6

Ureide (N solute) method

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

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

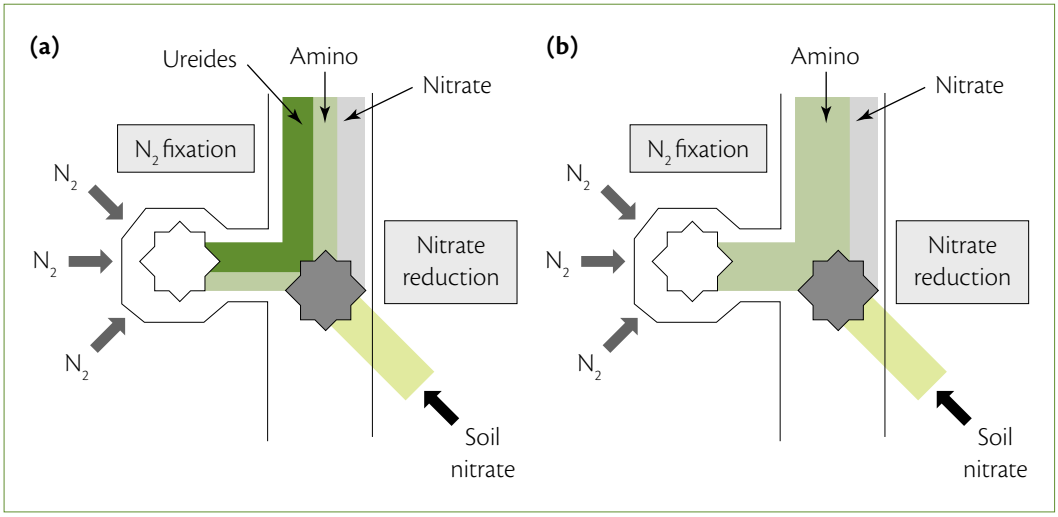


Figure 14. Assimilation and export of N from the nodulated roots of (a) ureide- and (b) amide-producing legumes. Note that the ureide species have three major groups of N solutes in the xylem stream—ureides, amino compounds and nitrate—while the amide legumes have only two—amino compounds and nitrate.

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

How it works

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

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

Ureide plants

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

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

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

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

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

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

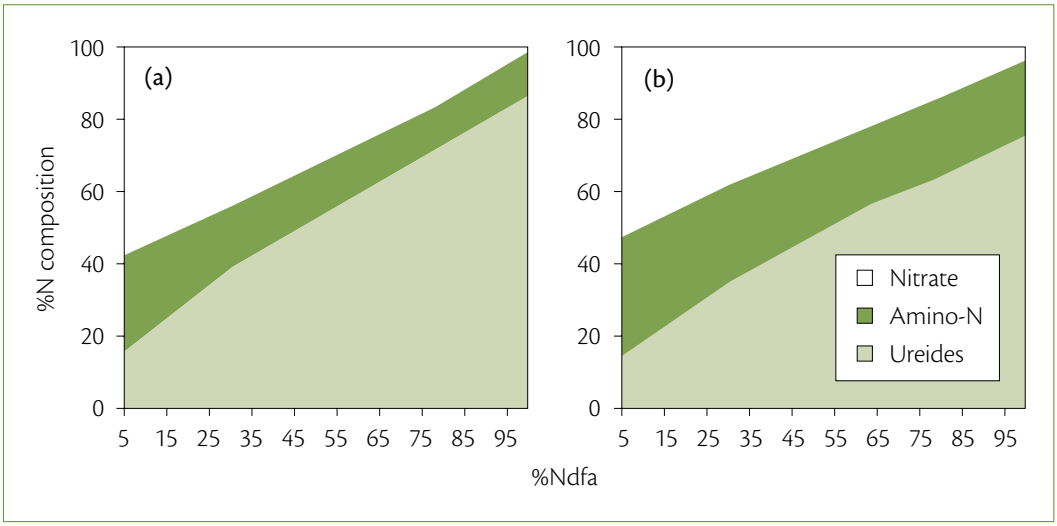


Figure 15. Changes in the composition of N solutes in (a) root-bleeding and (b) vacuum-extracted xylem sap of nodulated soybean, supplied with different concentrations of ^{15}N-labelled nitrate to generate different values of %Ndfa. Data are from Herridge and Peoples (1990).

Amide plants

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

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

6.2 Calibrations of relative ureide-N and %Ndfa

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

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

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

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

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

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

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

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

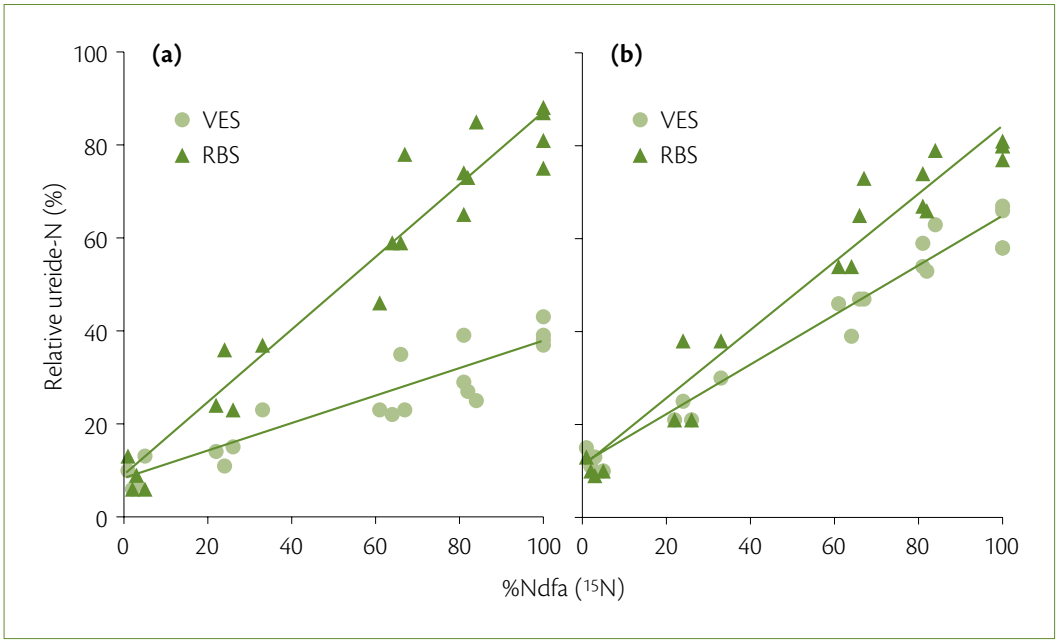


Figure 16. Relationships between %Ndfa and relative abundance of ureide-N in vacuum-extracted sap (VES) and root-bleeding sap (RBS) of (a) cowpea and (b) mung bean (green gram). Data are from Herridge and Peoples (2002a).

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

Perennial plants

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

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

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

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

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

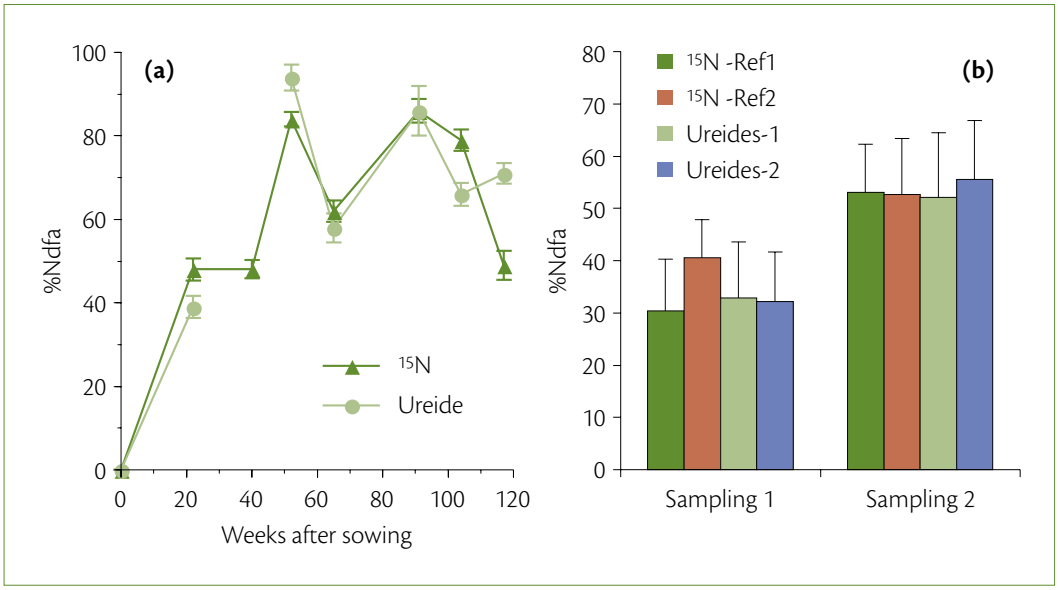


Figure 17. Crosschecking ureide and ^{15}N methods for determining %Ndfa for (a) *Codariocalyx gyroides* grown in the field in northern Australia (Peoples et al. 1996) and (b) *Desmodium ovalifolium* grown in the field in Brazil (^{15}N-Ref 1 *Brachiaria humidicola* used as the non N_2-fixing reference;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

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

Annual plants—estimating N_2 fixation inputs

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

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

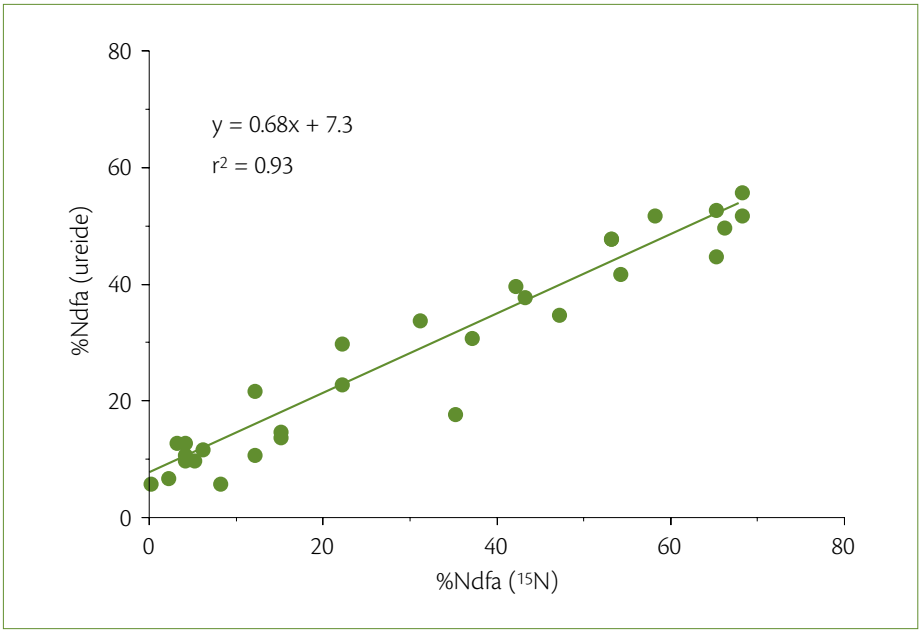


Figure 18. Correlation between %Ndfa of soybean genotypes, estimated using stem and xylem ureide (multiple samplings) and natural ^{15}N abundance (single sampling just prior to physiological maturity) techniques (see section 8.1). Source: Herridge et al. (1990)

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

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

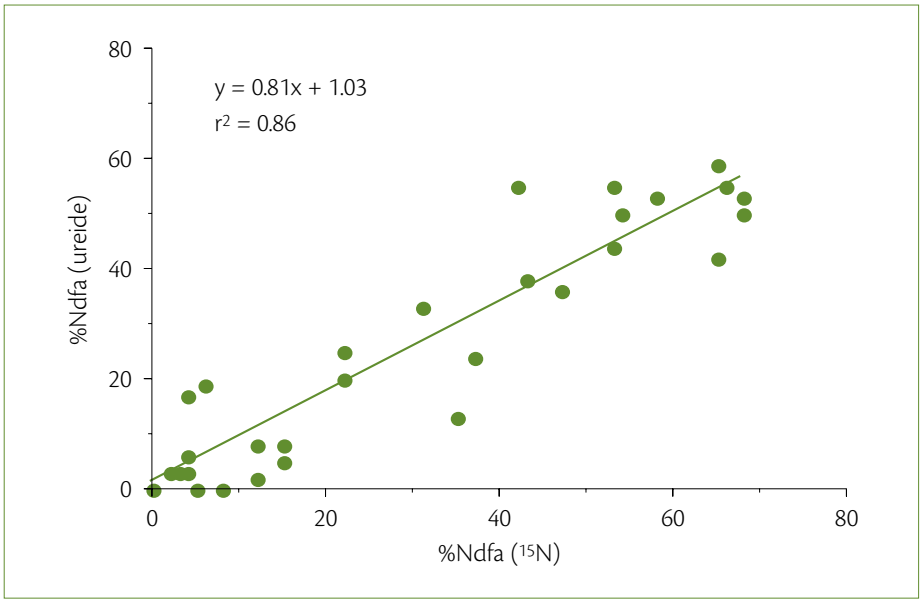


Figure 19. Correlation between %Ndfa of soybean genotypes, estimated using xylem ureide (single sampling at R3.5) and natural ^{15}N abundance techniques (single sampling at R6.5) (see section 8.1). Source: Herridge et al. (1990)

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

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

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

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

Annual plants—comparing treatments for N_2 fixation activity

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

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

6.3 Applications of the ureide technique

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

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

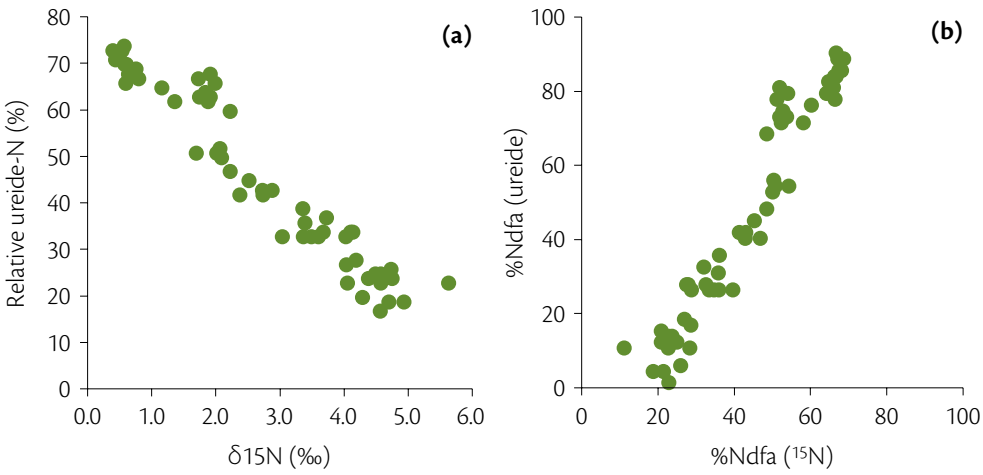


Figure 20. Correlations between (a) natural ^{15}N abundance values of harvested grain of field-grown soybean and relative ureide-N values of xylem sap collected during early pod-fill (physiological stage R3) and (b) %Ndfa estimates from grain ^{15}N and xylem sap ureides (see section 8.1). Source: Herridge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

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

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

Advantages and potential sources of error of the ureide technique

6.4

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

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

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

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

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

Table 11. (continued)

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

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

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

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

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

6.5 Setting up experiments and on-farm surveys

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

6.5.1 Glasshouse experiments

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

6.5.2 Field experiments

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

6.5.3 On-farm surveys

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

6.6 Sampling of N solutes

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

6.6.1 Root-bleeding sap

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

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

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

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

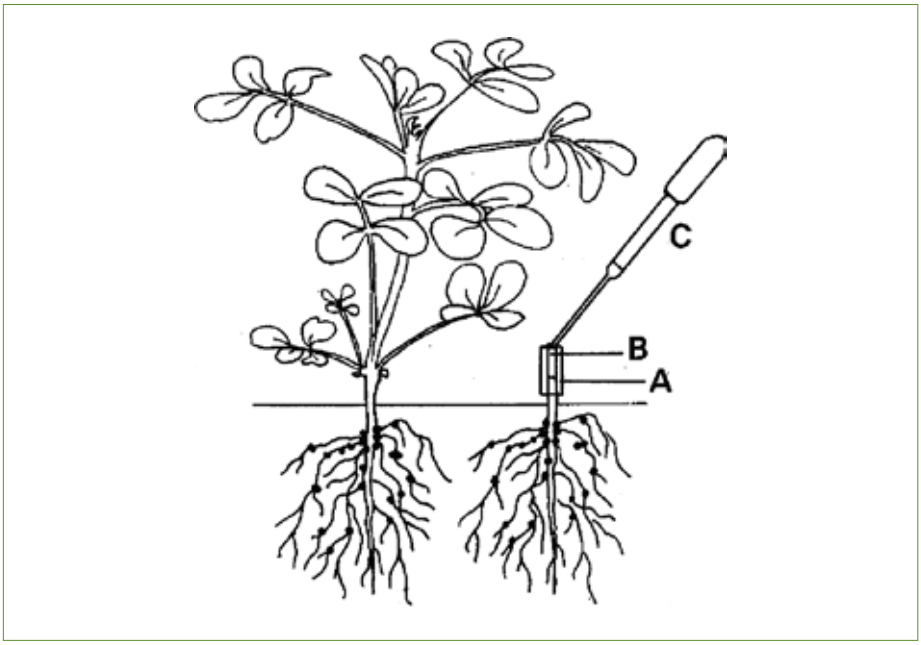


Figure 21. Sampling of root-bleeding xylem sap. A length of silicon rubber tubing (A) is placed over the root stump following decapitation of the shoot. The sap (B) collects in the tubing, out of which it can be drawn using a Pasteur pipette or syringe (C).

6.6.2 Vacuum-extracted sap

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

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

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

Equipment requirements

Items needed for vacuum-extraction of xylem sap are:

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

Procedure

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

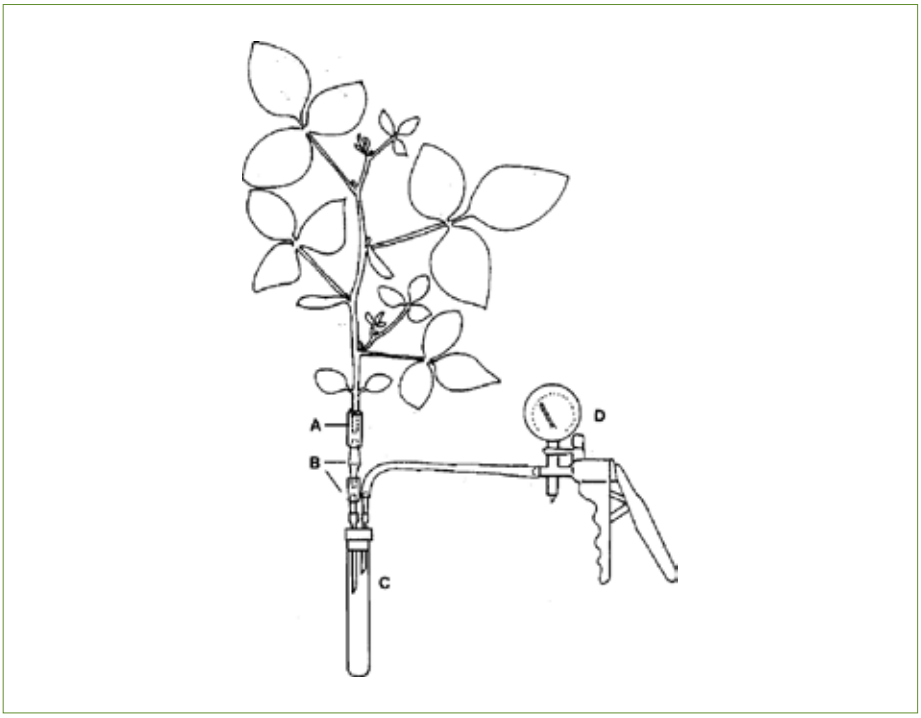


Figure 22. Apparatus for vacuum-extraction of xylem sap using a hand-held vacuum pump. The base of a freshly detached stem is placed into a sleeve of silicon-rubber tubing (A) of appropriate size fitted onto a syringe needle (using an adaptor (B) if necessary). The syringe needle is then inserted through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

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

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

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

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

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

6.6.3 Stems and stem segments

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

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

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

Extraction of solutes from stems and stem segments

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

Analysis of xylem saps and extracts

6.7

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

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

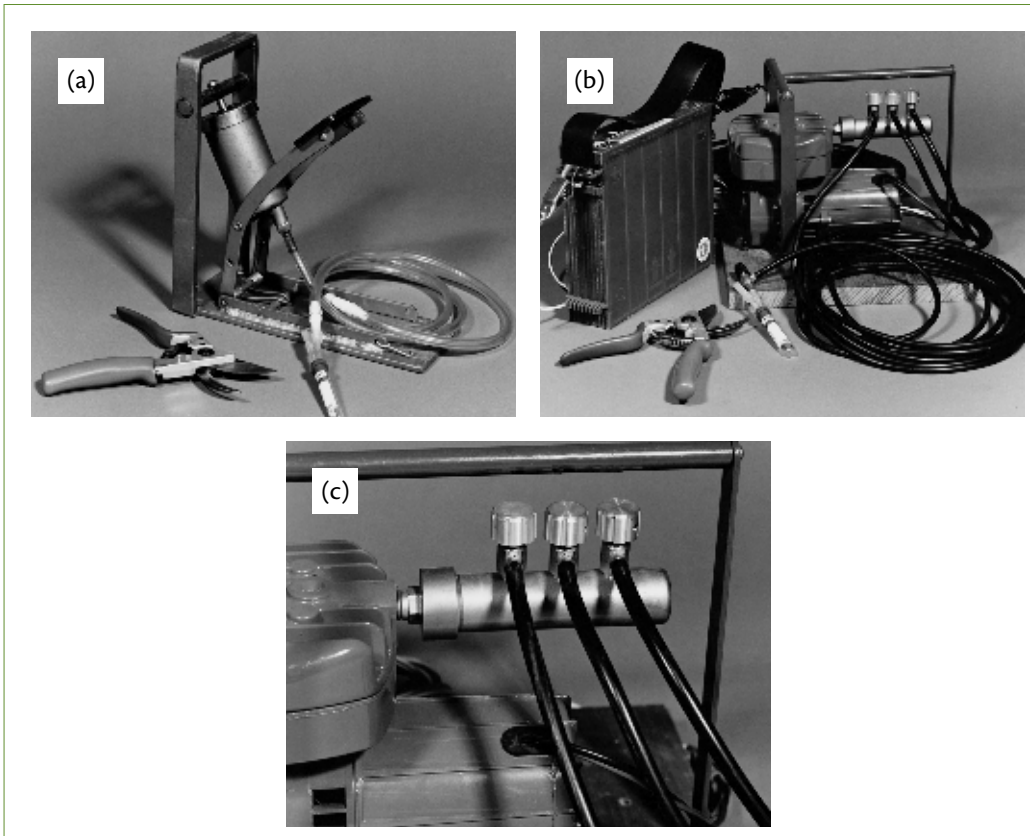


Figure 23. (a) Foot vacuum-pump, secateurs, Vacutainer® and fittings used to extract xylem sap from plant stems. The pump is a modified car-tyre pump with the plunger reversed and a one-way valve fitted in the plastic tube. (b) Battery-powered electric diaphragm vacuum-pump, fitted with a three-port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

6.7.1 Ureide assay

Reference: Young and Conway (1942)

Reagent preparation

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

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

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

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

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

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

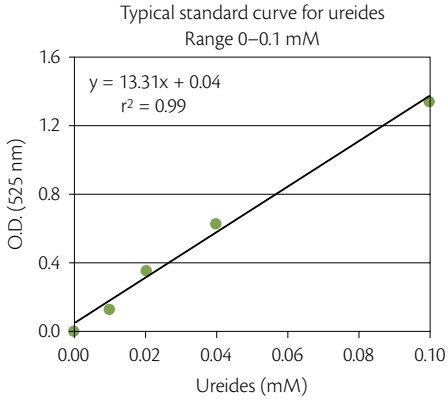
(g) The concentrated HCl and 1.67% KFeCn are mixed together—total volume 500 mL, and placed in a freezer (if possible) or a fridge/ice bath. (Pour the concentrated HCl and KFeCn mix into **Dispenser 3**; leave in fridge.) Make fresh daily.

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

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

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

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



Analysis

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

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

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

4. Remove from water bath and place on bench.

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

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

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

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

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

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

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

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

6.7.2 Amino-N (ninhydrin method)

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

Reagent preparation

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

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

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

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

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

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

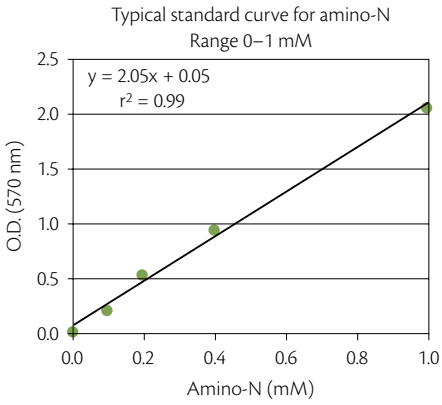
Vacuum extracting xylem sap from field-grown soybean using an electrical vacuum pump powered by a generator (foreground). Note that a manifold in the vacuum line allows at least two people to collect xylem sap at the same time.

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

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

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

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



Analysis

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

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

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

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

5. Remove from water bath and place on bench.

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

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

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

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

6.7.3 Salicylic acid method for nitrate determination

Reference: Cataldo et al. (1975)

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

Reagent preparation

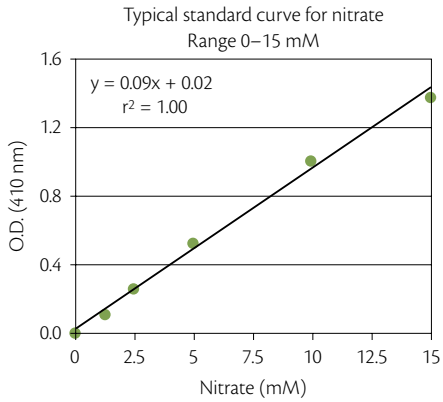
(a) Salicylic acid in concentrated sulfuric acid (H_2SO_4) (5% w/v). Leave for 24 hours before use.
Add 5 g salicylic acid to 100 mL concentrated H_2SO_4 (beaker).

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

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

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

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



Analysis

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

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

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

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

6.8 Calculations

6.8.1 Calculating concentrations of ureides, nitrate and amino-N

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

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

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

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

6.8.2 Calculating the relative abundance of ureide-N

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

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

6.8.3 Calculating %Ndfa

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

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

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

6.9 Interpretation of a typical dataset

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

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

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

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

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

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

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

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

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

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

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

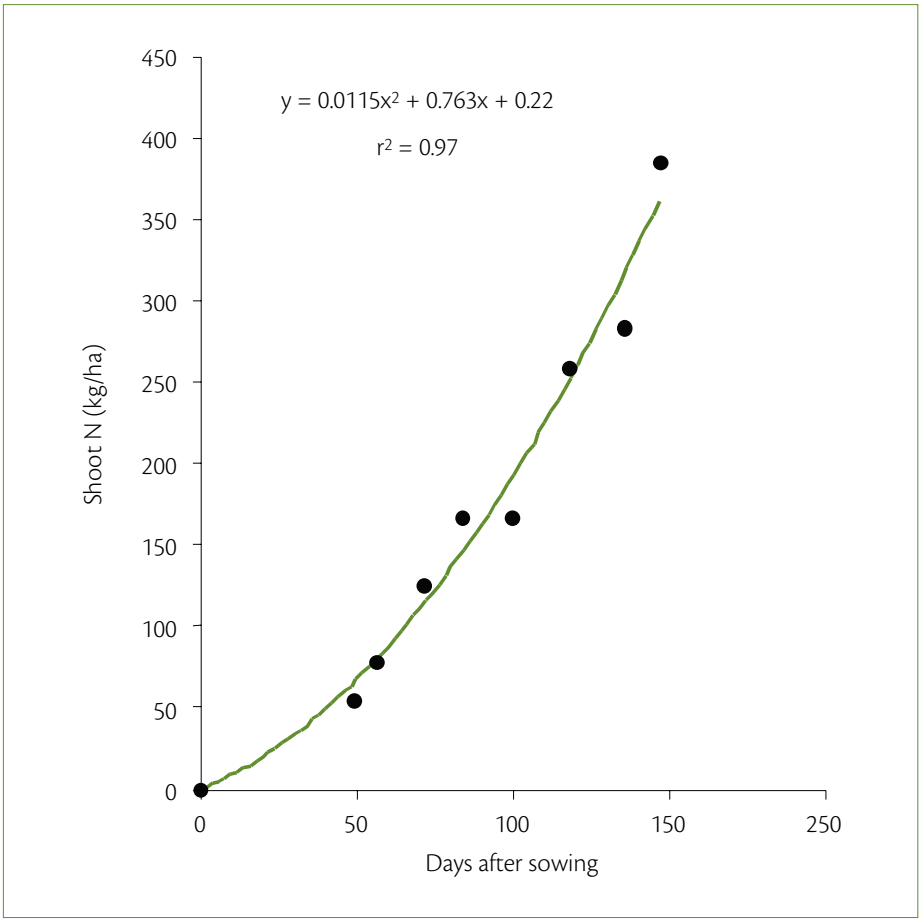


Figure 24. Accumulation of shoot N during growth of soybean in a high mineral N soil. The line of best fit is shown. Values are the means of four replicates.

6.10 Final word

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

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

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

7

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

7.2 The ^{15}N abundance of soil N

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

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

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

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

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

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

[b] nd = not determined.

$^{15}N_2$ feeding

7.3

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

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

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

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

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

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