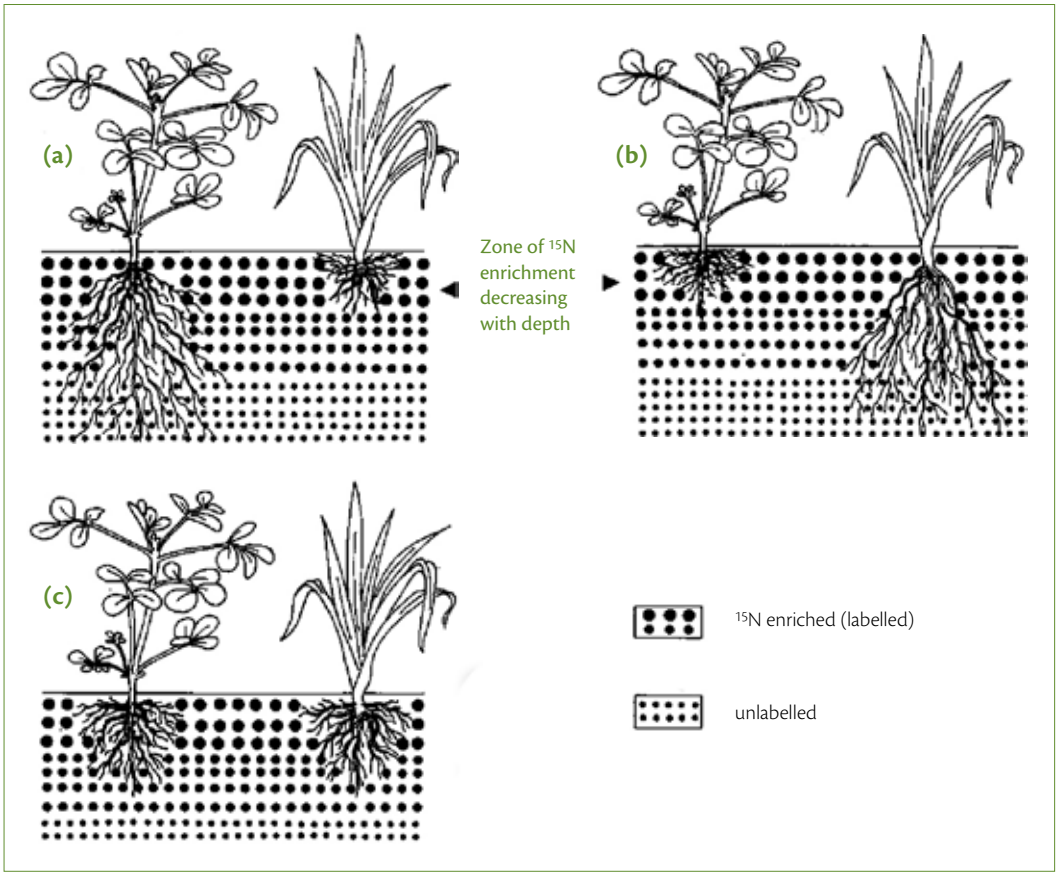

Figure 33. Scenarios that can arise in field experiments using ^{15}N labelling. The three examples show differences in root growth of N_2-fixing plants and non N_2-fixing reference plants (a and b), and differences in the distribution of the ^{15}N enrichment with depth (a, b and c). Scenarios (a) and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

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

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

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

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

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

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

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

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

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

9.2.3 Slow-release formulations

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

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

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

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

9.2.4 How much ^{15}N to add?

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

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

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

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

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

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

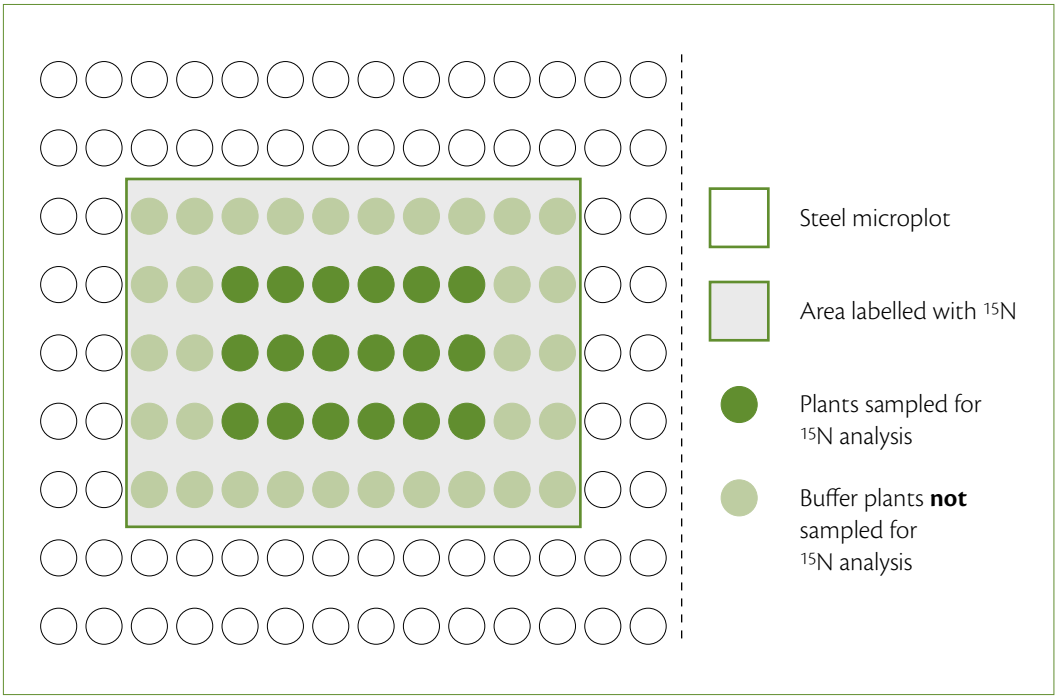

Figure 34. Typical microplot and sampling strategy for ^{15}N-labelling experiments. The ^{15}N application and sampling strategy could be the same with or without the steel microplot.

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

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

9.2.5 Preliminary calculations

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

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

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

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

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

Pot experiments

9.3

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

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

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

9.4 Choice of reference plants

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

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

9.4.1 Absence of N_2-fixing ability

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

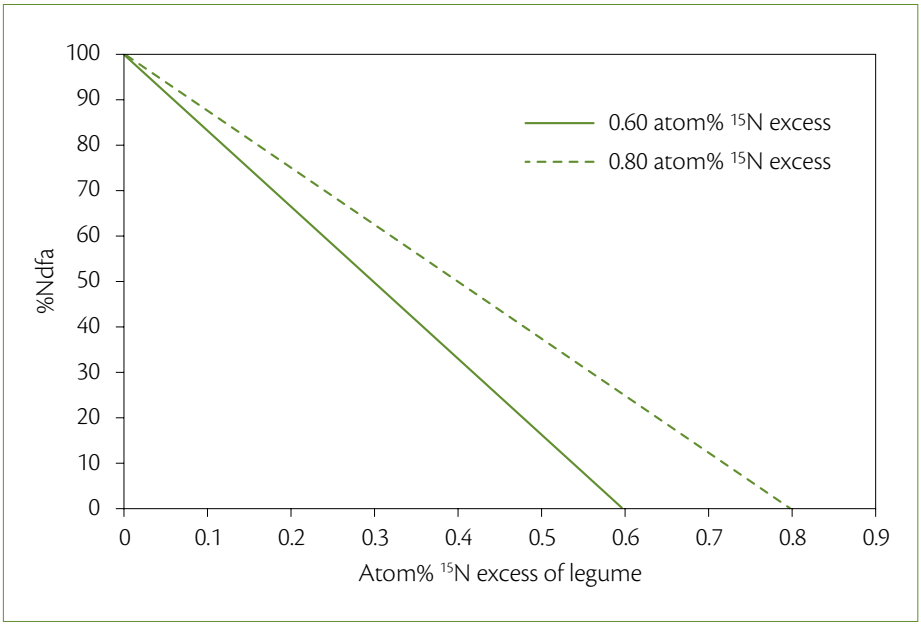

Figure 36. Relationships between N_2-fixing legume atom% ^{15}N excess (X axis) and estimates of %Ndfa (Y axis) calculated using two different atom% ^{15}N excess values (0.60 and 0.80) for the reference plants. Clearly, differences in reference plant ^{15}N have a much greater impact on estimates of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

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

9.4.2 Exploitation of the same soil rooting zone

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

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

9.4.3 Duration of growth

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

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

9.4.4 N transfer between species

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

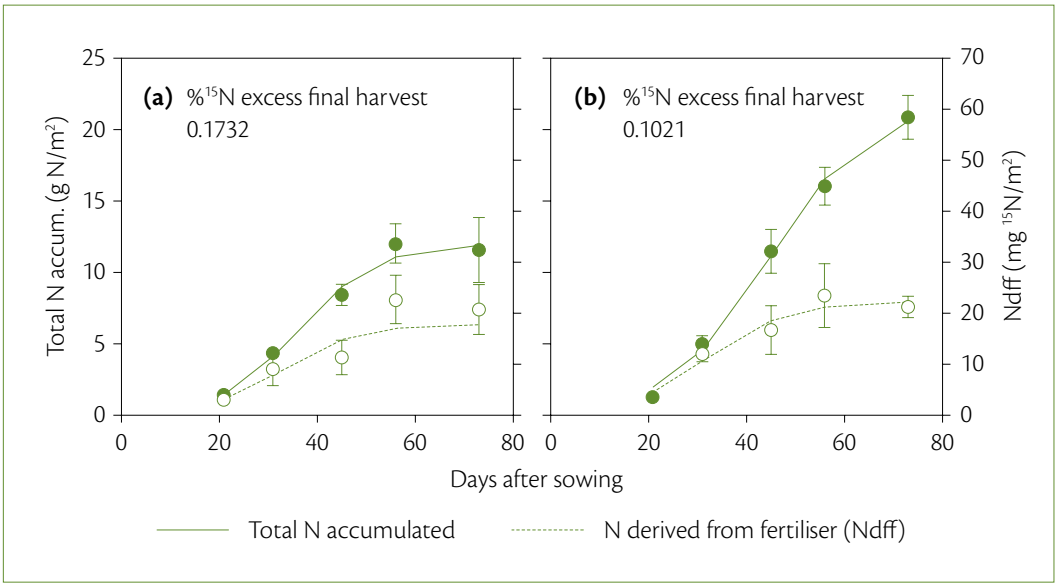

Figure 37. Total N accumulation and uptake of ^{15}N-labelled fertiliser for two non N_2-fixing reference plants fed 2 kg N/ha of 21 atom% ^{15}N-labelled ammonium sulfate: (a) non-nodulated soybean; (b) sorghum. Source: Boddey et al. (1995a)

Modelling soil ^{15}N enrichment

9.5

9.5.1 Background

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

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

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

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

9.5.2 Yield-independent model

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

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

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

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

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

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

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

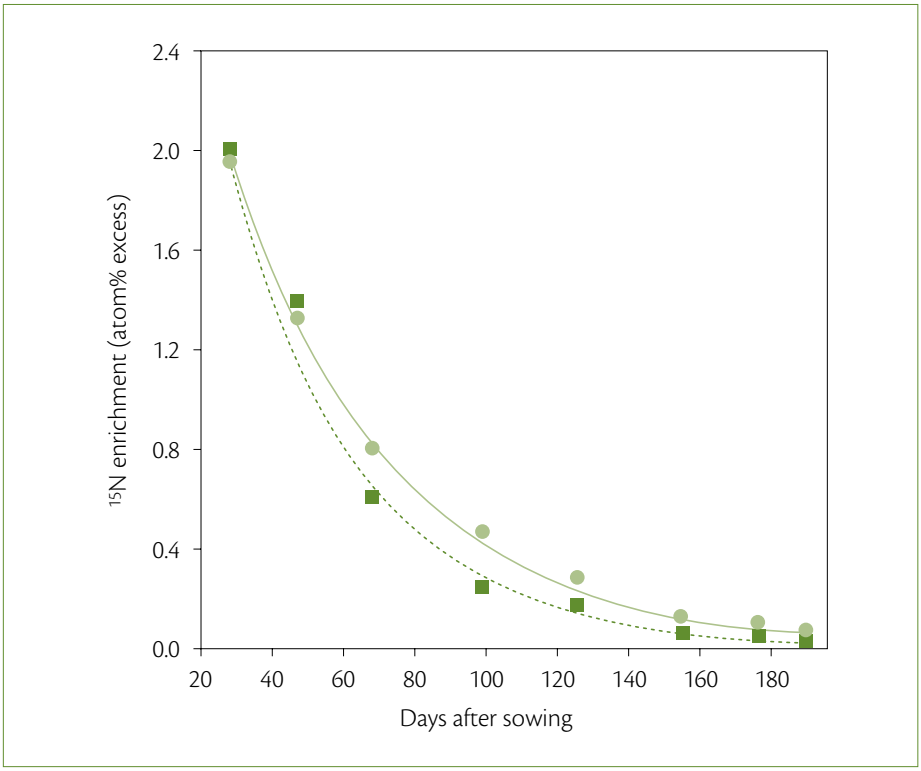

Figure 38. Temporal decline in the ^{15}N enrichment of the KCl-extractable soil nitrate (●) or mineral N (■) pools (0–15 cm) under lupin. Labelled fertiliser was applied at sowing at the rate of 2.5 g N/m^2 at 10 atom% ^{15}N. Curves represent the relationships described by equation (14). For the NO_3^-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

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

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

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

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

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

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

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

9.5.3 How it works

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

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

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

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

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

9.5.4 Attributes of the yield-independent model

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

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

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

9.6 Experimental plots

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

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

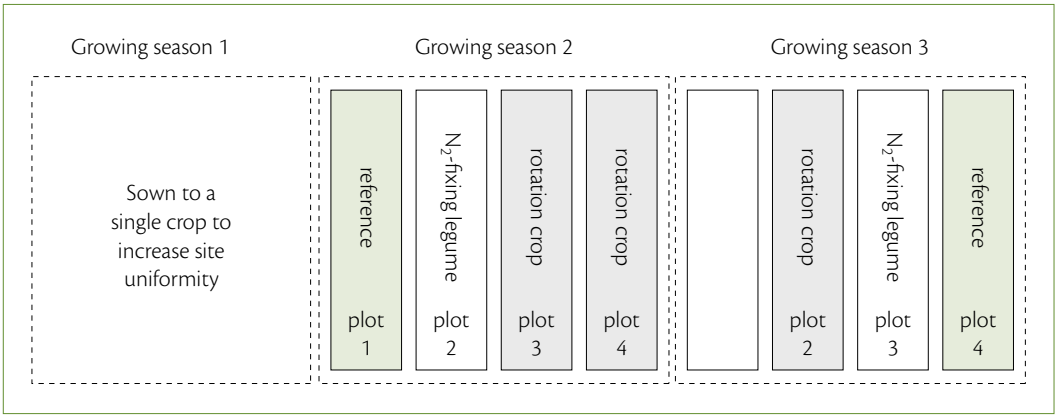

Figure 39. Layout of field plots through a two-crop sequence to ensure that non N_2-fixing reference plants are grown in soil with exactly the same history as the N_2-fixing legume under study. In this case plot 1 is of no use (redundant) for the final crop sowing. Replication of crops and rotational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

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

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

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

Source: Danso and Kumarishinge (1990)

9.7 Perennial tree systems

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

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

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

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

Evaluation of ^{15}N data and calculations

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

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

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

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

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

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

Source: Evans et al. (1987)

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

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

9.9 Final word

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

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