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Methods for Evaluating Nitrogen Fixation by Nodulated Legumes in the Field

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Methods for Evaluating Nitrogen Fixation by Nodulated Legumes in the Field

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Foreword

Nitrogen deficiency is a widespread problem in many soils used for the production of staple cereal crops. In high potential areas of the developing world, farmers can afford nitrogen fertilisers to increase cereal production. In the less-favoured areas, an alternative, cheaper source of nitrogen in the cropping system is that fixed by the root nodule bacteria in food and forage legumes. In addition to sparing existing soil nitrogen, nitrogen fixation by these legumes can provide significant inputs which enter the soil organic pool and are released to companion or subsequent cereal crops.

Although various aspects of biological nitrogen fixation have been researched for many years, the development of improved methods for enhancing and managing this process has been hampered by the inadequacy or sophistication of methods for measuring nitrogen fixation. In order to help overcome this constraint, ACIAR has, since 1984, funded research aiming to develop simpler, more reliable methods for measuring nitrogen fixation. The research has involved a partnership between scientists at Chiang Mai University in Thailand, the Rubber Research Institute of Malaysia, and the CSIRO Division of Plant Industry and NSW Agriculture & Fisheries in Australia.

This handbook describes methods that are particularly designed for use by developing country scientists. The authors are to be commended for the simple but complete manner in which the methods are presented. Thanks should also go to the ACIAR Communications Program, and especially Mr Reg MacIntyre who edited the publication, and to the anonymous peer reviewers who suggested a number of valuable and constructive changes.

We hope that the developing country scientists using the techniques described here will gain a better understanding of the role of nitrogen fixation in cropping systems. We also hope that they will use this knowledge to design better, more productive legume-based systems for the resource-poor farmers who are the ultimate clients for the research ACIAR supports.

E.T. Craswell
Research Program Coordinator
ACIAR

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1
INTRODUCTION

1.1 Why measure N_2 fixation?

The unique advantage of growing legumes in agricultural systems reflects their potential capacity to fix large amounts of atmospheric N_2. Yet the formation of the symbiosis between legume and *Rhizobium* spp. is dependent upon many factors and cannot be assumed to occur as a matter of course. Failure to achieve an effective symbiosis in a soil low in mineral-nitrogen (N) will result in a loss of legume production, and applications of N-fertiliser of up to 160 kg N/ha may be required to achieve seed yields similar to those of a well-nodulated crop (Gault et al. 1984). In a soil of higher mineral-N content, the legume may compensate for poor N_2 fixation by scavenging N from the soil. Although production in this situation may not be impaired, the net result of cropping with a legume deficient in nodulation is an exploitation of N reserves. Soil N fertility is lost and it represents a wasteful use of a legume in a cropping sequence (Table 1.1).

Table 1.1 Interaction between soil mineral nitrogen, N_2 fixation and legume production.

Soil mineral–N	N_2 fixation	Legume production	Potential benefit to soil
Low	Poor	Low	None
	Good	High	High
High	Poor	High	None
	Good	High	High

The relationship between N_2 fixation and plant or food production is direct and obvious in some cases (e.g. on a soil low in mineral-N, Table 1.1); however, the overall benefits of including N_2-fixing legumes in cropping systems cannot be assessed unless a reliable and accurate field measurement is made of the levels of fixation achieved. Any other parameter (e.g. nodule number, legume biomass) provides only a qualitative and descriptive assessment.

Below are listed several reasons why the measurement of N_2 fixation is important in many aspects of legume research.

(i) Ecological considerations require an understanding of the relative contribution of N_2-fixing components to the N-cycle.

(ii) Development of sustainable farming systems. Understanding of the amount of N_2 fixed by legumes as influenced by soil management or cultural practices allows development of efficient agricultural and agroforestry production systems.

(iii) Measurements of N_2 fixation enable an investigator to evaluate the ability of indigenous *Rhizobium* spp. to effectively nodulate newly introduced legumes, the symbiotic effectiveness of rhizobial inoculants and success of inoculation procedures, or the N_2-fixing capabilities of legume genotypes in plant breeding programs.

(iv) Once the user is satisfied that nodule development is adequate, measurement of N_2 fixation establishes whether a legume is achieving its potential, and thus becomes an added means of identifying constraints, such as nutrient limitations.

(v) Residual effects on subsequent crops following the growth of legumes are frequently attributed to N_2 fixation. There may be other benefits such as improvements to soil structure or control of pests or diseases. Measurement of N_2 fixation allows proper assessment of the potential benefits from the input of fixed nitrogen or sparing of soil N by the legume.

1.2 Methods to measure N_2 fixation

There is no single 'correct' way of measuring N_2 fixation. No one technique will provide an accurate measure of N_2 fixation for all legumes grown in any soil under diverse environmental conditions. Each technique has its own unique advantages and limitations, and these will be detailed in the following sections for all the most commonly used methodologies except the acetylene reduction assay.

The acetylene reduction assay is a useful diagnostic tool for the detection of nitrogenase activity and has been widely used in all areas of N_2 fixation research because of its high sensitivity and simplicity. However, its reliability is now questioned for even comparative studies with legumes. Acetylene reduction provides only an instantaneous measure of nitrogenase activity under the prevailing assay conditions, therefore its accuracy has always been restricted by the requirement for many repeated determinations to adjust for marked diurnal and seasonal changes in N_2-fixing activity. Further errors in the field can arise due to the use of an inappropriate calibration factor to relate ethylene production to N_2 fixation, incomplete recovery of a plant's total nodule population, nodule detachment or damage prior to assay, plant disturbance, or an acetylene-induced decline in nitrogenase activity during assay (Witty and Minchin 1988). Although in situ procedures with flow-through gas systems are being devised to overcome some of these technical problems, the procedure has only limited application for the measurement of N_2 fixation in the field.

It is intended that this publication will complement the many books and review articles that deal with the various technologies associated with symbiotic N_2 fixation. In particular, the principles behind xylem-solute techniques, N-difference procedures, and ^{15}N isotope dilution methods for quantifying N_2 fixation will be described. The application of these techniques in the field will be discussed as will the analysis and interpretation of experimental data. In each section the reader is provided with references to the most relevant literature citations for additional detail. Much of the text will refer to studies of food legumes, although the general concepts

can also be applied to other legume systems. Similar methodologies (N-difference, ^{15}N) are also utilised in studies of associative N_2 fixation with non-legumes; readers are referred to a comprehensive review of this topic by Boddey (1987).

2
FORMATION OF THE LEGUME – *RHIZOBIUM* SYMBIOSIS

The contribution of a legume in any production system is dependent on the formation of nodules by strains of *Rhizobium* spp. effective in N_2 fixation with the chosen legume host.

There are three major groups of legumes which can be distinguished on the basis of compatibility with a range of strains of *Rhizobium* (Table 2.1). At one extreme is a group of legumes which can form an effective symbiosis with a wide range of strains. Members of this group are the tropical legumes nodulated by cowpea-type *Rhizobium*. These *Rhizobium* spp. are so widespread in tropical soils that legumes of this group seldom respond to inoculation; yet nodulation failures may still occur because of low numbers of rhizobia in the soil or high levels of soil mineral N. At the other extreme are legumes with specific rhizobial requirements. These specificities are most relevant when the legume is being introduced into new areas. Response to inoculation of these legumes is usually successful provided adequate numbers of rhizobia are applied at sowing. The third and intermediate group of legumes nodulate with many strains of *Rhizobium*, but effectively fix N_2 with only a limited number of them. Thus inoculation and nodulation failures are more frequent because the inoculum strain is unable to compete with the ineffective but established soil populations of rhizobia.

Overall, it must be remembered also that environmental conditions (temperature, water availability, soil pH, etc.), the level of availability of mineral nutrients in the soil (reviewed by O'Hara et al. 1988) and diseases of legumes may affect nodulation and/or N_2 fixation, obscuring the beneficial effects which are being sought.

2.1 Inoculation

There are five conditions under which soils may be devoid of *Rhizobium* to form an effective symbiosis with a legume, and which may warrant inoculation.

(i) The absence of the same or a symbiotically related legume in the immediate past history.

(ii) Poor nodulation when the same crop was grown previously.

(iii) When the legume follows a non-leguminous crop in a rotation.

(iv) In land reclamation.

(v) When environmental conditions are unfavourable for *Rhizobium* survival (e.g. if soil pH is less than 5.5 for *R. meliloti* or greater than 7 for *R. lupini).* In these situations soil amendments will be required to ensure establishment of rhizobia.

Table 2.1 Legumes, grouped on the basis of nodulation and N_2 fixation with a range of *Rhizobium* species[a].

Nodulate effectively with a wide range of strains. Genera listed forming one loose group.		
Albizia	*Galactia*	*Psophocarpus*
Alysicarpus	*Gliricidia*	*Pueraria*
Arachis	*Indigofera*	*Rhynchosia*
Calliandra	*Lablab*	*Stylosanthes*
Calopogonium	*Lespedeza*	(several sub-groups)
Cajanus	*Macroptilium*	*Tephrosia*
Canavalia	*Macrotyloma*	*Teramnus*
Clitoria	*Mimosa*	*Vigna*
Crotalaria	*Pachyrhizus*	*Voandzeia*
Dolichos	*Pongamia*	*Zornia*
Erythrina	*Neonotonia*	
Nodulate with a range of strains but often ineffectively. Genera listed forming individual groups with some crossing between groups. Sub-groups distinguishable.		
Acacia	*Astragalus*	*Psoralea*
Adesmia	*Centrosema* (2 sub-grp)	*Sesbania* (2 sub-grp)
Aeschynomene	*Desmanthus*	
	Desmodium (2 sub-grp)	
Nodulate effectively with specific strains only. Genera listed forming specific groups.		
Cicer	*Lotononis-Listia* (3 sub-grp)	*Phaseolus*
Coronilla	*Lotus* (3 sub-grp)	*Pisum*
Glycine max	*Lupinus* (2 sub-grp)	*Trifolium* (many sub-grp)
Hedysarum	*Medicago*	*Trigonella*
Lathyrus	*Melilotus*	*Vicia*
Lens	*Onobrychis*	
Leucaena	*Ornithopus*	

[a] J. Brockwell, personal communication.

The rhizobia to be used for inoculation may be isolated by the researcher himself from nodules, dried root material, or soil, or be requested from a *Rhizobium* culture collection (see Appendix 10.1; Brockwell 1980; Somasegaran and Hoben 1985). In either case the culture will require further growth and multiplication until there are sufficient bacterial numbers present for inoculation. [Various aspects of inoculant production and uses are reviewed in Somasegaran and Hoben (1985), Thompson (1980), and Vincent (1982).] Alternatively, inoculant can be purchased from a commercial producer. In this instance it is desirable to select commercial inoculant of the highest quality possible. The minimum number of viable bacteria accepted in peat cultures by the Australian Inoculants Research and Control Service (Gosford, N.S.W.) is 10^9 rhizobia per gram at manufacture and 10^8 per gram at the time of inoculant expiry with less than 0.1% contamination. (Australian inoculant producers who meet this standard are listed in Appendix 10.2). Not all manufacturers routinely

produce inoculant for all legumes; however, it may be possible to arrange for a special batch of rhizobia to be fermented for an uncommon legume species.

Methods of inoculating legume seed are numerous and often determined by the aims of the experiment itself [see discussion in Brockwell (1980), Gault et al. (1982), and Vincent (1982)]. Some general principles apply whatever the technique chosen:

(i) If uninoculated control treatments are included, they should always be handled before treatments in which the seed is inoculated. This reduces the risk of contamination and subsequent nodulation of the controls.

(ii) Inoculation levels should always be as high as feasible within the objectives of the experiment. The presence of large numbers of inoculant rhizobia reduces the scope for contamination and nodulation by naturally occurring rhizobia or strains from other inoculation treatments.

(iii) Rhizobia are mobile organisms and are easily transferred by water movement, human or animal agency, or accidentally from treatment to treatment or plot to plot. Awareness of this is needed in setting up the experiment, fencing to exclude animals, weeding it, and even in walking across it.

(iv) Rhizobia are incompatible with many agrochemicals applied as seed dressings. Even a fertiliser such as superphosphate can be toxic to rhizobia when mixed in direct contact with inoculated seed, because superphosphate is very acidic.

(v) Rhizobia can easily be killed by heat. Inoculant therefore must be kept cool before use. Environmental temperature at sowing, however, is also important. Some 4.6% of a soybean inoculum can be recovered from the soil 24 hours after sowing at 28°C; but only 0.2% or less might survive sowing at 38°C (Brockwell et al. 1987).

2.2 Evaluation of nodulation

Many traits which provide a subjective evaluation of the response to inoculation or the capacity of a crop to fix N can be assessed during field experimentation. These are useful when taken in conjunction with field measurements of N_2 fixation and can often assist in the interpretation of the data obtained. Such parameters include earliness of nodulation, nodule number, mass and colour, distribution and longevity of the nodule population, and visual nodulation scores.

Nodulation is generally assessed periodically after sowing by digging up a number of plants at random from each treatment. Nodule number and nodule mass or nodule weight per unit dry weight of the whole plant or root system are often used in trial comparisons; however, similar information can be obtained by visually scoring nodulation on a 0–5 basis taking into account nodule number, size, pigmentation and distribution.

The system devised for soybean in Figure 2.1 represents an adaptation of the classification criteria used by Corbin et al. (1977) when visually ranking nodulation in field-grown chickpea. The nodule score for soybean is determined by the number of effective nodules in the crown-root zone (regarded as the region 5 cm below the

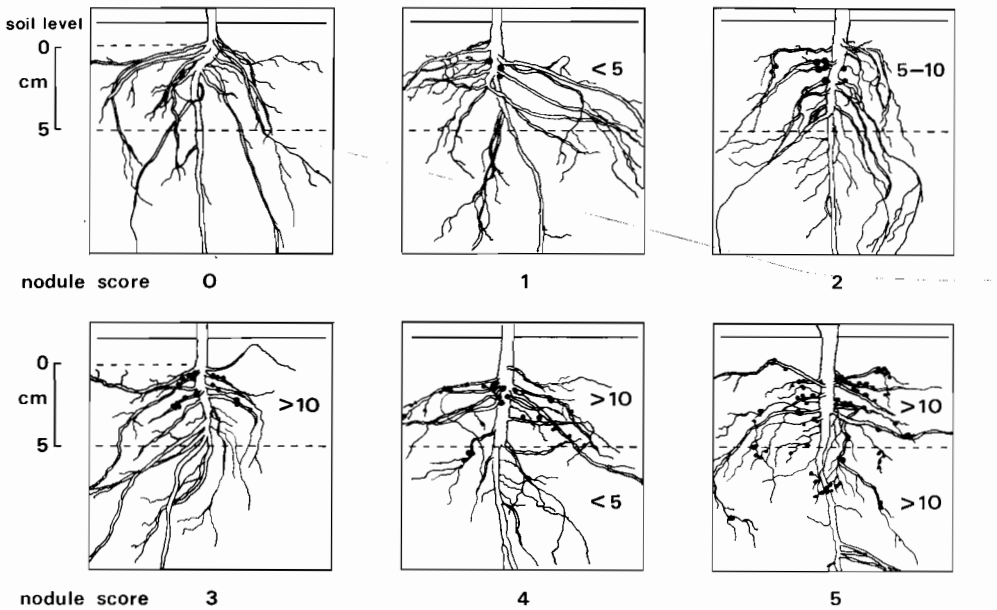

Fig. 2.1 Diagrammatic representation of the visual classification criteria used to evaluate the root system of soybean. Nodule score is judged by the number of effective nodules in the crown-root zone (regarded as the region 5 cm below the first lateral roots) and elsewhere on the root system. After J. Brockwell and R.R. Gault (personal communication) adaptation of the scheme devised by Corbin et al. (1977) for chickpea.

first lateral roots; this may be as much as 10 cm below soil level, depending on sowing depth) and elsewhere on the root system (Fig. 2.1).

Effectiveness of nodules can generally be gauged by the degree of pink or red coloration of N_2-fixing bacteroid tissue inside each nodule. As a general rule, white or green nodules are inactive and would not be considered when classifying currently active nodulation. The rankings depicted in Figure 2.1 and described in Corbin et al. (1977) should be regarded as a guide only; they should be reconsidered for other species in different environments. Ideally, visual ratings should be consistently done by one person throughout an experiment, but if more than one person is involved, division of labour should be on the basis of replicates and not treatments. The procedure involves carefully digging-up 20 plants at random across a crop (ensuring the root system and nodules are recovered) and scoring each plant using a predetermined classification criteria. The scores from all plants are added and then divided by 20 to obtain a mean nodule score. A mean nodule score of:

4 – 5 represents excellent nodulation; excellent potential for N_2 fixation
3 – 4 represents good nodulation; good potential for N_2 fixation
2 – 3 represents fair nodulation; N_2 fixation may not be sufficient to supply the N demand of the crop.
0 – 2 represents poor nodulation, little or no N_2 fixation.

3
ANALYSIS OF NITROGEN

Regardless of the method used to measure N_2 fixation, it is necessary to determine amounts of total plant and crop N if inputs of N by N_2 fixation are to be quantified in terms of kg N/ha. Evaluation and interpretation of N_2 fixation data can also be assisted by measurements of soil N status. Procedures which have been found to be most useful in the authors' laboratories for accurate and reproducible measures of plant and soil N will be detailed in this chapter. The analytical methods described have been adapted to include precautions necessary to allow the precise measurement of low levels of ^{15}N (Table 6.1; see Bergersen 1988), but they are also suitable for N analysis for all purposes.

3.1 Total plant nitrogen

There are two methods upon which all commonly used analyses are based. The first is the oxidative method based on the original Dumas technique, in which organic material is oxidised in the presence of copper oxide to produce N_2 gas, the volume of which is measured. Incomplete combustion can be a problem with this method (only 70–80% of the sample is converted to N_2 without mixing during combustion, 90% with mixing, and up to 99% conversion with addition of potassium perchlorate; Fielder 1984) and it may not be suitable for very precise determinations of low levels of ^{15}N, particularly if there is variation in ^{15}N abundance or dry matter N content between different plant tissues and there is insufficient care taken in subsampling (section 3.1.2.2). Commonly, the 'wet' Kjeldahl digestion is used. In this, organic and mineral N is reduced to NH_3 in hot, concentrated sulphuric acid in the presence of a catalyst. The NH_3 is recovered by distillation or diffusion and estimated by titration or colorimetrically (Bergersen 1980).

3.1.1 Sampling

Difficulties in obtaining accurate estimates of crop dry matter and N content generally result from sampling errors rather than errors associated with subsequent analyses. The principles of plant sampling and subsampling are discussed by Hunt et al. (1987).

Plant samples should be collected from the field using a predetermined pattern, which should be followed for all plots or sampling areas. Generally, it is preferable to collect samples from treatment replicates as fixed lengths of rows (e.g. 1 m) or quadrat areas (e.g. 300 × 300 mm). Sometimes it will be necessary to use individual plants, but this usually gives less precision and if small numbers are involved, can bias the estimate (see Hunt et al. 1987). It is desirable also to include roots in the analyses. However, in many soils, recovery of roots may be so difficult that the analysis must be confined to above-ground plant parts, all of which must be included.

3.1.2 Preparing samples for analysis

3.1.2.1 Drying

Plant samples must be dried to constant weight at 75°C (usually for 48 hours) in an air circulating oven. Very bulky samples should be spread on metal trays and returned to the bags when dry. Dry weight of the total sample is immediately recorded.

3.1.2.2 Subsampling and preparation for digestion

Weighed, dry samples should be chopped or coarsely ground. A suitable implement is a Wiley Mill No. 3, fitted with a 1 mm screen and a hopper for feeding bulky samples. The total ground sample is then *thoroughly mixed* on a sheet of paper or plastic and a sub-sample of 5–10 g stored in a screw-capped container. The entire sub-sample is later ground, using a finer screen on the above mill, or preferably, ground to a fine powder in a hammer mill fitted with a 0.2 mm screen. Alternatively, a laboratory ring grinder (e.g. Roklabs Pty Ltd, Auckland, N.Z.) produces very finely ground samples. Finely-ground sub-samples are returned to the container and stored dry until analysed.

Mills and grinders should be cleaned between samples using an air jet or brushed out carefully. This is particularly important when ^{15}N analysis is to follow. *Samples for determination of natural abundance of ^{15}N should be ground in batches at separate times from ^{15}N enriched samples, and extreme care taken to avoid contamination between samples* (see Bergersen 1988).

In wet or humid climates, finely ground samples should be again dried to constant weight and thoroughly mixed before analysis. Well-mixed sub-samples of about 300 mg of finely ground plant material are weighed to within ±0.001 g and transferred to the digestion vessels (see Table 3.1 for appropriate sample size for different tissues).

At all stages in preparation of samples, it must be remembered that non-uniform sub-samples are a major source of error. *All material should be thoroughly mixed before sub-samples are taken.* Vibration during storage leads to non-uniformity within containers, even when the contents were originally well-mixed. The fine and coarser material which separate upon storage often differ in both N concentration and ^{15}N abundance (G.L. Turner, unpublished data).

3.1.3 Digestion

3.1.3.1 Reagent

Concentrated analytical grade sulphuric acid (from a freshly-opened or protected bottle)	1 L
Potassium sulphate (to raise the boiling point)	100 g
Powdered metallic selenium	1 g

Place the solids in a 5 or 10 L Erylenmeyer flask. Selenium is toxic and care must be taken to avoid breathing fumes and to use safe disposal practices. Add the acid slowly, avoiding drips. The normal procedures for use with strong, fuming acids are

usually adequate. Heat the flask to 200°C in a large tray of sand, of sufficient volume to retain all the acid if the flask breaks. When dissolved completely, the liquid is straw coloured. Turn off the heat and stopper the flask with a rubber stopper bearing an air inlet tube filled with acid-soaked glass beads, to protect the flask contents from contamination with NH_3 which is always present in laboratory air drawn into the flask as it cools. When cool, transfer the reagent to a tightly-stoppered bottle. *(Note: Smokers are a hazard: tobacco smoke contains free NH_3 and other N-containing materials. Also, volatile nitrogenous compounds should be stored well away from digestion reagent.)* The reagent described above has been found to be as good as, or better than any published formulations containing other catalysts such as Hg or Cu. Complete recovery of N from compounds such as tryptophane or nicotinic acid is usual when digestion is continued for 1 to 2 hours after clearing.

3.1.3.2 Apparatus

Although conventional digestions in long-necked flasks are still used, as well as small-scale digestions in 100 mL Erylenmeyer flasks on temperature-controlled, electrically-heated hot plates (Bergersen 1980), the following methods are preferred when ^{15}N analysis is to follow.

Digestion vessels for plant material are standard Quickfit Pyrex tubes with standard taper (24/29 mm) tops and ground stoppers. The internal diameter is approximately 25 mm, the outside diameter 27 mm and the height 170 mm. *(Note:* Some manufacturers supply tubes of different dimensions; use the appropriate tubes for the hole dimensions of the digestion block.) It is convenient to transfer weighed (approximately 300 mg) samples of finely ground plant material to a set of numbered tubes; stopper and store them in a rack until digestion begins.

The tubes are heated in a metal block, bored to accept the digestion tubes with a clearance of about 1 mm when cold, and to a total depth about 30 mm. The metal blocks may be heated directly from an embedded heating element as in several commercially-available units (e.g. Fig. 3.1), or a block may rest on an electric hot plate. If workshop facilities are available, digestion blocks can be constructed of brass (more resistant to spilt acid than aluminium) or of cast iron and the surface covered with compressed insulating material to minimise radiant heating of the upper parts of the digestion tubes. Photographs and the circuit diagrams of the electronic controllers of such a unit can be obtained on request from the authors.

Digestions should be carried out in a well-ventilated fume hood. As will be seen below, well-conducted digestions should generate little acidic vapour once initial oxidation is completed. The digestion block temperature should be adjusted with full load when the fume hood is operating with the door lowered to the level of the tops of the tubes (i.e. maximum cooling, see Fig. 3.1).

3.1.3.3 Procedure

(a) Switch on the digestion block heater and bring it to the digestion temperature, as indicated by a thermometer placed in an unused tube near the centre of the block. The tube temperature is usually a few degrees below the block temperature. *Control of digestion temperature is very important.* On no account

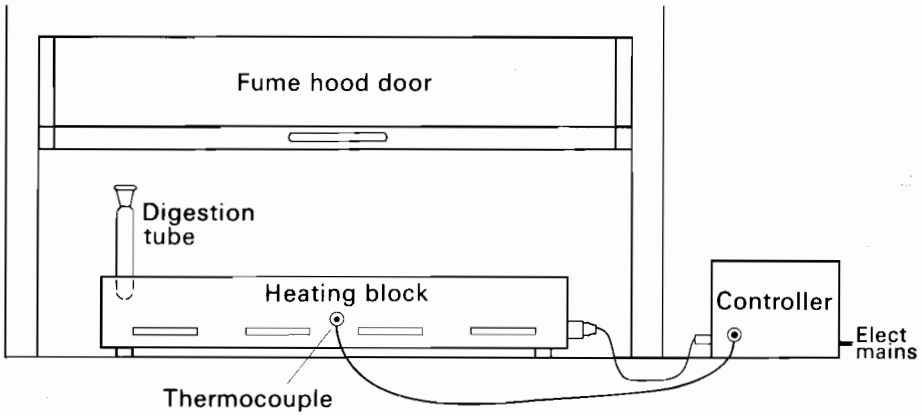

Fig. 3.1 Apparatus used in the digestion of plant material for total nitrogen and ^{15}N determinations. See text for description.

should 320°C be exceeded and 280–310°C has proved best. Higher temperatures than these may produce faster digestion, but there is loss of N due to thermal decomposition of $(NH_4)_2SO_4$ in the acid, even when the refluxing of acid is controlled well below the top of the tubes. Such losses, even if barely detectable, discriminate isotopically and lead to raised ^{15}N analyses in the digests (i.e. the N lost is depleted in ^{15}N; Bergersen 1988). Digestion of 300-500 mg of plant material usually takes approximately 16 hours at 300°C.

(b) When the block is hot, remove the stoppers from 4 tubes and add an appropriate volume of digestion reagent (Table 3.1) to the weighed, finely ground sample in the digestion tube, using a bulb pipette or glass dispensing unit (such as the EMIL Pressmatic Dispenser, Mk2); *do not pipette digestion acid by mouth,* or allow drips to run down the outside of the tubes.

(c) *Place the tubes in the hot digestion block* and watch carefully because initially there will be charring and frothing. Control of frothing is best achieved by adding a drop of cold distilled water from a clean glass pasteur pipette fitted with a rubber bulb. If the drop is allowed to fall into a rising froth plug, near the wall of the tube, the plug is broken or collapsed and rises no further. If this does not succeed lift the tube from the block before the upper half of the tube is contaminated. When the froth collapses, continue heating. It may be necessary to repeat the water treatment once or twice, until the initial frothing ceases. Then proceed with the next 4 samples. *(Note:* frothing is greater if the tubes are placed in the block before it reaches operating temperature.)

(d) When the entire batch of samples is digesting smoothly, check that the acid is refluxing no higher than 70 mm above the surface. If it is higher, reduce the digestion temperature slightly, or preferably, increase the draught of air by adjusting the fume hood opening and/or adjusting the fume hood exhaust fan speed; this will decrease the temperature of the upper half of the digestion tubes.

Once these adjustments have been made, they should be the same for all subsequent digestions.

There may be some charred material adhering to the glass above the area of the tube wall which is being washed with refluxing acid. If so, remove the tube from the block and carefully swirl the contents to wash down the charred material. This is hazardous and not usually successful without practice. Therefore, cool the tube in a rack and then wash down the walls of the tube with several minimal quantities (1–2 mL) of distilled water directed at the contaminated surface. Do not allow the tip of the wash bottle to contact the inner surface of the tube (i.e. avoid contamination). After each small volume of water, swirl the digesting fluid gently to mix with the acid, which may splutter dangerously if the water directly penetrates the surface, instead of running down the tube wall. Return the tubes to the digestion block and continue heating. If the volume of added water is too great, avoid excessive boiling by reducing the block temperature to 150°C until all the water is evaporated, then return to 310°C and continue the digestion for 1–2 hours after the digest becomes colourless.

For some plant samples which contain much mineral material (especially Fe), the digest may remain yellow-straw coloured even when digestion is complete. This colour is readily distinguishable from the pale brown colour due to traces of undigested organic material which is seen just before digestion is complete.

(e) When digestion is complete, remove the tubes, stopper with clean, dry stoppers and cool.

(f) Next add 10 mL of distilled water gradually, with swirling, to prevent overheating. Transfer the diluted digest quantitatively to the volumetric flask (Table 3.1), using a small glass funnel (well washed between samples) and several small volumes of distilled water washed down the sides of the tube and

Table 3.1 Sizes of plant samples, digestion, dilution and distillation volumes for the provision of at least 4 replicate distillates containing about 1 mg of plant nitrogen for mass spectrometer analysis. (Based on field-grown soybeans.)

	% N	Dry sample (mg)	Digestion reagent (mL)	Diluted digest[a] (mL)	Volume distilled[b] (mL)	N in distillate (mg)
Seed (3.5–6)[c]	6	250	5	50	5	1.5
	5	250	5	50	5	1.25
	4	300	5	50	5	1.2
Leaves (1.5–4.5)	3	300	5	50	5	0.9
Entire shoot (1.5–3.5)	2	300	5	25	5	1.2
Entire plants (1–3)	1	500	7	25	5	1.0
Roots (0.5–1.5)	0.5	1000	10	25	5	1.0

[a] Volumes of dilutions prepared in volumetric flasks.

[b] Accurate pipetted volumes (5±0.05 mL.).

[c] Range of expected %N values for each plant tissue.

swirled to transfer every trace of acid from the tube to the flask. Finally wash the funnel into the volumetric flask and add water to the mark. Stopper the volumetric flask and thoroughly mix the contents.

3.1.3.4 Adjustments for nitrate

Plants usually contain nitrate. When its amount is small, there is usually sufficient carbon present to effect its reduction to ammonia during digestion. However, this may be variable and if there is reason to suspect that significant amounts of nitrate are present it is advisable to modify the digestion procedure. There are several suitable methods—the simplest is to convert the nitrate into nitrosalicylic acid, which digests readily to produce ammonia.

The weighed sample in the digestion vessel is well mixed with 1–3 mL of a solution of pure salicylic acid (5% w/v in concentrated sulphuric acid) for at least 20 minutes. Sodium thiosulphate (0.3–1 g) is then added and the mixture gently heated until fuming. Then it is cooled, digestion reagent is added and digestion is carried out as described previously.

3.1.4 Recovery of ammonia by distillation and estimation by titration

3.1.4.1 Reagents

A. Boric acid/indicator

(This reagent contains less boric acid than usually described. It is designed for distillations of 1–5 mg of NH_3–N when mass spectrometer analysis is to follow.)

Boric acid (analytical grade)	10 g
Distilled water	1 L
Mixed indicator	10 mL

(4 mL of 0.1% methyl red + 20 mL of 0.1% bromcresol green, each dissolved in 95% ethanol).

When dissolved, adjust the bulk solution to neutrality with 1% NaOH (i.e. until the indicator is the grey-red end point colour).

B. 10M NaOH

NaOH (analytical grade)	400 g
Distilled water	1 L

C. Phenolphthalein (1% w/v in 50% ethanol).

D. Standard borax

Water at 55°C is saturated with borax (Na-borate; Analytical grade), filtered and allowed to crystallise. The crystals are recovered, redissolved in water at 55° to saturation and again crystallised. The crystals are recovered, and successively washed with ice-cold distilled water, dry ethanol, and dry diethyl ether, before drying on a filter paper. The crystals are finally equilibrated over a solution of

distilled water, saturated with NaCl and sucrose, in a closed container for at least 24 hours. They should be stored also in the same conditions.

E. Standard HCl.

Approximately N/28 HCl: 31.8 mL of analytical grade HCl diluted to 1 L with distilled water and stored in a tightly stoppered bottle. One mL of exactly N/28 HCl (0.03571 N) is equivalent to 0.5 mg N. It should be standardised as follows:

Dissolve approx. 0.3 g of standard borax (weighed to ±0.0005 g) in 50 mL of distilled water in a volumetric flask. An aliquot of 10 mL of this solution is titrated with the acid to be standardised, using one drop of 0.1% ethanolic methyl red as indicator.

$$\text{Normality of acid} = \frac{\text{weight of borax} \times 200}{190.72 \times \text{mL of acid used}}$$

Alternatively, a commercial standard solution of HCl may be used after accurate dilution to give the desired normality.

3.1.4.2 Apparatus

Several commercially available stills are suitable for recovery of NH_3 from Kjeldahl digests (e.g. Fig. 3.2a and b). The NH_3 passing from the condenser in a stream of condensed steam is trapped in boric acid-indicator solution. An adaptation of the commercial designs which has proved superior for use when ^{15}N analysis is to follow, is shown in Fig. 3.2d. The increased volume of the sample chamber diminishes the hazards of alkaline mist, entrapped in the steam flow, affecting the titration which follows distillation.

The other stills are available as standard items from suppliers of Quickfit glassware. The steam generator is assembled with Fig. 3.2c as a guide, from any standard laboratory glassware. If ground glass joints are not available, improvisations with rubber stoppers and glass tubing are quite satisfactory. Although an electric heating mantle is preferred because boiling can be better-controlled (C, Fig. 3.2c) heating with a gas burner is acceptable, providing boiling is steady. This is assisted by the use of oven-dried anti-bumping granules added to the steam generator as required. Flow and temperature of the condenser cooling water should be such that condensate drips from the condenser at about 30°C. If this cannot be done easily, reduce the flow of steam by adjusting the heating rate, or by partially venting the steam from the generator.

Titration is done in the standard way with a suitable burette, graduated to read in units of 0.01 mL. Suitable types may also be fitted with a reagent reservoir and filling mechanism. While this is an advantage, it is not an essential feature. Alternatively an electronically or manually controlled piston burette may be used. These can give more precision to titrations, making titration error a negligible part of the overall error of estimating N. A suitable instrument is the Dosimat (655 Dosimat; Metrohm, Herisau, Switzerland, which can if desired be coupled to an automatic unit for pH titration; Metrohm Titrator E526).

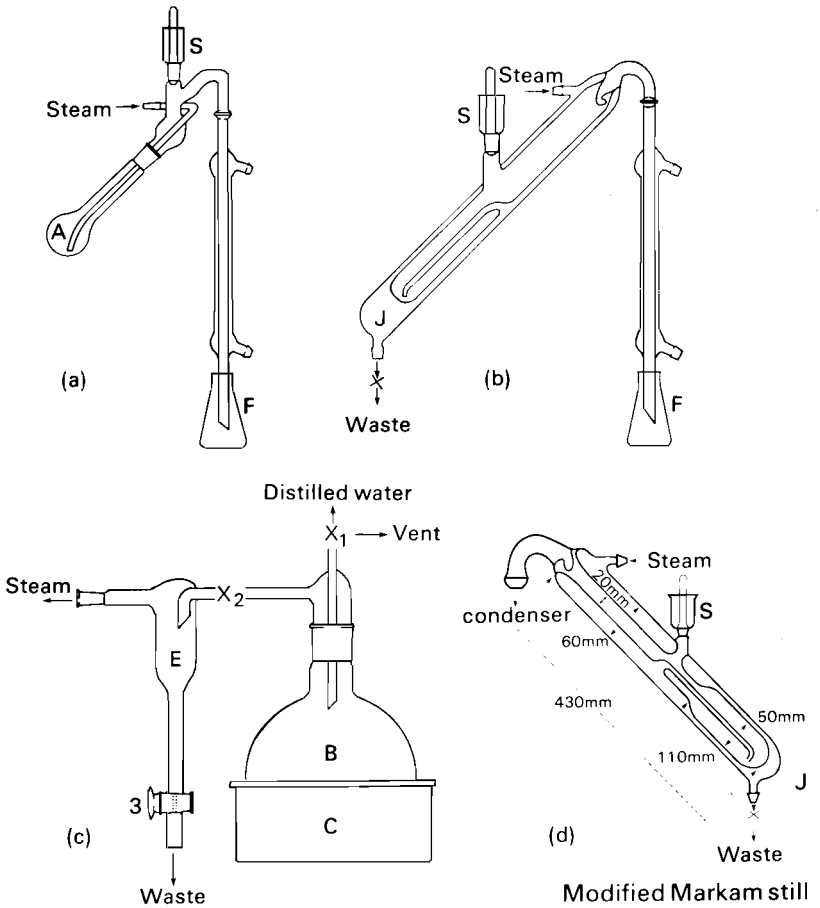

Fig. 3.2 Apparatus used in the distillation and recovery of ammonia (after Bergersen 1980). (a) Quickfit semi-micro Kjeldahl assembly (digestion flask (A) of 50 or 100 mL capacity). (b) Quickfit Markham still (type 46 Mc). (c) A steam generator assembly including a 2–3 litre distillation flask (B) heated with an electric mantle (C), and a supply of distilled water connected through a two-way stopcock (X_1). (d) Modified Markham still. See text for description.

3.1.4.3 Distillation

(a) Before use, the still should be thoroughly cleaned. A chromic acid wash, followed by rinsing in 10% (v/v) HCl and several washes in hot water, followed by several rinses in distilled water, is suggested. The steam generator flask should be cleaned in similar fashion, before filling with distilled water to half its volume. Add 0.5 to 1 mL of concentrated H_2SO_4/L of water to prevent contamination of the steam from traces of dissolved NH_3. The level of water in the steam generator should not be allowed to fall below 50% of the initial volume. Pass steam through the distillation assembly for 10 minutes before the first distillation.

(b) Place 10 mL of boric acid-indicator in a 150 mL Erylenmeyer flask (F, Fig. 3.2) and place under the condenser outlet with the tip immersed in the solution.

(c) Allow steam to flow through the still continuously by opening X_2 and closing X_1 (Fig. 3.2c) on the steam generator, until the entire still is hot and all condensed droplets on the inner surfaces have been vaporised or flushed off with freshly condensing water.

(d) Place 5 mL (see Table 3.1, so that sample contains at least 1 mg N) of diluted digest in the entry port (S, Fig. 3.2a, b, d). Run the liquid into the still by raising the stopper slightly and rinse in slowly with water and a drop of phenolphthalein indicator solution.

(e) Slowly and as in (d) add sufficient 10M NaOH to render the digest alkaline to phenolphthalein (pH > 11). For 5 mL of digested plant material, 8 mL is usually sufficient.

(f) Continue passing steam until *at least 80 mL* of distillate has collected in the receiving flask. Then lower the flask and rinse off the tip with distilled water from a wash bottle. This large collection volume ensures completeness of recovery, but it should be increased if the volume of sample is greater than 5–10 mL. Observe the precautions noted above, with reference to condensate temperature.

(g) Remove the flask for titration of the distillate.

(h) Vent the steam generator by opening X_1 and close the steam line X_2 (Fig. 3.2c). Fill the entry port with distilled water. As the steam condenses in space E (Fig. 3.2c), the partial vacuum which is generated sucks the residue of the distillation into E. Immediately lift the stopper (S, Fig. 3.2a, b, d) and run in the distilled water and repeat this washing twice. Finally, discharge waste and washings collected in J (Fig. 3.2b, d) through the waste lines and restart the steam flow as soon as possible. *Note:* This procedure may leave some residual NaOH adhering to the glass inside the apparatus. Because of this it is essential that the condenser tip be immersed before adding the next sample so that loss of NH_3 by premature distillation is avoided (see (b) above). It is also essential that all condensed steam in the upper part of the apparatus be removed in the flow of hot steam before adding the next sample. Hang-up of NH_3 dissolved in condensed water can be a problem, slowing completion of recovery.

When samples containing ^{15}N are being distilled, additional precautions must be observed in order to avoid possible contamination from trace amounts of highly labelled NH_3 remaining in the still. Some workers advise the distillation of about 15 mL of ethanol between samples to remove traces of NH_3 adhering to the glass surfaces of the still. In our laboratory, no cross-contamination between enriched samples has been encountered with the modified Markham still (Fig. 3.1 d), provided a total of about 80 mL of distillate is collected for each sample. Samples for measurement of natural abundance of ^{15}N should be distilled in apparatus dedicated to this purpose. Samples enriched in ^{15}N should be processed with a different still and it is also advisable to distil a blank between samples: the upper parts of the apparatus should not be allowed to cool whilst contaminated with residual NH_3 from an imperfectly recovered sample.

3.1.4.4 Titration

When sufficient distillate has been collected (80–100 mL), the tip of the condenser is rinsed into the flask and the contents immediately titrated with standard HCl until the indicator reaches the grey-red end point. The titration volume is recorded and one more drop added, after which the indicator should have reached a definite pink-red colour.

The calculation of N present is:

$$\text{mgN} = \frac{\text{normality of standard. HCl}}{0.03571} \times 0.5 \times \text{titration (mL)}$$

The titrated distillate is a source of N for estimation of ^{15}N. If this is to follow, each distillate should be acidified with 1–2 drops of 1% H_2SO_4 and its volume reduced by boiling until it contains approximately 1 mgN/mL. *(Note:* avoid concentrating samples to dryness; see Table 6.1.) Samples may then be stored in sealed containers until analysis.

3.1.5 Colorimetric estimation of N in distillates

The most convenient colorimetric assay for ammonia–nitrogen is described below. This procedure reliably measures up to 14 µg N in a 1 mL sample.

3.1.5.1 Reagents

A.	Phenol	50 g
	Na nitroprusside	0.25 g
	Distilled H_2O	1 L
B.	NaOH	25 g
	Na hypochlorite	2.1 g
	(28.2 mL of B.D.H. Analar solution containing 1N hypochlorite in 0.1N NaOH or an equivalent volume of another convenient source of hypochlorite in NaOH.)	
	Distilled H_2O	1 L

For use, dilute both A and B (1 part) with 4 parts of distilled H_2O.

C. Standard $(NH_4)_2SO_4$ (20 µg N/mL)

$(NH_4)_2SO_4$ (analytical grade)	0.0943 g
H_2O	1 L.

3.1.5.2 Procedure

Make the distillate to 100 mL in a volumetric flask. If the quantities recommended in Table 3.1 have been used, the distillate will contain about 1 mg of N, so 1.0 mL of the distillate should contain about 10 µg of N. Place 1.0 mL in a 20 mL volume test

tube. Prepare a set of standards in similar tubes by pipetting 0 (blank), 0.25, 0.50, 0.75 and 1.0 mL of solution C and adding sufficient water to bring volumes to 1.0 mL. Add 5 mL of each of diluted reagents A and B to each tube and leave the colour to develop for at least 30 minutes. Record the optical absorbance at 625 nm with a suitable spectrophotometer, prepare the standard curve and read off the values of the distillates from this, and calculate the N present in the distillate as follows:

$$\text{mg N in distillate} = \frac{\mu\text{g N from standard curve}}{10}$$

3.2 Total soil nitrogen

Studies of N_2 fixation in the field are frequently aided by knowledge of the soil N status. The following methods are suitable for use when ^{15}N analysis is to follow.

3.2.1 Sampling

These analyses are similar to analysis of total N in plant samples and the same principles of sampling and subsampling apply. There are however, some important differences. They are based on weighed oven-dried samples of soil, but in addition the relationship between bulk density and moisture content needs to be recorded. To do this, samples are usually taken with a cylindrical coring device of known diameter to a defined depth. This volume of soil is weighed accurately, broken up and well mixed, sieved and a weighed sub-sample (3–5 g for top soil) is taken for drying, weighing and digestion. Samples from lower in the profile (below 30 cm) will usually contain less N. Soil samples which are too wet for sieving and sub-sampling are usually slurried with water and a sub-sample of known proportion by volume used for the analysis.

3.2.2 Digestion

Digestion reagent (see Section 3.1.3.1) (5–10 mL) is added to the dry soil sample in a standard Kjeldahl flask and digested with frequent agitation to break up the mineral material which remains as a dense mass in the bottom of the vessel. Tube digestions are only possible if a digestor block is available with large holes to accept tubes of 40–50 mm diameter. When digestion is complete, the entire contents are washed into a 500 mL round-bottom flask with a single side-arm (A, Fig. 3.3) and the residue extracted with several portions of water, allowed to settle and the supernatant decanted and added to the digest.

3.2.3 Distillation and estimation of N

Distillation and titration or colorimetric analysis are done as described above (Sections 3.1.4 and 3.1.5).

3.3 Extractable mineral nitrogen

Of greater importance in N_2 fixation studies is the level of soil mineral N (nitrate and ammonium). Mineral N is labile and subject to isotopically discriminating biological and chemical reactions during transport and storage. Furthermore, the levels of N

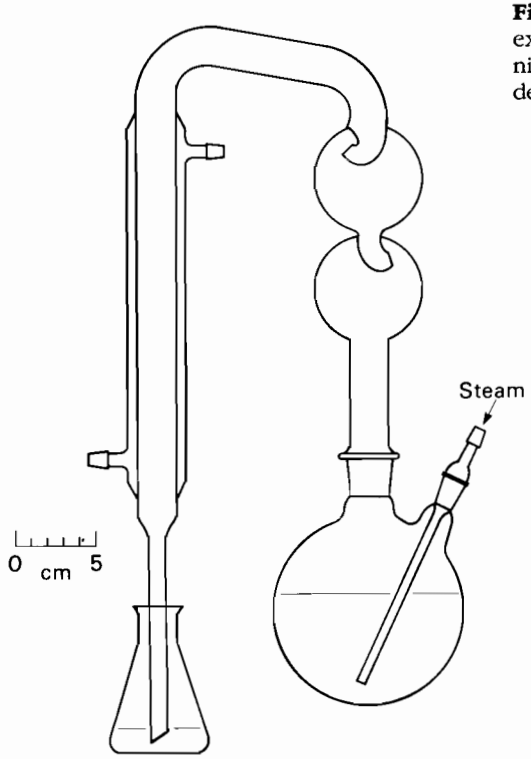

Fig. 3.3 Modified apparatus used in the extraction and steam distillation of nitrogen from soil. See text for description.

extracted can change rapidly following sampling (Westfall et al. 1978). The following procedures have been found to minimise these effects and give good isotopic agreement between the mineral N extracted from soil and the N assimilated by plants from the same soil.

3.3.1 Sampling

Samples are collected as cores as described (3.2.1) above. They are immediately chilled in ice, stored at –10°C as soon as possible thereafter and kept frozen until thawed for analysis. Alternatively, the biological and chemical reactions referred to above may be prevented by adding the extraction reagent and mixing thoroughly immediately after sampling (e.g. see Herridge et al. 1984). The salts in the extractant effectively prevent biological modifications of the mineral N. In this case, parallel cores are taken for estimations of fresh weight, dry weight and bulk density.

Note: Air-dried samples should be avoided, since air-drying elevates the ammonium to nitrate ratio (which may be differentially enriched in ^{15}N) and increases analytical errors by substantially raising the levels of mineral N extracted (see Westfall et al. 1978).

If soil samples are too wet for sieving and mixing when taken from the coring device, a 300 g bulk sample is mixed with 100 mL of distilled H_2O to form a

slurry. Two aliquots (140 g each) of the slurry are weighed and 60 mL of 3.5M KCl added to bring the aqueous suspension to 2M. Record the total volume of liquid added.

3.3.2 Extraction

3.3.2.1 Reagents

A. Extractant

2M KCl (74.55 g/L)

B. Alkali

Prepare a quantity of freshly-calcined heavy magnesium oxide (heat to 400-450°C overnight, in shallow layers in ceramic dishes. Cool and store in a tightly-stoppered bottle in a desiccator for no more than 1 week to avoid formation of $MgCO_3$). Immediately before use, prepare a suspension, 121% (w/v) in distilled H_2O.

C. Devarda's Alloy (BDH Laboratory Chemicals, Poole, England)

Grind finely in a pestle and mortar. *(Note:* this material is sometimes of variable quality and the amount used may need to be increased in order to ensure completion of the reduction of nitrate to ammonia. Grinding finely increases the effectiveness.)

D. Boric Acid/Indicator:

As described in 3.1.4.2A.

3.3.2.2 Apparatus

A. Erlenmyer flasks (500) mL with rubber stoppers.

B. Reciprocating or wrist-action shaker.

C. Glass funnels and Whatman No. 1 filter paper..

D. Steam distillation apparatus such as illustrated in Figure 3.3. Alternatively an equivalent apparatus may be assembled from Quickfit components.) The double splash-head shown in Figure 3.3 minimises the danger of alkaline mist entering the steam flow during Devarda's alloy reductions. The steam generator used with the Markham still (Fig. 3.2c) is used to supply steam at the port indicated.

E. Wide-mouthed 10 mL delivery pipette made by filing away the tip of a standard pipette.

3.3.2.3 Procedure

Shake 100 g of sieved dry soil (or equivalent weight of wet soil) with 100 mL *(v)* of

2M KCl for 1 hour in stoppered flasks. Filter the slurry and measure the volume of the extract (*x* mL and correct all results on the basis of *v/x).* If the soil is infertile, or is from lower levels of the soil profile, it may be necessary to increase the weight extracted in order to recover sufficient N for isotopic analysis. Keep the ratio of soil to extractant as above (100 g/100 mL).

3.3.3 Distillation of NH_3

Place the extract in the flask of the distillation apparatus and place 10 mL of boric acid-indicator in a 150 mL Erlenmeyer flask beneath the condenser outlet. Then add by wide-mouthed pipette, through the steam side-arm 10 mL of the MgO suspension, so that it runs beneath the surface of the extract. Connect the steam line and pass steam vigorously until at least 80 mL of distillate is collected. Remove the collecting flask and replace with another.

3.3.4 Reduction of nitrate and distillation of NH_3

With the condenser tip immersed, carefully add 0.5 g of Devarda's Alloy through the steam sidearm. Immediately connect the steam line and recommence distillation until a further 80 mL is collected. *Caution:* At this stage it is important to avoid rendering the steam flow alkaline with mist caused by the effervescence. This will produce titration errors. The problem is usually indicated by unexpectedly large titrations and/or unusual colour changes in the indicator during distillation. If these are noted, redistillation of the distillate as for ammonia (Section 3.3.3) is indicated.

3.3.5 Estimation of N

This is done by titration (as in Section 3.1.4.4) or colorimetrically (3.1.5).

4
XYLEM – SOLUTE TECHNIQUE

4.1 Principles behind the method

Xylem sap carries N-containing compounds from the roots to the shoots of field-grown legumes originating from (i) nodules as assimilation-products of N_2 fixation, and (ii) soil mineral N taken up by the roots (Fig. 4.1). If there are well established differences in xylem N-solute composition between fully symbiotic plants and non-nodulated plants which are totally dependent upon soil N, it should be possible to devise an assay system based on analysis of xylem sap to assess the extent to which plants rely on N_2 fixation or soil mineral N.

Nitrate and ammonium ions are the two most important forms of N taken up by plant roots in both fertilised and unfertilised soils. In most arable, agricultural soils where nitrification takes place rapidly, nitrate is believed to be the predominant N source for plant growth. Solutes derived from soil mineral N under these conditions will be transported in the xylem as free nitrate or as organic products of nitrate reduction (principally the amino acid asparagine) in the root (Fig. 4.1). Ammonium nutrition, however, could become important when legumes are used as catch-crops in rice-based cropping systems or when grown in acid soils or in soils with a high C : N ratio. In these instances ammonium must be rapidly incorporated into organic compounds (largely amino acids) to avoid its toxic action in cells and will be exported to the shoot in the xylem in this form.

A wide range of different N-solutes have been identified in xylem exudates collected from effectively nodulated legumes solely dependent upon N_2 fixation for growth. However, generally one or two N-rich molecules, characteristic of the species, dominate the spectrum of organic N compounds transported to the shoots in xylem sap (Fig. 4.2). Many legumes of tropical origin (Table 4.1) transport the bulk of their fixed N from nodules in the form of the ureides, allantoin and allantoic acid (e.g. soybean, Fig. 4.2). Ureides have also been detected as minor components of xylem sap in some species (Table 4.1), but since exudate samples were collected in some instances from field-grown plants whose symbiotic status was unknown, the significance of ureides to the N-economy of these plants remains uncertain. In most of the other legumes which have been studied, nodule products are exported predominantly as the amides, asparagine and glutamine (e.g. groundnut, Fig. 4.2).

4.1.1 Ureide-exporters

Root nitrate reductase activity characteristically assumes a minor role in assimilating nitrate in legume species which export ureides from their nodules. As a

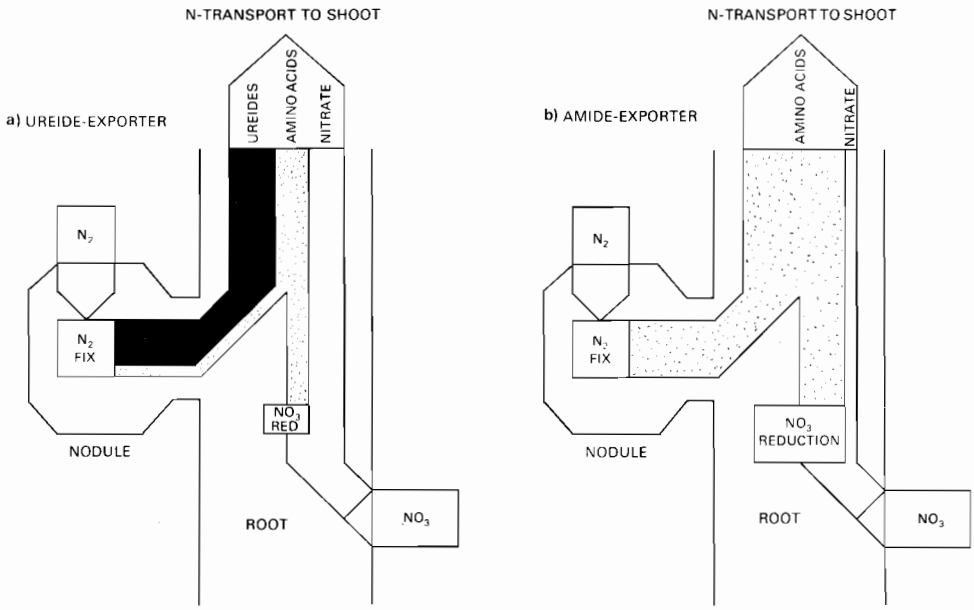

Fig. 4.1 Pathways of nitrogen transport from the nodulated root systems of (a) ureide-exporting, and (b) amide-exporting legumes reliant on both symbiotic N_2 fixation and nitrate uptake from soil for growth. The areas indicating nitrate reduction are proportional to the relative extents of nitrate metabolism occurring within the roots of species from each class of legume (after Ledgard and Peoples 1988).

consequence, much of the nitrate taken up is transported to the shoot in an unreduced form (Fig. 4.1.a). The composition of xylem exudate of nodulated plants therefore changes progressively from one dominated by ureides to one dominated by nitrate and amino compounds as the plant's dependence upon N_2 fixation declines in response to increased uptake of nitrate by the roots (Fig. 4.3a, b). Such changes in xylem N-solute composition have been found to be so predictable that the relative abundance of ureides (estimate of ureide-N as a proportion of total xylem sap N, e.g. Fig. 4.3c) of field-collected samples has been used as a measure of the N_2-fixing status of a number of legumes within this broad group of plant species (Herridge et al. 1984; Norhayati et al. 1988; Rerkasem et al. 1988).

4.1.2 Amide-exporters

The response of amide-exporting legumes to changes in N-source is less well defined than ureide-producing species since the products of N_2 fixation and nitrate uptake are essentially the same (Fig. 4.1b). 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 source of N for growth is changed 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 content as plants increasingly rely upon uptake of nitrate from the rooting-medium (Fig. 4.4a). Under these conditions, the relative level of

Table 4.1 Occurrence of ureides in xylem sap of nodulated legumes[a].

Species in which ureides are major components of solute N[b]	Species in which ureides have been detected as a minor component[c]	Species in which ureides have not been detected
Albizia lophantha	*Albizia falcataria*	*Acacia alata*
Cajanus cajan	*Bossaiae aquifolium*	*auriculaformis*
Calopogonium caeruleum	*Erythrina variegata*	*extensa*
Centrosema spp.	*Flemingia congesta*	*insauvis*
Codariocalyx gyroides	*Gliricidia sepium*	*pulchella*
Cyamopsis tetragonoloba	*Pisum arvense*	*Arachis hypogaea*
Desmodium discolor	*Sesbania rostrata*	*Bauhinia* spp.
rensonii	*sesban*	*Caesalpinia*
uncinatum	*Stylosanthes hamata*	*calothyrsus*
Glycine max	*Vicia ervilia*	*Calliandra* spp.
Hardenbergia spp.	*sativa*	*Cicer arietinum*
Lablab purpureus	*Viminaria juncea*	*Clitoria* spp.
Macroptilium atropurpureum		*Derris elliptica*
Macrotyloma uniflorum		*Juncea* spp.
Pueraria javanica		*Lathyrus cicera*
phaseoloides		*sativus*
Phaseolus vulgaris		*Leucaena* spp.
lunatus		*Lens culinaris*
Psophocarpus tetragonolobus		*Lotus corniculatus*
Tedhegi spp.		*Lupinus albus*
Vigna angularis		*angustifolius*
mungo		*cosentinii*
radiata		*mutabilis*
triloba		*Medicago minima*
unguiculata		*sativa*
umbellata		*Mimosa pigra*
Voandzeia subterranea		*Pisum sativum*
		Sesbania grandiflora
		Trifolium pratense
		subterraneum
		repens
		Vicia monantha
		faba
		Zornia spp.

[a] Information adapted from Norhayati et al. 1988; Peoples et al. 1988b; unpublished data of M.B. Peoples, R.R. Gault, D.F. Herridge and B. Palmer.

[b] 40% or more of total N of xylem sap estimated to be in ureides.

[c] 10–25% of total N of xylem sap collected from glasshouse-grown or field plants estimated to be in ureides.

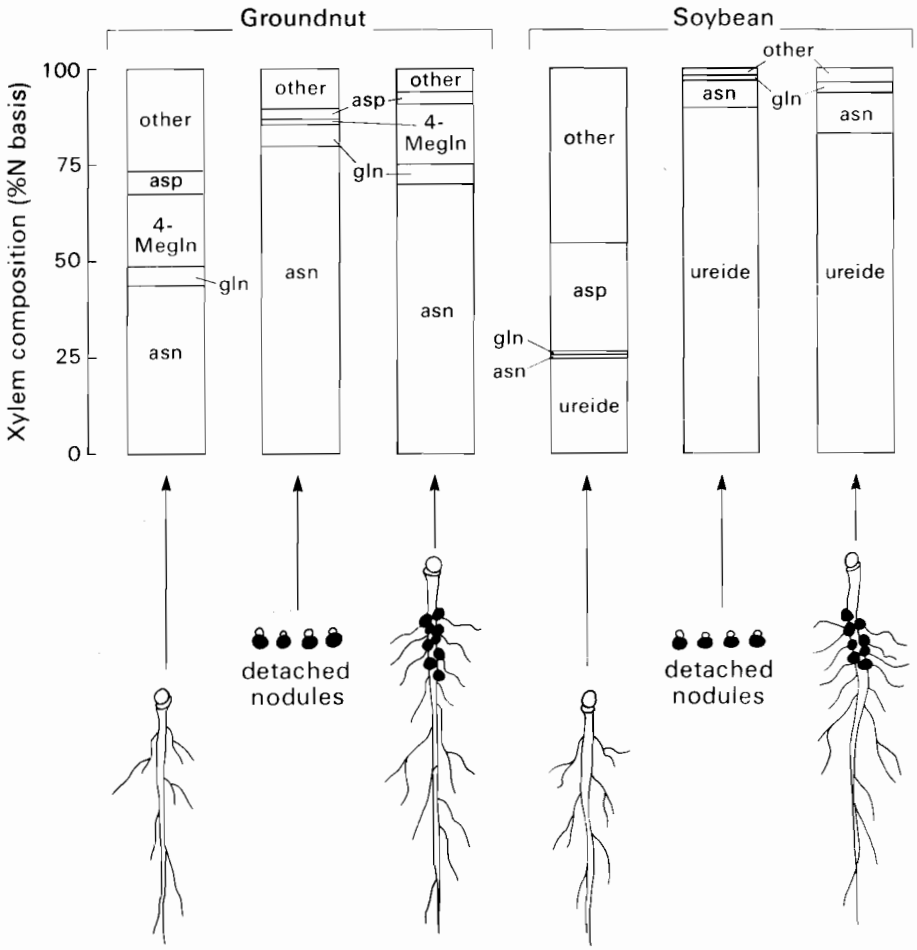

Fig. 4.2 The proportional composition of nitrogenous compounds in xylem exudates of fully symbiotic groundnut *(Arachis hypogaea* L.) and soybean *(Glycine max* [L] Merr.) grown in water-culture. Xylem exudates were collected as bleeding sap from plants decapitated at either the top of the root, or at a site on the root below the lowest nodule, or from detached nodules (after Peoples et al. 1986, 1988a). Asn = asparagine, gln = glutamine, asp = aspartic acid, 4-Megln = 4-Methylene glutamine, other = other amino acids.

nitrate detected in xylem exudate may be regarded as being indicative of the contribution of soil N to plant growth (e.g. Fig. 4.4b).

Before xylem–solute techniques can be utilised in the measurement of N_2 fixation by field-grown legumes, three basic requirements are essential:

(i) a means of sampling xylem N-solutes;

(ii) a calibration curve which relates xylem solute composition to legume reliance on N_2 fixation in the presence of soil N; and

(iii) an understanding of the advantages and possible limitations in the application of the methodology.

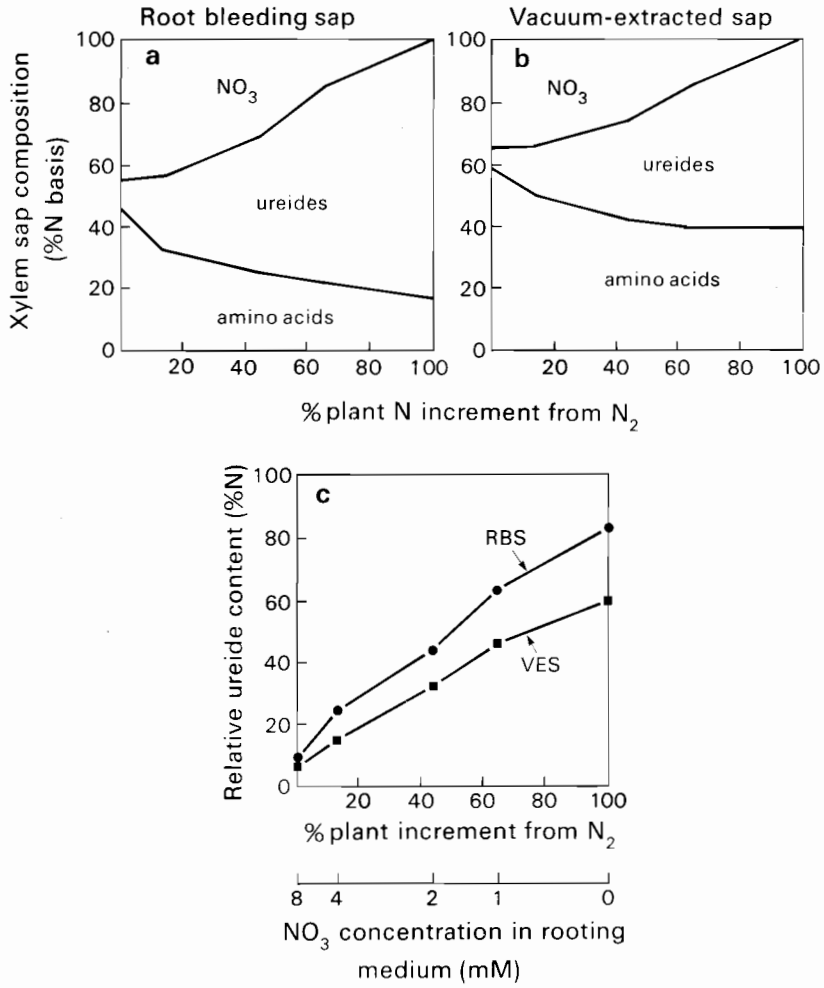

Fig. 4.3 Changes in the N-solute composition of xylem sap collected as (a) root-bleeding exudate, or (b) vacuum-extracted from stems of nodulated soybean fed a range of constantly maintained levels of nitrate, and (c) the relationship between the abundance of ureides and plant dependence upon N_2 fixation. Relative ureide contents of root-bleeding sap (RBS) and vacuum-extracted sap (VES) are expressed as a proportion of total sap N (ureide-N + α-amino-N + nitrate-N). Derived from the data of Herridge (1984), after Peoples et al. (1988a).

4.2 Sampling of N-solutes

It is possible to collect xylem exudate 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 recover root-bleeding exudate from field-grown plants in other environments. Pre-watering field plots may assist plants to bleed but this is not always convenient nor does it guarantee success. As a result, alternative methods for sampling xylem exudate have been examined. One technique for sampling N-solutes involves preparing aqueous extracts from plant tissue (Herridge 1982; Peoples et al. 1987). However, the procedure requires

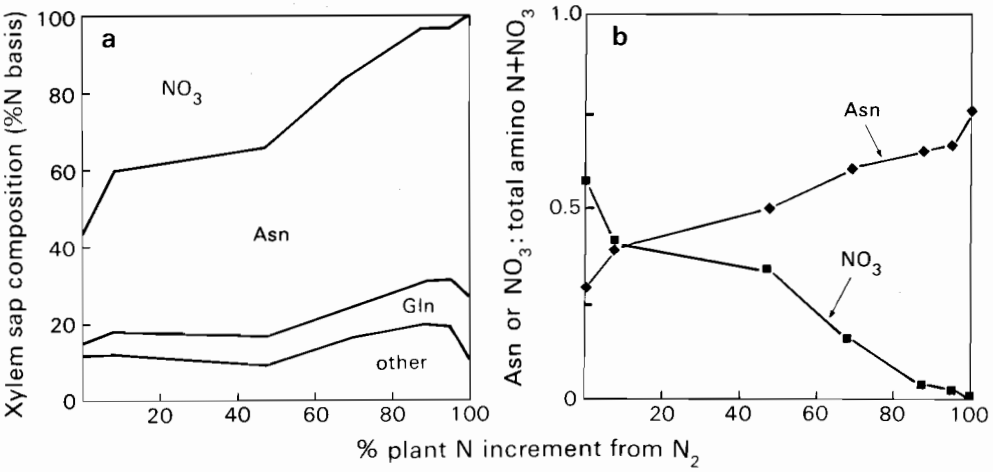

Fig. 4.4 Changes in (a) the xylem exudate N-solute composition of nodulated groundnut fed a range of constantly maintained levels of nitrate, and (b) the relationships between xylem asparagine or nitrate contents (expressed as a fraction of total sap N) and plant dependence upon N_2 fixation. Derived from Peoples et al. (1986). Asn = asparagine, Gln = glutamine, other = those amino acids listed in Peoples et al. (1986).

the collection, drying, grinding, and laboratory extraction of plant material prior to analysis. These laborious and time-consuming steps are avoided by another technique in which the xylem contents of the stem are extracted in the field using a mild vacuum (Herridge et al. 1984, 1988). The sap displaced from the stem xylem strands is available for immediate analysis. The technique has been found to be applicable to many different field-grown legumes under diverse (e.g. drought and water saturated) environmental conditions. Procedures for the three methods of sampling N-solutes are described in the following sections.

4.2.1 Root-bleeding exudate

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

(b) Place a silicon or latex rubber tubing sleeve, 2 to 4 cm long with an internal diameter slightly smaller than the stem, over the exposed root stump (A, Fig. 4.5).

(c) The sap exuding under root-pressure (B, Fig. 4.5) can be easily collected from the tubing sleeve reservoir using a Pasteur pipette or syringe (C, Fig. 4.5). It is advisable to discard the sap exuding during the first minute of bleeding to minimise contamination from cut cells of the stem. It is also recommended that root stumps be allowed to exude for no more than 20-30 minutes before collection since changes in N-solute composition which might occur over longer periods of bleeding could introduce errors in the subsequent analyses and interpretation of xylem data. The accumulated exudate should be collected every few minutes and placed in a sealable tube or vial kept on ice to minimise potential decomposition or metabolism of xylem N-components.

(d) 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.

4.2.2 Tissue extraction

The stem, because it is essentially involved in transport and to a lesser extent storage of solutes, is the most suitable target organ when this technique is used to assess legume N_2-fixing status. Leaves are not suitable. They rapidly metabolise incoming N-compounds; variation in tissue N-solutes can therefore result from changes in plant metabolism which are not related to N_2 fixation. The relative composition of the stem's soluble N component is insensitive to diurnal fluctuations and is unchanged by storage at ambient temperatures of 20–30°C for up to 24 hours before being placed in an oven for drying (Herridge 1982).

The disadvantage of the technique is that cellular-contents and stored N-compounds are extracted in addition to xylem constituents. This method therefore, may not provide as sensitive a measure of symbiotic performance as an equivalent assay developed solely on the basis of xylem sap analysis. Nonetheless, the procedure may represent the only available method of field-sampling N-solutes from very small plants or legume species in which xylem sap cannot be easily recovered by vacuum (e.g. groundnut, lentil, pea) or spontaneous bleeding.

4.2.2.1 Procedure

(a) Harvest shoot material and remove leaves. *(Note:* retain leaves in a separate labelled bag if total crop N determinations are required; Section 3.1.)

(b) Place stem samples in clearly-labelled bags and dry at 75–80°C in a forced-air oven for 2 days.

(c) Record total dry weight if Kjeldahl analysis is required later, and grind tissue to pass a 60-mesh (1.0 mm) screen. Store in a dry place until extraction.

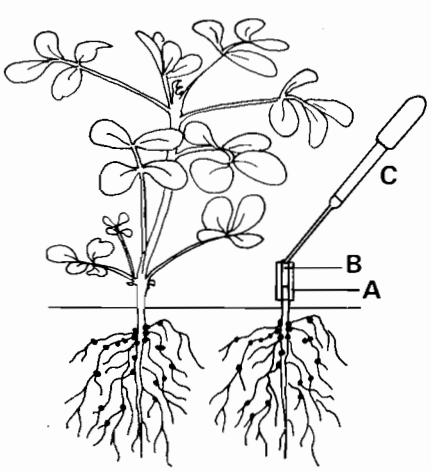

Fig. 4.5 Collection of root-bleeding xylem sap. An appropriately-sized silicon rubber tubing sleeve (A) is placed over the root-stump exposed following the detachment of the shoot. The sap exuding into the tubing (B) can be collected with a Pasteur pipette (C).

4.2.2.2 Extraction

(a) Weigh 0.5 g subsamples of dried and ground stem material and transfer to 100 mL beakers or Erlenmeyer flasks.

(b) Add 25 mL distilled water to each subsample and boil for 1–2 minutes.

(c) Filter whilst 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.

(d) When contents of flask cool, make volume up to 50 mL with distilled water.

(e) The eluant can be stored indefinitely in small vials or flasks in a freezer until analysis of N-solutes.

4.2.3 Vacuum-extracted exudate

The technique has been most extensively characterised for the ureide-exporters soybean (Herridge 1984; Herridge et al. 1988) and pigeon pea (Peoples et al. 1989). The relative N-solute composition of field-extracted xylem samples does not change during the day between 9 a.m. and 4 p.m. and is unaffected by the level of applied vacuum. Generally each replicate sampling represents the bulked collections from 8 to 12 plants; however, sufficient xylem exudate can often be recovered from each stem of mature, field-grown legumes (0.5–1 mL/plant) for complete N-solute analysis (Section 4.3.1) so that the method may also be used on individual plants (Herridge et al. 1988).

4.2.3.1 Equipment requirements

The following items are required:

- 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) and appropriate sized fittings or adaptors (e.g. disposable micropipette tip cut to size).
- 5 mL Vacutainers (Becton Dickinson, Rutherford, New Jersey, USA).
- A source of vacuum. In the field this may be a hand-held vacuum pump (e.g. Nalgene, Sybron Corp., Rochester, New York, USA, as depicted in Fig. 4.6), a foot pump (e.g. Plate 4.1) or consist of 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 Plate 4.2). 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 (Plate 4.3).

4.2.3.2 Procedure

(a) Cut the stem (of greater than 3 mm in diameter to ensure collection of

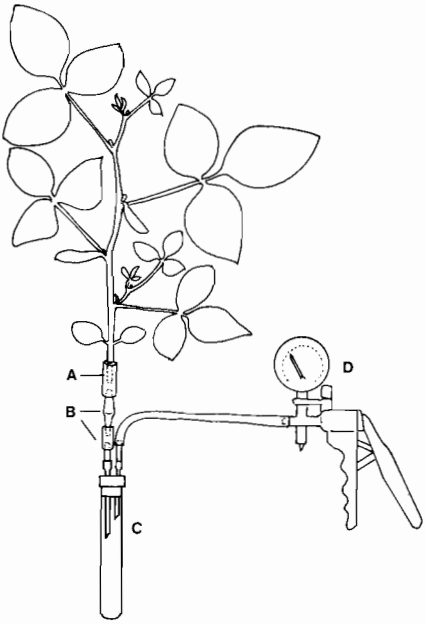

Fig. 4.6 Vacuum-extraction procedure of xylem exudates from shoots. The base of a freshly detached stem is placed into an appropriately-sized silicon rubber tubing sleeve (A) and fitted onto a syringe needle (using an adaptor (B) if necessary). The syringe needle is then inserted through the lid of a Vacutainer (C) connected to a vacuum pump (D) via another syringe needle connection.

reasonable volumes of sap) close to ground-level with secateurs. If the nodes at the base of the stem are compacted (internode lengths of less than 10 mm) 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 added xylem resistance occurring at such vascular junctions.

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 from step (d) can be collected after each replicate sampling and retained.

(b) The detached stem is then immediately inserted into a silicon or latex rubber tubing sleeve with an internal diameter slightly smaller than the stem (A, Fig. 4.6), and fitted onto a syringe needle using an appropriately sized adaptor (B, Fig. 4.6).

(c) The needle is then pushed through the rubber stopper of a 5-mL Vacutainer (C, Fig. 4.6) which has been linked to a vacuum pump (D, Fig. 4.6) via another syringe needle connection and a flexible plastic-tubing line.

Note: The base of the syringe needles must not be level otherwise sap may be sucked into the vacuum pump line rather than collect in the Vacutainer.

(d) Partial vacuum is applied and short segments (3 to 4 cm) of the stem are then cut with secateurs successively from the top to the bottom of the shoot (Plate 4.4) 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.

Note: (i) 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.

(ii) 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.

(iii) It is essential that vacuum extraction of the stem xylem contents commence *immediately after detachment of stems* from the root. A time-delay of more than 5 minutes can introduce errors into subsequent analysis (see Herridge et al. 1988; Peoples et al. 1989).

(iv) A greater volume of sap can be collected if leaves are removed before extraction (Peoples et al. 1989).

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

4.3 Analyses of N-solutes in xylem exudates and tissue extracts

4.3.1 Equipment requirements

The following items are required before commencing the N-solute assays described below.

- Weighing balance accurate to 0.1 mg
- Metal test-tube racks and glass test-tubes to match (e.g. 150 × 18 mm)
- Micropipettes and tips (e.g. Gilson Pipetman, France), and/or dispensers (e.g. Wheaton Zippette, England) to cover the following desired ranges
  - 2–20 µl
  - 50–200 µl
  - 0.2–1 mL
  - 1–5 mL
- Vortex mixer
- Boiling-water bath
- Cold-water ice bath (e.g. ice in a foam box), or refrigerated water bath
- Spectrophotometer or colorimeter.

4.3.2 Total ureides

(Reference: Young and Conway, 1942)

4.3.2.1 Reagents

A. 0.5 M NaOH

NaOH (analytical grade)	20 g
Distilled water	1 L

Plate 4.1 Equipment used to vacuum-extract xylem sap from stems. Foot vacuum-pump, secateurs, Vacutainer and fittings. The foot pump was constructed from a car-tyre pump by reversing the plunger so that the pump draws in air rather than blowing it. A non-return valve was inserted in the plastic tubing leading to the Vacutainer so that vacuum can be maintained during extraction.

Plate 4.2 Equipment used to vacuum-extract xylem sap from stems. Diaphragm vacuum-pump with manifold and three vacuum lines, rechargeable 12 volt battery, secateurs, Vacutainer and fittings.

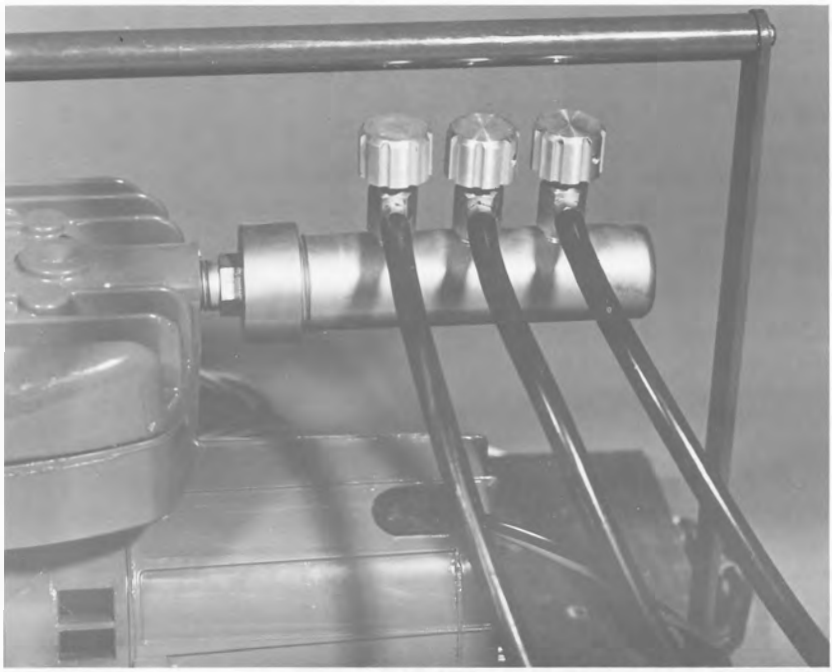

Plate 4.3 Detail of manifold on laboratory vacuum-pump. Suction through each of the three vacuum lines may be controlled by valves on the manifold and/or needle valves at the end of each line.

Plate 4.4 Vacuum-extraction of xylem sap from shoots. Once a vacuum is applied to the base of the cut stem, leaves are removed and short segments of stem are cut with secateurs successively from the top to the bottom of the shoot. The xylem sap recovered from the stem collects within the Vacutainer.

B. Phenylhydrazine hydrochloride

Phenylhydrazine hydrochloride*	0.33 g
Distilled water	100 mL

To be made fresh on each day of analysis. Keep flask containing stock solution covered with tin-foil once prepared. *Store with desiccant in freezer.

C. 0.65 M HCl

(32% w/w) concentrated hydrochloric acid	6.5 mL
Diluted with distilled water to	100 mL

D. Potassium ferricyanide

Potassium ferricyanide	0.833 g
Distilled water	50 mL

To be made fresh on each day of analysis. Keep flask containing stock solution covered with tin-foil once prepared.

E. Concentrated hydrochloric acid (32% w/w = 10M)

F. Allantoin standards

Prepare freshly for each day's analyses a 1 µmole/mL allantoin stock solution

Allantoin (store with desiccant)	15.8 mg
Distilled water	100 mL

Dilute stock—

1 mL to 100 mL with distilled water (10 nmole/mL), 2.5 mL sample for analysis	= 25 nmole
2 mL to 100 mL with distilled water (20 nmole/mL), 2.5 mL sample for analysis	= 50 nmole
3 mL to 100 mL with distilled water (30 nmole/mL), 2.5 mL sample for analysis	= 75 nmole
5 mL to 100 mL with distilled water (50 nmole/mL), 2.5 mL sample for analysis	= 125 nmole
10 mL to 100 mL with distilled water (100 nmole/mL), 2.5 mL sample for analysis	= 250 nmole

Note: Always include 2.5 mL distilled water blanks with standards during analysis. Before proceeding with the ureide assay, a full set of standards should be run through (0–250 nmole) to check linearity of response.

4.3.2.2 Procedure

Since colour development is not stable (see below) it is advisable to analyse ureides in batches of 20–30 samples including 2 water blanks and at least 3 ureide standards (e.g. 10, 20 and 50 nmole/mL).

(a) Place 0.2 mL of hot water tissue extract or 0.05–0.1 mL xylem exudate sample into each test tube and dilute to 2.5 mL with distilled water. (Use 2.5 mL of each ureide standard and 2.5 mL for water blanks.)

(b) Add 0.5 mL of 0.5 M sodium hydroxide

(c) Mix and place tubes in a boiling water-bath for 10-15 minutes

(d) Remove tubes and allow to cool to room temperature, then add 0.5 mL of 0.65 M HCl and 0.5 mL of phenylhydrazine solution to each tube.

(e) Mix and place tubes in a boiling water-bath for 2–4 minutes.

(f) Remove from boiling water-bath and immediately plunge tubes into an ice-bath for 15 minutes. *The rapidity of cooling is an important factor in development of final colour. If possible an ice-salt mixture should be used.* (The reaction may be left at this stage for a short time if necessary.)

(g) Remove from ice bath and add 2 mL of concentrated HCl (also chilled to 0°C) and 0.5 mL potassium ferricyanide.

(h) Mix immediately after each addition of potassium ferricyanide.

(i) Read optical density (absorbance) at 525 nm on a spectrophotometer after 10 minutes at room temperature. *The colour development is not stable. There is a 10–15% fading of colour intensity by 60 minutes. It is advisable therefore to only assay as many samples at one time that can be comfortably read within about 20 minutes.*

(j) Total ureide contents of samples are determined from the curve prepared from allantoin standards (the 250 mole standard should give an optical density reading of between 1.0 and 1.4), and a correction factor used (i.e. if 0.05 mL samples were used, the factor is 1.0/0.05 = × 20) to convert sample nmole determinations to nmole/mL.

4.3.3 Total amino acids ninhydrin method

[Reference: Yemm and Cocking (1955). An adaptation of the method detailed below is described in Herridge (1984)].

4.3.3.1 Reagents

A. 0.2 M citrate buffer

Citric acid	21 g
NaOH (analytical grade)	8 g
Distilled water	500 mL
Check pH and adjust if necessary to pH 5.0.	

B. Ninhydrin reagent

0.01 M potassium cyanide (65 mg in 100 mL distilled water) (stable 3 months at 20°C, *do not pippette by mouth)*	10 mL
Methoxy-ethanol (analytical grade)	590 mL
ninhydrin	5 g

The ninhydrin reagent should be prepared at least 24 hours before use. The reagent is very light-sensitive and should be stored away from light in a brown-glass bottle. Stable for only 2 weeks at room temperature. Stability can be prolonged by storage at 4°C.

C. Amino acid standards

Prepare freshly for each day's analyses a 2.5 µmole/mL, 50:50 asparagine:glutamine (most common amino compounds in xylem sap) stock solution.

Asparagine (store with desiccant)	16.5 mg
Glutamine (store with desiccant)	18.2 mg
Distilled water	100 mL

Dilute stock—

0.1 mL to 10 mL with distilled water (25 nmole/mL) 0.5 mL sample for analysis = 12.5 nmole
0.2 mL to 10 mL with distilled water (50 nmole/mL), 0.5 mL sample for analysis = 25 nmole
0.4 mL to 10 mL with distilled water (100 nmole/mL), 0.5 mL sample for analysis = 50 nmole
1.0 mL to 10 mL with distilled water (250 nmole/mL), 0.5 mL sample for analysis = 125 nmole
2.0 mL to 10 mL with distilled water (500 nmole/mL), 0.5 mL sample for analysis = 250 nmole

Note: Always include 0.5 mL distilled water blanks with standards during analysis.

4.3.3.2 Procedure

(a) Place 0.5 mL sample (20–50 µl xylem sap + 450–480 µL distilled water) in each test-tube. (For preparation of amino standard curve use 0.5 mL of each amino acid standard and duplicate 0.5 mL water blanks.)

(b) Add 1.0 mL 0.2M citrate buffer (pH 5.0), and

(c) add 1.2 mL ninhydrin reagent.

(d) Mix well and place in a boiling water-bath for 10–15 minutes.

(e) Remove from water-bath and cool to room temperature.

(f) Read optical density (absorbance) at 570nm on a spectrophotometer.

(g) Total amino acid content of samples is determined from a curve prepared from amino acid standards (the 250 nmole standard should give an optical density reading of around 1.2), and a correction factor used (i.e. if 0.05 mL samples used, the factor is = 1.0/0.05 = × 20) to convert sample nmole determinations of nmole/mL.

4.3.4 Salicylic acid method for nitrate determination

[Reference: Cataldo et al. (1975). Suitable for all legume xylem sap samples tested to date except pigeonpea, which requires a metal reduction procedure such as described by Herridge (1984) for accurate nitrate determinations.]

4.3.4.1 Reagents

A. Salicylic acid (5% w/v)

Salicylic acid	5 g
Concentrated sulphuric acid	100 mL

It is best if the salicylic acid reagent is prepared a few days before use. Once prepared it should be stable for several weeks.

B. 2 M NaOH

NaOH (analytical grade)	40 g
Distilled water	500 mL

C. Nitrate standards

Prepare a 25 µmole/mL potassium nitrate stock solution)

KNO_3	0.253 g
Distilled water	100 mL
(sodium nitrate may be used if KNO_3 not available)	

Dilute stock—

1 mL to 10 mL with distilled water (2.5 µmole/mL), 0.05 mL sample for analysis	= 0.125 µmole
2 mL to 10 mL with distilled water (5 µmole/mL), 0.05 mL sample for analysis	= 0.25 µmole
4 mL to 10 mL with distilled water (10 µmole/mL), 0.05 mL sample for analysis	= 0.5 µmole
6 mL to 10 mL with distilled water (15 µmole/mL), 0.05 mL sample for analysis	= 0.75 µmole
8 mL to 10 mL with distilled water (20 µmole/mL), 0.05 mL sample for analysis	= 1 µmole.

Note: Always include 0.05 mL distilled water blanks with standards during analysis.

4.3.4.2 Procedure

(a) Place 0.05 mL sample of xylem exudate or hot water tissue extract in each test-tube. (For preparation of nitrate standard curve use 0.05 mL of each nitrate standard plus duplicate 0.05 mL water blanks.)

(b) Add 0.2 mL 5% salicylic acid and mix.

(c) Stand at room temperature for 20 minutes, then add 4.75 mL 2 M NaOH (to raise pH > 12).

(d) Cool to room temperature and read optical density (absorbance) at 410 nm on a spectrophotometer.

(e) Nitrate contents of samples are determined from the curve prepared from nitrate standards (the 1 µmole standard should give an optical density reading of around 1.2), and a correction factor used (1.0/0.05 = × 20) to convert sample µmole determinations to µmole/mL.

4.4 Calibration curves

Quantitative relationships between the composition of N solutes in xylem exudates and proportional plant dependence upon N_2 fixation can be prepared in the glasshouse by growing a population of inoculated legumes in pots containing inert, N-free rooting medium (e.g. organic matter-free washed river sand, or a 1:1 mixture of sand:vermiculite). The effectively nodulated plants are supplied throughout growth with either N-free complete nutrient solution [e.g. as described by Gibson (1980) or Herridge (1984)], or nutrient solution supplemented with one of a range of concentrations of mineral N (e.g. 1,2,4,8 or 15 mM nitrate). This results in a series of plants whose source of N for growth declines progressively from full dependence upon atmospheric N_2 (plants fed N-free nutrient solution) to almost complete reliance upon mineral N (high inorganic N levels e.g. as depicted in Fig. 4.3). Xylem exudate collected from these plants or tissue extracts of harvested stem material are analysed for N-solute composition. Plant reliance upon N_2 fixation or root uptake of N at each level of mineral N may be assessed by procedures such as ^{15}N-isotope dilution (by supplying inorganic N of known ^{15}N-enrichment); relationships between N-solute composition and N_2 fixation are then prepared.

Because of its importance in most agricultural systems, nitrate has invariably been used to generate different degrees of legume dependence upon N_2 fixation in such calibration experiments. Ammonium nutrition, however, has been considered for both ureide- (Peoples et al. 1989) and amide-exporting legumes (M.N. Sudin and M.B. Peoples, unpublished data). Those studies indicated that the accuracy of N_2 fixation measurements would not be influenced by using nitrate-derived calibration curves in ureide-producing species and would be little affected in amide-producers provided less than 50% of the N uptake by roots was in the form of ammonium.

4.4.1 Ureide-exporters

The ureide method, using root-bleeding exudate, extracts from plant parts and vacuum-extracted exudate has been used in field studies for several years to assess N_2 fixation (principally with soybean). In most of the investigations, the technique has provided an index of fixation activity, rather than a quantitative estimate of N fixed (e.g. Neves et al. 1985; Patterson and La Rue 1983; Thomas et al. 1984; van Berkum et al. 1985). However, where estimates of seasonal fixation have been made using glasshouse prepared calibration curves, there has been reasonable agreement between ureide derived determinations and estimates using N-difference and ^{15}N procedures (e.g. Herridge et al. 1984; Rerkasem et al. 1988).

The absolute solute concentrations in the xylem and in extracts of the stem are determined by plant water status and are related to transpiration rate. As a consequence, the concentrations of individual N-compounds can vary considerably from plant to plant. For this reason, the ureide content of a sample is more accurately expressed in terms of a relative proportional value or ratio, rather than as a single component molarity. The relative ureide index therefore is used as a measure of the proportion of total xylem sap or extract N in the form of allantoin or allantoin acid, when relating ureide contents to symbiotic reliance in calibration curves:

For xylem exudate:

$$\text{Relative ureide index (\%)} = \frac{\text{Ureide N} \times 100}{\text{Total sap N}}$$

Since one ureide molecule contains 4N atoms, ureide N is calculated as 4 × ureide molar concentration. Total sap N is estimated as (4 × ureide + amino acid + nitrate) molar concentrations determined by colorimetric analysis (section 4.3.1). The relative ureide index can be calculated as:

$$\text{Relative ureide index (\%)} = \frac{4 \times \text{ureide}}{(4 \times \text{ureide} + \text{amino acid} + \text{nitrate})} \times 100$$

For extracts of shoot axes:

Amino-N is not measured. Therefore

$$\text{Relative ureide index (\%)} = \frac{4 \times \text{ureide}}{(4 \times \text{ureide} + \text{nitrate})} \times 100$$

Experiments calibrating relative ureide index against ^{15}N-derived estimates of N_2 fixation have been undertaken for soybean, cowpea (Pate et al. 1980), ricebean (Rerkasem et al. 1988), and pigeonpea (Peoples et al. 1989). Preliminary investigations comparing a range of legume species (soybean, green gram, black gram, cowpea, pigeonpea and navy bean) indicated that differences in ureide relationships between species are likely to be minor. Therefore, only calibration functions prepared for soybean have been presented below. However, in principle, calibration experiments should be completed for each species before the ureide-technique can be used to quantify N_2 fixation with confidence.

In soybean (and pigeonpea; Peoples et al. 1989) there were discernible effects of plant age on N-solute composition resulting in two sets of calibrations; one for vegetative and flowering plants (V-R2 stage according to the developmental scheme of Fehr et al. 1971), and another for plants during pod-fill (R3–R7). The relationships between relative ureides and plant dependence on N_2 fixation appeared unaffected by cultivar/genotype or rhizobial strain. Therefore, the following values should be appropriate for any soybean cultivar/strain combination (Table 4.2).

4.4.1.1 Root-bleeding exudate

The functions describing the relationship between P (the proportion of plant N derived from N_2 fixation) and the relative abundance of ureides in root-bleeding exudate (x) are:

$P = 1.2\ (x - 4.8)$ for plants in the vegetative and floweringstages
$P = 1.5\ (x - 21.3)$ for plants during pod-fill.

Note: Xylem solute concentrations ***must not*** be multiplied by the rate of bleeding

from the root stump (determined by the volume of sap collected over a period of time) to calculate translocation rates [e.g. as have Neves et al. (1985) and Thomas et al. (1984)]. The flux of N in xylem exudate *does not* provide a quantitative estimate of the N transported from root to shoot of intact plants (Rufty et al. 1982), and may be only loosely related to translocation if at all (M.B. Peoples, unpublished data).

Table 4.2 Relative abundance of ureides in root-bleeding and vacuum-extracted exudates and in extracts of shoot axes of soybean for different values of P[a]. All values as percentages.

Proportion of plant N from N_2 fixation	Root-bleeding exudate		Vacuum-extracted exudate		Shoot axes extracts	
(P)	Veg.,fl[b]	Pod-fill[c]	Veg.,fl	Pod-fill	Veg.,fl	Pod-fill
0	5	21	8	16	1	11
5	9	25	11	19	3	13
10	13	28	14	22	5	16
15	17	31	17	25	7	19
20	21	35	20	29	10	22
25	26	38	24	32	13	25
30	30	41	27	35	16	29
35	34	45	30	38	19	33
40	38	48	33	41	23	36
45	42	51	36	45	27	40
50	46	55	40	48	31	44
55	50	58	43	51	36	49
60	54	62	46	54	41	53
65	59	65	49	57	46	58
70	63	68	52	61	51	63
75	67	72	56	64	57	68
80	71	75	59	67	63	73
85	75	78	62	70	69	78
90	79	82	65	73	76	84
95	83	85	68	76	82	89
100	88	88	72	79	90	95

[a] Derived from experimental data.
[b] Vegetative and flowering stages of development up to R2 according to the scheme of Fehr et al. (1971).
[c] Reproductive stages of development after R2.

4.4.1.2 Vacuum-extracted exudate

The functions describing the relationship between P (the proportion of plant N derived from N_2 fixation) and the relative abundance of ureides in vacuum-extracted exudate (x) are:

$P = 1.6\,(x - 7.7)$ for plants in the vegetative and flowering stages

$P = 1.6\,(x - 15.9)$ for plants during pod-fill.

4.4.1.3 Extracts of the shoot axis (stems plus petioles)

Tissue extracts would be most relevant for young (small) vegetative plants. With larger plants, it is more convenient to collect xylem exudates (either vacuum-extracted or root-bleeding).

$x = 1.4 + 0.31\ P + 0.0057\ P^2$ for plants in the vegetative and flowering stages

$x = 10.7 + 0.50\ P + 0.0034\ P^2$ for plants during pod-fill

where P = the proportion of plant N from N_2 fixation, and x = relative abundance of ureides in extracts of the shoot axis.

4.4.2 Amide-exporters

The development of calibration curves for amide-exporting legumes relies on the xylem nitrate content as an index of changing symbiotic reliance. Glass-house studies indicate that N-solute procedures may be applicable for groundnut, chickpea, lentil and pea (Peoples et al. 1986, 1987) and lupin (tissue extracts only; Herridge 1988). However, Hansen and Pate (1987) suggest that the technique would be unsuitable for the *Acacia* spp. found in West Australian forests. Nor does there appear to be sufficient change in faba bean xylem composition to allow for the preparation of reliable relationships between N-solutes and N_2 fixation (Peoples et al. 1987).

The field potential of N-solute analysis methods in non-ureide food legumes has to date been evaluated only in lupin and groundnut. The former gave similar estimates of N_2 fixation to other methods (Herridge and Doyle, 1988). The groundnut study (Norhayati et al., 1988) showed that symbiotic dependence could be derived from the calibration curve depicted in Figure 4.4b when either nitrate was used as an indicator of soil N use, or when asparagine was used as an indicator of nodule activity; however, the accuracies of such estimates have yet to be validated by other techniques.

Since the N-solute relationships with N_2 fixation appear to be very species-specific in amide-exporters, readers are referred to Peoples et al. (1987) and Herridge (1988) for further examples.

4.5 Evaluation of analytical data and estimation of N_2 fixation

In this section, data from a field experiment will be used to quantify the effects of two treatments (in this case tillage) on N_2 fixation by soybean.

Protocols for sampling of crop dry matter and N are discussed in Section 3.1.1. The values of total crop N in Fig. 4.7 are the means of four replicates, each involving 15-plant samples taken at random from the replicate plots (Table 4.3). Therefore each estimate of crop N in Fig. 4.7 involved the sampling of 60 plants (4 replicates of 15 plants). This should be regarded as a minimum number (see Hunt et al., 1987, for further details). The values for the relative abundance of ureides in extracts of the shoot axis and in xylem exudates (Fig. 4.8) are the means also of the four replicates, in this case involving 10-plant samples taken at random from the replicate plots. To save on the total number of plants taken from the plots, thereby minimising competition effects, the 10-plant samples used for extraction of xylem exudate can form part of the 15-plant sample required to estimate crop dry matter and N by simply retaining the clippings once the exudate has been extracted (see Section 4.2.3.2). Because the total number of samplings is large (7 plus a final sampling for grain), the plots should be substantial in size also i.e. approximately 20 m of row of harvestable plants; buffer areas will be additional to this.

Table 4.3 Crop N (kg/ha) for the four replicate plots of the two tillage treatments at each sampling time. Crop N was calculated as:

[(shoot wt × %N shoots)/100 + (root wt × %N roots)/100] × no. plants/ha × 10^{-3}
(where shoot wt. and root wt. are in g/plant)

Physiological stage[a]	Days from sowing[b]	Cultivated					No-tillage				
		Replicate				Mean	Replicate				Mean
		1	2	3	4		1	2	3	4	
V6	30	9.6	10.8	14.2	14.6	12.3	8.6	7.9	12.6	9.8	9.7
R1	45	41.5	38.6	48.7	41.8	42.7	29.8	34.5	28.4	35.8	32.1
R2	53	76.9	74.1	85.7	72.8	77.4	58.9	64.1	65.2	58.1	61.6
R4	65	125	148	136	158	141.8	115	139	142	137	133.3
R5	72	167	181	175	169	173.0	164	171	183	157	168.8
R6	83	202	196	215	242	213.8	223	218	231	199	217.8
R7	98	213	219	202	229	215.8	241	202	228	215	221.5

[a] According to the scheme of Fehr et al. (1971).
[b] Note: Sampling age shown here should be used as a guide only. The actual timing of harvests for crop N and ureides should be determined by stage of physiological development, and the test-legume's characteristic pattern of N-accumulation and rate of maturity in any particular environment.

Table 4.4 Calculations for estimating N_2 fixed by the cultivated soybean crop.

Days after sowing	Crop N cumulative[a] (kg/ha)	Crop N increment (kg/ha)	Crop N from N_2 fixation proportion[c] (%)	Crop N from N_2 fixation increment[d] (kg/ha)	Crop N from N_2 fixation cumulative (kg/ha)
0	3[b]				
		9.3	6	0.5	
30	12.3				0.5
		30.4	22	6.7	
45	42.7				7.2
		34.7	35	12.1	
53	77.4				19.3
		64.4	48	30.9	
65	141.8				50.2
		31.2	59	18.4	
72	173.0				68.6
		40.8	77	31.4	
83	213.8				100.0
		2.0	85	1.7	
98	215.8				101.7
Total	215.8	215.8			101.7

[a] From Table 4.3
[b] Seed N
[c] From Fig. 4.9
[d] Column 3 × Column 4/100

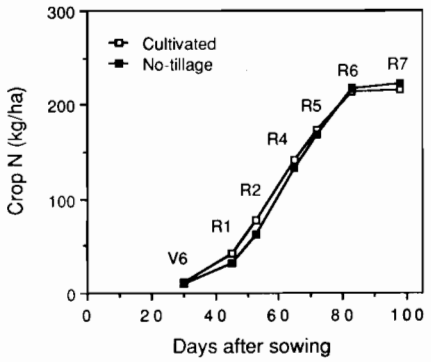

Fig. 4.7 Accumulation of crop N over time. Both shoots and roots are sampled to determine crop N. Stages of plant growth (V6–R7) are shown and correspond to sampling times (see Fehr et al. 1971 for descriptions of plant developmental stages.

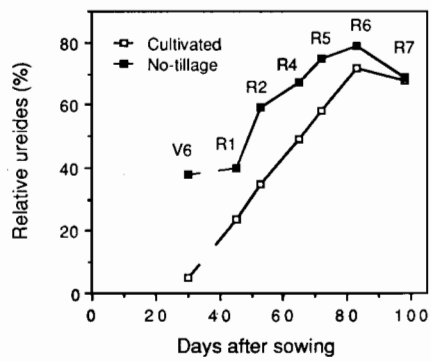

Fig. 4.8 Changes in the relative abundance of ureides in extracts of shoot axes and in vacuum-extracted xylem exudate of soybean with time. Each point is the mean of four replicates, each involving 10-plant samples.

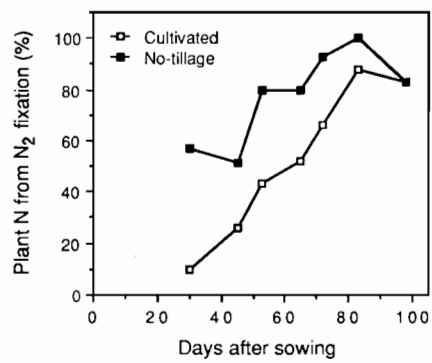

Fig. 4.9 Changes with time in the proportions of plant N derived from N_2 fixation as determined from ureide calibartion curves (section 4.4.1).

The treatment means for crop N can be presented in graphical form (Fig. 4.7). Xylem exudate could not be collected either as root-bleeding exudate or by vacuum-extraction from the small plants at the first sampling. Therefore, shoot axes (stem plus petioles) were sampled and hot water extracts made (see Section 4.2.2). Xylem exudate was vacuum-extracted from plants in the following samplings (i.e. R1 and R7). Data are presented in graphical form as Fig. 4.8.

These data for relative ureides are now matched with the calibration tables (Table 4.2) to determine the proportions of crop N derived from N_2 fixation for each of the two tillage treatments at each sampling time (Fig. 4.9).

Data on the accumulation of crop N (Table 4.3) may be used to calculate the incremental increase in total plant N between harvests (e.g. column 3, Table 4.4). The estimates of symbiotic dependence shown in Fig. 4.9 (column 4, Table 4.4) can then be used to derive the inputs of fixed N between samplings (column 3 × column 4/100, Table 4.4).

The calculations in Table 4.4 indicate that, over the entire growing season, the cultivated soybeans fixed 102 kg N/ha (column 6) or 47% of total crop N. A similar table of data for the no-tillage treatment resulted in a seasonal estimate of 175 kg N/ha fixed, equivalent to 79% of total crop N.

Because of the 'point-in-time' nature of ureide-derived estimates of N_2 fixation, the intensity of sampling described above is necessary to provide an accurate measure of the seasonal contribution by N_2 fixation to legume N yield. However, less frequent sampling could be used for comparative purposes when assessing treatment effects on legume reliance upon N_2 fixation. Such comparisons can be undertaken either with or without associated measures of crop N.

4.6 Advantages and possible applications of N-solute methods

Technically the field-sampling of xylem contents is simple and the analysis of N-components (i.e. ureides, α-amino N and nitrate) can be done by colorimetric assays in a test tube (see section 4.3). As a consequence there is no need for expensive or sophisticated equipment, and many analyses may be performed daily. It is not necessary to dig out legume roots and recover nodules to obtain measures of N_2 fixation, nor is it necessarily a totally destructive technique as sufficient sap can be collected from stem segments and laterals of mature plants for complete analysis (Herridge et al. 1988). Since sampling is confined to the accessible aerial parts of the plant, the solute method may potentially overcome many problems associated with measuring N_2 fixation by twining ground-cover or forage legumes (Norhayati et al. 1988), or woody perennial legumes (Peoples et al. 1988b).

Some studies have already examined the forms of N transported in the xylem of woody species (Hansen and Pate 1987; van Kessel et al. 1988). A comprehensive survey of important tree and shrub legumes is currently under way to identify the principal N-solute compounds present in the xylem of both N_2-fixing and unnodulated nitrate-dependent plants (D.F. Herridge, T. Ibrahim, D.P. Nurhayati, B. Palmer, M.B. Peoples, unpublished data). Once these compounds have been clearly identified the relationships between xylem composition and symbiotic dependence will be calibrated and N-solute methods will be available for use in field investigations with N_2-fixing trees.

4.7 Potential limitations

Apart from species differences in the forms of transport N, the effect of sampling procedures, physiological, environmental and nutritional variables should be considered before glasshouse-derived relationships can be legitimately applied to field-grown crops. Various factors which should be considered when using the technique have been identified (Table 4.5). Of the potential sources of error listed, time delays between harvesting the shoot and vacuum-extracting the xylem contents

Table 4.5 Potential limitations in the use of xylem N-solute techniques to evaluate N_2 fixation by legumes in the field[a].

Variable	Comments
Plant species	Principal N-compounds transported from nodules are characteristic of a species. Relationships between N-solutes and N_2 fixation are often similar in ureide-exporters, but in theory should not use xylem sap or extract analysis for inter-specific comparisons without first calibrating each species. Relationships vary between amide exporting legumes. Method may not be suitable for some species. Method is an indirect measure — must establish relationship between xylem composition and N_2-fixing status.
Cultivar/genotype	Appear to be insignificant, although differences in location of nitrate reductase may be important in amide-producers.
Rhizobium strain	Conflicting reports on the effects of *Rhizobium* strain on xylem composition, requires further testing. Significance in estimating N_2 fixation yet to be evaluated, but probably minor.
Plant age	Little effect with indeterminate species. May require several calibration curves to cover all stages of growth in some legumes, e.g. soybean, pigeonpea.
N-stress and senescence	N-solute relationships invalid in N-deficient or senescing plants (total xylem solute concentrations of less than 1-2 µmole/mL). Since sap collections should be taken in conjunction with periodic harvests for plant total N, severe stress should become apparent.
Source of soil N	Apparently insignificant in ureide-exporters. Important effect on xylem composition in amide-exporters only if ammonium is the major form of mineral N taken up by roots, i.e. >50%.
Collection of xylem samples by vacuum	Relative solute content constant between 9 a.m. and 4 p.m. and unaffected by source of vacuum or vacuum strength. Increase in N-solute composition if time delay of more than 5 minutes between shoot detachment from root and vacuum-extraction of xylem from stem. Potential source of error if recommended sampling protocol not followed.
Storage of xylem samples	Stable for 4 hours at 25–30°C. Stable in 50% ethanol for 14 days. Preferable to keep cold until placed in freezer.

[a] Prepared from Herridge et al. (1988), Peoples et al. (1988b; 1989) and unpublished data of D.F. Herridge, M.B. Peoples and M.N. Sudin.

appear the most serious. However, such time-related changes in N-solutes can be avoided by vacuum-extracting sap within 5 min. of stem decapitation (Herridge et al. 1988; Peoples et al. 1989).

The major disadvantage of the N-solute technique is that it provides a short-term rather than time-integrated measure of symbiotic dependence. If an estimate of seasonal fixation is required rather than a comparative measure of treatment effects on N_2 fixation, repeated measurements must be combined with sequential sampling from the crop for dry matter and total N contents (Section 4.5).

5
NITROGEN-DIFFERENCE TECHNIQUE

5.1 Principles behind the method

The simplest field estimates of N_2 fixation reported in the literature have been obtained by measuring total amount of N in the legume crop. Such determinations are based on the arbitrary assumption that these crops derive all their N from symbiotic fixation. This suggestion is unrealistic and unsubstantiated and values calculated just on the basis of legume N yield would almost always overestimate fixation. A true measure of fixation based on crop N accumulation can be obtained only when the contribution of soil N to the total legume N is determined. This may be estimated by growing a companion non-N_2-fixing control crop in the same soil and under identical conditions as the legume (usually in an adjacent plot). The difference in total shoot N accumulated on a per-plant or per-unit-area basis between the legume and control crop is then generally regarded as the contribution of symbiotic fixation to the legume.

5.2 Applications, advantages and limitations

The N-difference method is a relatively simple procedure. The principal assumption is that both legume and control contain the same quantity of soil-derived N in their shoots. For this to be valid the two plant types should explore the same soil-rooting volume, have the same ability to extract N, and accumulate soil N over the same period of time (this is particularly important if several estimates of N_2 fixation are desired during a growing season). The distribution of N between tops and roots of the two plant types should also be similar. A non-N_2-fixing control plant may be:

(i) A non-legume.

(ii) An uninoculated legume of the same species (requires soil to be devoid of effective *Rhizobium* spp.).

(iii) A non-nodulating legume genotype.

Unfortunately, differences between the fixing and non-fixing plants in their capacities to use soil N often occur, and the underlying requirement that there is identical usage of soil N by the legume and control is not always easy to attain. Even when the supposed 'ideal' control plant (a non-nodulating isoline of the test legume)

is used, results may be incorrect due to difference in root morphologies (e.g. Boddey et al. 1984). The importance of choosing a particular control plant depends on the level of plant-availble soil N. If levels of soil N are low and control plants accumulate much less N than the legume test-plant, error due to plant type will be minimal.

5.3 Methodology

5.3.1 Analysis of legume and non-N_2-fixing control plant nitrogen.

Appropriate sampling of plant material (and for soil mineral N) and subsequent Kjeldahl analysis are described in Section 3. If more than one estimate of N_2 fixation is required during a season, plot size for both legume and control should allow sufficient plants to be harvested to provide a reliable estimate of crop N (see Hunt et al. 1987; Section 3.1.1). Similar areas would be harvested from both legume and control plots and the experiment would be laid out in a randomised complete block design with at least four replicates (depending on soil homogeneity). Plot design is discussed further in Section 6.3.3.

5.3.2 Evaluation of analytical data and estimation of N_2 fixation.

Several variations of the N-difference method exist. Generally the quantity of legume N derived from N_2 fixation (Q) is calculated as:

$$Q = \text{N yield (legume)} - \text{N yield (control)} \quad (A)$$

However, a modification of the procedure has been suggested which is proposed to improve the accuracy of measurements when the legume and control are not well-matched (Evans and Taylor 1987). In this method the differences in post-harvest soil mineral N are also determined in the fixing and non-fixing plots and added to the differences in total N yields of the two crops. Thus equation (A) becomes:

$$Q = [\text{N yield (legume)} - \text{N yield (control)}] + [\text{N soil (legume)} - \text{N soil (control)}] \quad (B)$$

The use of this second expression assumes that mineralisation, leaching and denitrification are identical under each crop. The validity of these assumptions has yet to be demonstrated under field conditions.

Examples of the use of field data to calculate N_2 fixation by N-difference methods are presented in Table 5.1. In these examples the choice of non-N_2-fixing plant species (wheat or linseed) as the control crop for lupin greatly affected the estimate of N_2 fixation obtained. The inclusion of post-harvest levels of soil mineral N to 20 cm depth in equation (B) did not greatly change the disparity between the two control crops (accuracy may have been improved by measuring soil mineral N to 100 cm depth; Evans and Taylor 1987). However, comparison of the amounts of N accumulated by the two control species (Table 5.1) suggests that linseed growth was limited by factors other than N, indicating that in this instance wheat was the more appropriate control. To avoid using mismatched controls it is desirable to include and compare a number of different control species in experimentation where crop N yield is to be utilised to calculate legume N inputs from N_2 fixation.

Table 5.1 Shoot N contents of lupin and two non-N_2-fixing control crops (wheat and linseed), levels of post-harvest soil mineral N, and estimates of N fixed by N-difference techniques[a] (kgN/ha)

Species	Crop N	Postharvest mineral N[b]	Estimate of N fixed Q[c]	Q(modification)[d]
Lupin	192	30	73 (W) 125 (L)	85 131
Wheat (W)	119	18	–	–
Linseed (L)	67	24	–	–

[a] Derived from the data of Evans et al. (1987). Lupin and the control species were sown so that lupin plots were flanked by wheat and linseed. Each plot was 8 m long containing 24 rows, 18 cm apart. The crop N data represent the mean of 0.25 m^2 from each of 4 replicates and includes all fallen leaves

[b] Taken 0–20 cm depth. Calculated on the basis of concentration of NO_3 + NH_4 as ppm, and adjusted by soil bulk density and cross-sectional area of soil-core used to collect soil samples.

[c] Calculated from equation (A), using wheat (W) or linseed (L) as the non-N_2 fixing control.

[d] Calculated from equation (B), using wheat (W) or linseed (L) as the non-N_2 fixing control.

6
^{15}N-ISOTOPIC TECHNIQUES

6.1 Principles behind the method

There are two stable isotopes of nitrogen, ^{14}N and ^{15}N. The heavy isotope, ^{15}N, occurs in atmospheric N_2 at a constant abundance of 0.3663 atoms % (total variation ranges from 0.36628 to 0.36632; Mariotti et al. 1983). If the ^{15}N abundance in plant-available soil N is higher than this, an estimate of the proportions of legume N derived from each source can be made. For these estimates, the abundance of ^{15}N in plant-available soil nitrogen is obtained by analysing a non-N_2-fixing reference plant which is totally dependent on soil N for growth (Fig. 6.1).

With increasing N_2 fixation, the abundance of ^{15}N in the N_2-fixing plant declines as nitrogen assimilated from the soil is 'diluted' by atmospheric N_2 of lower ^{15}N abundance fixed in its root nodules (Fig. 6.1). The extent of N_2 fixation is calculated from simple algebraic expressions (8, 9 in section 6.3.6). Assumptions inherent in the method are that:

(i) the $^{15}N/^{14}N$ ratio of the non-N_2-fixing reference plant is the same as that of the soil N, and

(ii) the legume and reference plant explore a soil N pool of identical $^{15}N/^{14}N$ composition.

In many cases the very small differences in natural abundance of ^{15}N between soil N and N_2 can be used, provided a suitably precise mass spectrometer is available. More usually, the difference between soil N and N_2 is extended by incorporation in the soil of ^{15}N enriched nitrogenous compounds (e.g. of between 5 and 95 atoms % ^{15}N).

6.2 Applications, advantages and limitations

6.2.1 ^{15}N-enrichment

The use of methods involving artificial adjustment of soil ^{15}N enrichments to measure N_2 fixation have been extensively reviewed, (e.g. Chalk 1985; Danso 1988; Hauck and Weaver 1986; Ledgard and Peoples 1988; Witty et al. 1988). A major assumption is that the legume and reference plant absorb the same *relative* amounts of N from added ^{15}N and soil N. The main advantage is that the method provides a

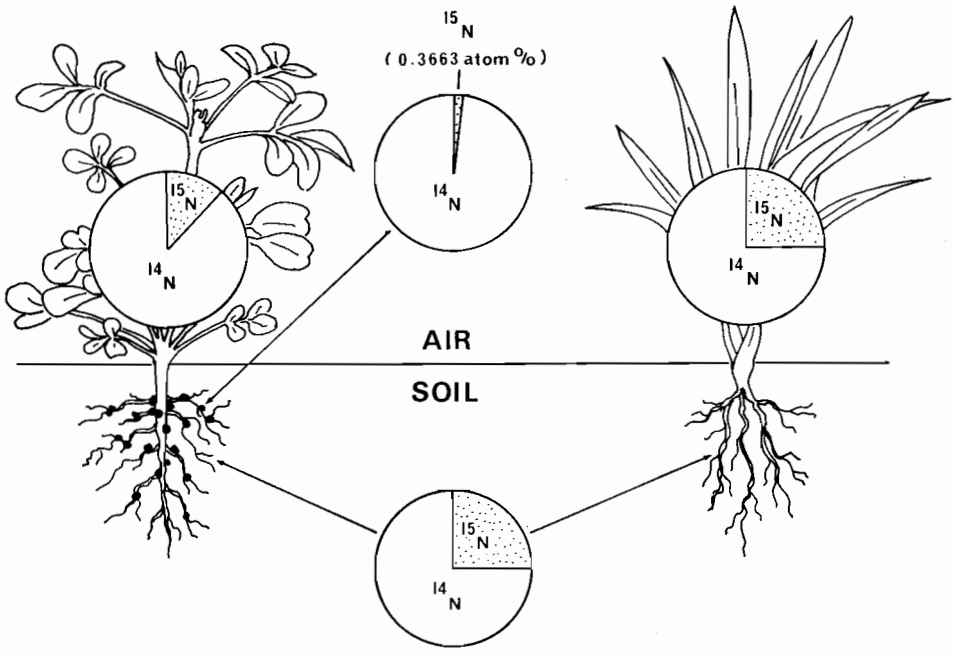

Fig. 6.1 Diagrammatic representation of the principles involved in the ^{15}N dilution technique. Dilution of soil N by atmospheric N_2 results in a lower ^{15}N composition in the legume's products of growth than measured in the reference crop and is represented as a reduced area in the ^{15}N sector of the leguminous plant. After Peoples et al. (1988b).

'time-averaged' estimate of the proportion *(P)* of legume N derived from N_2 fixation which is the integral of any changes in *P* that may have occurred during the measurement period. The estimate of *P* is independent of measurements of yield, although it is necessary to measure dry matter and N yield to determine the amount of N fixed.

Apart from the high cost of instrumentation to measure ^{15}N and expense of ^{15}N-labelled material (around US$10 per gram of ^{15}N-nitrate of 10% enrichment) there are other limitations which should be considered when undertaking ^{15}N investigations:

(i) It is essential that addition of ^{15}N-labelled material does not affect N_2 fixation. The effect of mineral N on N_2 fixation is well established and therefore the use of low rates of N application (e.g. < 5 kgN/ha) are preferable. If a carbon source is being used to immobilise added ^{15}N or if ^{15}N-labelled organic matter is being added, it is also important that there is not a stimulation of N_2 fixation due to an effect of labelling treatment on soil N availability.

(ii) The choice of an appropriate non-N_2-fixing reference plant is the most important factor affecting the estimate of *P*. Errors in ^{15}N-derived measures of N_2 fixation most often occur because the legume and reference plant differ in the ratio of N assimilated from added ^{15}N to N taken up from indigenous soil N. This can be due to: (1) the legume and reference plant differing in their uptake of N from different

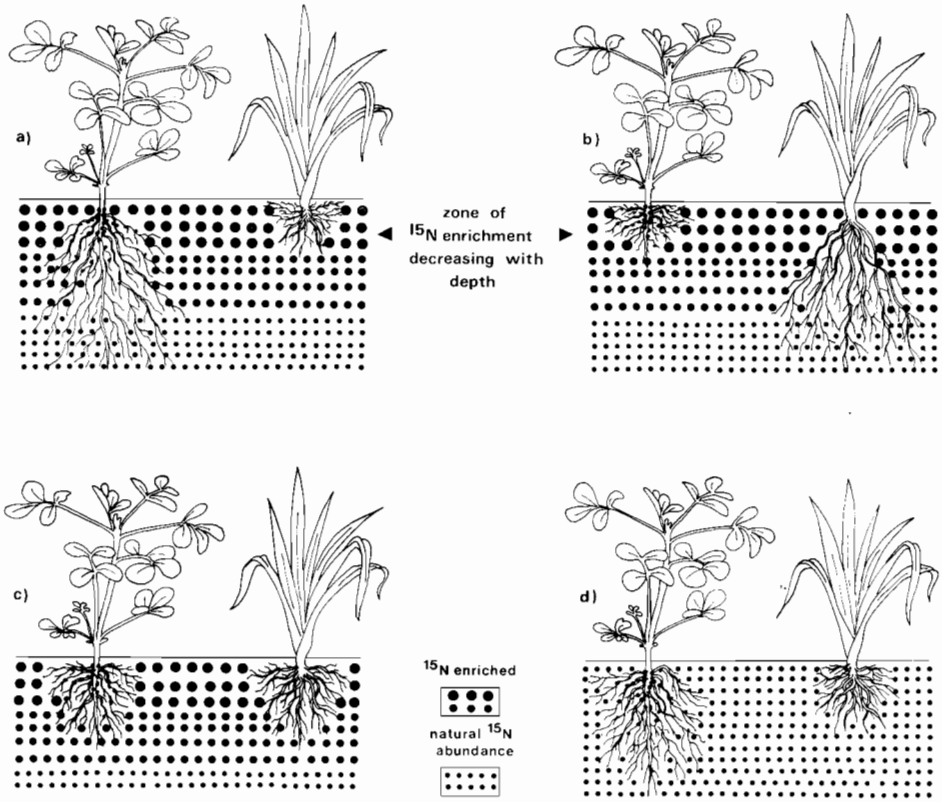

Fig. 6.2 Diagrammatic representation of four situations which may arise during ^{15}N isotope dilution experimentation in the field. Figures (a) to (c) illustrate differences in the comparative root growth of a nodulated legume and a non-N_2-fixing reference plant in hypothetical ^{15}N enrichment studies. Application of ^{15}N enriched material to the soil surface has created a zone where the plant-available soil nitrogen is artificially enriched in ^{15}N. The ^{15}N enrichment within this zone however, decreases down the soil profile until natural abundance levels of ^{15}N are reached at depth. The fourth case-study (d) illustrates a nodulated legume and non-N_2 -fixing reference plant in ^{15}N natural abundance study where the ^{15}N abundance of plant-available soil nitrogen is uniform down the soil profile.

soil depths where there are differences in the isotopic composition of plant-available soil N, or (2) the plants differing in their pattern of N assimilation with time in association with time-related changes in the isotopic composition of soil N.

Figures 6.2a-c illustrate three possible legume–reference plant relationships which can occur in field experiments using ^{15}N enrichment. When ^{15}N-labelled material has been applied to soil a highly ^{15}N enriched layer is created at the surface; however, the ^{15}N enrichment of plant-available soil N declines rapidly with depth. In figure 6.2a the roots of the legume have explored deep into the region which has not been artificially enriched with ^{15}N, while the roots of the non-N_2-fixing reference have been restricted to the uppermost enriched zone. The uptake of N by the legume from the unenriched soil will, when the $^{15}N/^{14}N$ composition of the legume and reference plant are compared, lead to an overestimate of N_2 fixation. In the reverse situation (Fig. 6.2b), where the roots of the reference plant have extensively grown below the ^{15}N-enriched zone, the isotopic dilution of the ^{15}N content of the reference plant by N taken up from unenriched soil will result in an underestimation

of N_2 fixation by the legume. Only in the ideal situation, where the roots of the legume and reference plant explore a similar volume of soil and utilise soil mineral N of similar ^{15}N enrichment (Fig. 6.2c), can accurate determinations of N_2 fixation be expected (see Section 6.3.2). Similarly where there is a changing isotopic composition in the soil N pool, precise measurements of fixation can only be obtained if the legume and reference have the same seasonal pattern of N uptake. The decline in $^{15}N/^{14}N$ ratio in soil with time presumably results from a loss of plant–available ^{15}N due to uptake, leaching or immobilisation, and by the continuing release of soluble ^{14}N-compounds by mineralisation of soil organic matter.. Witty (1983) concluded that this decrease in soil ^{15}N enrichment coupled with differences in growth rate and patterns of N accumulation between legume and reference crops were major factors causing field estimates of fixation to vary so widely with the choice of different reference plants. The extent of the error in estimating P where plants are imperfectly matched will depend on the uniformity of ^{15}N application, the rate of change in ^{15}N concentration of plant-available soil N with time, and the difference in ^{15}N enrichment with depth. This is largely affected by the form of ^{15}N material used and method of application (discussed in section 6.3.1).

6.2.2 Natural ^{15}N abundance

Almost all N transformations in the soil result in isotopic fractionation. The net effect is a small increase in the ^{15}N abundance of soil mineral N (e.g. to between 0.368 and 0.373 atoms % ^{15}N) compared with atmospheric N_2 (0.3663 atoms % ^{15}N). In looking at small differences in ^{15}N concentration, data are commonly expressed in terms of parts per thousand ($\delta^{15}N$ or ‰; see Section 6.3.6). The method gives an integrated estimate of *P* over time as in ^{15}N-enriched experiments, but it can be applied to established experiments because no pretreatment (i.e. ^{15}N application) is necessary. Although the principles of the technique are similar to those of ^{15}N enrichment studies, the main limitations are quite different (reviewed by Bergersen 1988; Ledgard and Peoples 1988; Mariotti et al. 1983; Shearer and Kohl 1986). An isotope ratio mass spectrometer capable of accurately measuring differences of 0.1‰ (about 0.00004 atoms % ^{15}N) is needed and sample preparation requires great care to avoid (a) losses of N which change the ^{15}N abundance and (b) contamination from ^{15}N-enriched material (see Table 6.1). It is also preferable to have the $\delta^{15}N$ of plant-available soil N above about 6‰ because the accuracy in estimating *P* decreases markedly at values below this.

Use of the natural ^{15}N abundance method assumes that isotopic fractionation during N_2 fixation is nil or a known constant value. The $\delta^{15}N$ of the total N of plants fully dependent on N_2 fixation for growth (Table 6.4) may be different from that of atmospheric N_2 (which by definition is zero). In *Lupinus* spp., the relative enrichment or depletion of ^{15}N in plant parts can be influenced by *Rhizobium* strain. Certain strains of rhizobia produce nodules with raised $\delta^{15}N$ when grown with atmospheric N_2 as their sole source of N; however, the shoots of these plants have a lowered $\delta^{15}N$. The net result is little effect on the value for whole lupin plants regardless of the symbiotic association (Bergersen et al. 1986). Similar rhizobial strain-induced changes in $\delta^{15}N$ enrichment or depletion of different parts of fully symbiotic plants has not been observed in other species (e.g. soybean, pigeonpea, or peanut; M.B. Peoples and D.M. Hebb, unpublished data). Nonetheless, there can be dynamic changes in $\delta^{15}N$ of individual plant parts during organ development so

estimates of *P* should be based on $\delta^{15}N$ of whole plants or total shoot N, and not on $\delta^{15}N$ of single leaves or other plant parts (Bergersen et al. 1988). Isotopic fractionation during uptake of soil N has been examined for a large number of legumes and non-legumes and found to be insignificant. Thus, there appears to be no need to allow for this in estimating *P* using natural ^{15}N abundance (Shearer and Kohl 1986).

Low and/or variable soil $\delta^{15}N$ values have been detected in some intensively-grazed pastures (Steele 1983) and in a natural forest ecosystem (Hansen and Pate, 1987). The ^{15}N-natural abundance methodology would therefore be unsuitable for quantifying N_2 fixation at these particular sites. However, $\delta^{15}N$ has been found to be high and relatively uniform in a number of cropping soils enabling accurate estimates of symbiotic N_2 fixation (e.g. Bergersen et al. 1989; Ledgard and Peoples 1988; Rerkasem et al. 1988). Since natural abundance of ^{15}N in plant-available soil N can also be uniform with soil depth and does not appear to change rapidly with time (as depicted in Fig. 6.2d; see also Bergersen 1988; Bergersen et al. 1989; Ledgard et al. 1984), the major limitation of ^{15}N enrichment techniques (i.e. choice of reference plant) may be relatively less important with the natural ^{15}N abundance method . Several studies have found that calculations of *P* were not influenced by the use of different reference plants (Ledgard and Peoples 1988). Where natural abundance and ^{15}N enrichment methods have been compared, field estimates of N_2 fixation have been similar, with similar precision, regardless of which technique was used (e.g. Table 6.6; Bergersen and Turner 1983; Ledgard and Peoples 1988).

Table 6.1 Precautions required for the accurate measurement of natural abundance levels of ^{15}N.[a]

1. *Uniform samples* — to avoid variation due to non-uniform ^{15}N abundance in different plant tissues.
2. *Reagents* — protected from contamination from NH_3 in laboratory air.
3. *Digestion temperature* — to minimise possible losses of ^{15}N-depleted N resulting from thermal decomposition of ammonium sulfate during digestion.
   - use tall glass tubes in electrically-heated blocks, **NOT** uncontrolled conventional Kjeldahl flasks.
   - temperature controlled at a maximum of 310–315°C. Higher temperatures lead to non-uniform ^{15}N-enriched replicates.
4. *Distillation* — standard collections of only 30 mL of distillate may leave behind minute traces of ^{15}N-enriched ammonia. Routinely collect 80 mL distillate to ensure complete recovery of N.
5. *Concentration of distillates* — avoid evaporation to the point at which crystallisation of boric acid occurs, or the distillate becomes dry. Drying samples of $(NH_4)_2SO_4$ in boric acid, even at only 55°C, results in some thermal decomposition and loss of ^{15}N-depleted NH_3.
6. *Avoid contamination with ^{15}N-enriched material* — hygiene, separation in time and space between natural abundance and ^{15}N-enriched materials.

[a] If the described precautions are observed, it should be possible to match within 0.1–0.3‰, $\delta^{15}N$ values for samples of the same solution of $(NH_4)_2SO_4$ which have been analysed directly in a mass spectrometer, or analysed after each and all of the steps involved in sample preparation. Information presented here represents a summary of discussions in Section 3 and Bergersen (1988).

6.3 Methodology

6.3.1 Labelling techniques

No standard approach has been adopted for the application of ^{15}N-labelled materials. Methods have included broadcasting, banding, physical mixing with soil and application of solution by spraying or injection into soil (reviewed by Chalk 1985; Danso 1988; Witty et al. 1988). The types of ^{15}N enriched compounds used can be broadly classified as inorganic N and immobilised ^{15}N sources.

Immobilised forms of ^{15}N are used to try to ensure a gradual release of labelled N and provide a more stable enrichment of plant-available soil N. One slow-release form that has been used is ^{15}N-labelled plant material (e.g. Boddey et al. 1984). This may be suitable where crop residues are returned to soil as a normal management practice but is likely to be least useful where plants are already established (e.g. perennial pastures) and where soil disturbance should be avoided. One further disadvantage in the use of labelled organic matter is that, ideally, the site should be left to stabilise for several months after incorporation. This requires experiments and field sites to be planned and allocated one year or more in advance. Alternative approaches have used slow-release ^{15}N formulations (e.g. ^{15}N incorporated into gypsum pellets), or have added a soluble ^{15}N salt with a readily available carbon source (e.g. sucrose) so that the ^{15}N is bound in the soil biomass (see Giller and Witty 1987; Witty 1983). While pelleted fertilisers have proved better than soluble ^{15}N fertilisers in some field experiments, the pellets are difficult to incorporate into the soil and an uneven distribution of ^{15}N may occur with depth. The use of carbon substrates to immobilise ^{15}N fertiliser has been recommended by some authors, but care should be taken to ensure that the addition of an exogenous carbon source does not also immobilise indigenous soil mineral N and/or significantly alter soil microbial activity.

The most commonly utilised method of artificially enriching soil is by the direct addition of inorganic ^{15}N fertilisers. Many reports (e.g. Chalk 1985; Danso et al. 1988; Rennie 1986; Witty et al. 1988) have compared methods of labelling the soil which minimise variability and temporal changes in ^{15}N enrichment. Several investigators favour adding small consecutive amounts of ^{15}N to soil at intervals over the growing season. Others believe that such a procedure can result in differences in ^{15}N enrichment of the soil N pool under the legume and non-N_2-fixing reference if the two plant types do not assimilate mineral N at the same rate, and they prefer to use a single application of ^{15}N at the beginning of the growing season. Some researchers have applied a higher rate of fertiliser N (often at different ^{15}N enrichment) to the reference plant than the legume. This technique was introduced by Fried and Broeshart (1975) to allow the non-fixing plant to grow at a comparable rate to a legume on soils of low N status and so provide a more appropriate reference. This approach is based on the premise that a plant with more than one source of available N will absorb from each of these in proportion to their relative amounts; i.e. there will be no preference for one source of N over another. This so-called 'A-value' modification has been widely used in many legume studies; however, because the technique is dependent on fertiliser addition rates and fertiliser N recovery, errors could be expected if fertiliser N losses occur during experimentation (Chalk 1985). Recently, considerable doubt has been cast on the fundamental assumptions inherent in the method; i.e. that there is equal fertiliser

use efficiency by the heavily fertilised reference and lightly fertilised legume, and that the A-value is independent of N application rate for all plants (Smith et al. 1989).

The choice of labelling technique will influence the level of ^{15}N-enrichment of the labelled material used, as will the instrumentation used in the subsequent analysis of ^{15}N (section 6.3.4). Since emission spectrometry is less accurate than mass spectrometry, the levels of ^{15}N-enrichment applied to soil (0.15–0.2 g $^{15}N/m^2$) need to be higher than in equivalent experiments where a mass spectrometer is to be used (0.05–0.10 g $^{15}N/m^2$).

6.3.2 Selection of suitable reference plants

The only definitive way of checking whether a non-legume, an uninoculated legume (requires soil to be devoid of effective *Rhizobium*), or a non-nodulating legume genotype represents a suitable reference plant for isotope-dilution experiments is to estimate whether the legume and reference assimilate added ^{15}N and indigenous soil N in the same ratio. Wagner and Zapata (1982) estimated this ratio indirectly by measuring the relative uptake of labelled and indigenous soil sulfur (assuming similarities in N and sulfur assimilation from soil). Ledgard et al. (1985) described a regression method for assessing the relative uptake of ^{15}N and soil N. This involved two or more treatments with the same rate of N addition but at different ^{15}N concentrations, and a natural ^{15}N abundance treatment. When such checking procedures are not possible, the following criteria should be considered when selecting reference crops (after Chalk 1985; Danso 1988; Witty et al. 1988):

(i) The absence of N_2-fixing ability. In temperate climates, N_2 fixation associated with non-legumes appears to be insignificant; but in tropical environments, N_2 fixation associated with some non-legumes can supply up to 40% of the plant's N (Boddey 1987). It is important to check non-legume reference plants for such activity (e.g. using acetylene reduction) or to compare several different reference plants. Even a non-nodulating legume genotype should not be fully relied upon. Often a non-nodulating trait is *Rhizobium* strain-specific, and a reversion of genotypes to a nodulating type is not uncommon. Excavation of root-systems of 'non-nodulating' legume lines and examination for nodules should be routine when harvested as a ^{15}N reference.

(ii) Similarity in the rooting zone of the reference and legume. This can be examined by assessing root lengths and habits, or indirectly through comparisons of ^{15}N enrichments in different potential reference crops; particularly if a non-nodulating line of the test legume can be included. Differences in rooting patterns are often indicated by different ^{15}N recoveries.

(iii) Relative N uptake patterns of reference 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 and legume should at least start to accumulate N and reach their maximum N contents at the same time.

(iv) 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), it is

essential that both crops take up similar ratios of $^{15}N/^{14}N$ from the soil during the entire growing season. This implies that the reference plant must not mature or cease accumulating N before the final legume harvest.

(v) The cropping system used. The choice of reference plant is limited in grass-legume pastures or in intercropping studies. In such intimate mixtures of N_2-fixing and non-N_2-fixing species, it is important that there should be no significant transfer of N between plant types as this would lead to an underestimate of N_2 fixation by the legume. In practice, N transfer appears to be a relatively slow process and the effects on estimating *P* can be limited by restricting the measurement period.

It should be noted that errors associated with mismatching of the reference and N_2-fixing plants become most important when only small proportions of legume N are derived from the atmosphere. Under these conditions, minor variations in ^{15}N enrichment can be greatly magnified when calculating N_2 fixation. However, errors become smaller and mismatching less important as the amount of N_2 fixed by the legume increases.

6.3.3 Plot design and sampling procedures

It is not possible to define a single experimental protocol which gives an accurate estimate of N_2 fixation with ^{15}N under all circumstances. What is appropriate to one crop,or environment may not be appropriate elsewhere.

Perhaps the most important factor influencing the design of a field trial will be the objective of the investigation. A clear, concise reason for conducting a study is essential for successful planning. The purpose of the study will affect selection of the field site, the treatments employed, and the interpretation of the data. The site should be nearly level with a homogeneous soil type of uniform fertility and N status (Reichardt et al. 1987). The area must be big enough to provide adequate buffer zones between plots, drainage ditches to prevent cross-contamination by movement of surface water in *Rhizobium* inoculation or strain trials, fencing against animals, and, in large experiments, to manoeuvre machinery. Further guidelines for planning and conducting field experiments are given by Brockwell (1980) and Wynne et al. (1987).

Errors in measuring symbiotic N_2 fixation arise because basic theoretical requirements of the isotope dilution method have not been fulfilled. These requirements (Table 6.2) should be carefully considered before experiments are undertaken. The more nearly they can be met, the more accurate will be the resulting estimates of N_2 fixation.

The design of experiments to measure N_2 fixation from the natural abundance of ^{15}N, is essentially the same as for ^{15}N enrichment studies. That is, it is necessary to have the non-N_2-fixing reference plant grown in close proximity to the N_2-fixing plants to minimise the effect of any site variability on measurements (Bergersen 1988; Reichardt et al. 1987). Ideally, plants immediately adjacent to, and growing in the same soil as the N_2-fixing legume test-plant should be harvested. This may be achieved by utilising non-nodulating lines or uninoculated legumes in treatment guard rows in cropping experiments, or using the non-legume in intercropping or mixed pasture studies as the reference (e.g. Bergersen and Turner 1983; Bergersen et

al. 1989; Rerkasem et al. 1988). Where reference plants cannot be so conveniently included within legume test-plots, separate reference plots sited as close as practicable to the test-plots will be required in the experimental design (e.g. Evans et al. 1987; Reichardt et al. 1987).

Since the proportion of N_2 fixed can be measured without measuring plant yield, ^{15}N-derived estimates of N_2 fixation usually have smaller coefficients of variation than those obtained by N-difference methods. Because of this it is not necessary in ^{15}N-enrichment experiments to label large areas of a field with isotope. However, ^{15}N subplots and harvest areas should be large enough to avoid edge effects due to the migration of ^{14}N and ^{15}N in the soil (for discussion of errors associated with plot size and lateral movement of ^{15}N from unconfined plots see Sanchez et al. 1987). Such edge effects can be minimised by enclosing the ^{15}N subplots with metal plates inserted into the soil (e.g. Bergersen and Turner 1983; Evans et al. 1987). Apart from cost of isotope, the choice of area for ^{15}N application is governed by row spacing and crop density which in turn will be determined by the growth habit of the particular legume under test (Brockwell 1980). It is convenient to discard the outer one or two rows of a subplot and to harvest only central rows. With many food legumes, for example, appropriate areas might be 1.5 to 2 m^2 for ^{15}N addition, with an area (encompassing three or four rows of crop) of 0.6 to 1 m^2 being taken (equivalent to 15 to 30 plants in most crops) for measurement of ^{15}N enrichment (Witty et al. 1988). The use of such

Table 6.2 Requirements of ^{15}N dilution techniques to measure N_2 fixation[a].

1. ^{15}N enrichment of reference plant = ^{15}N enrichment soil mineral N
   True if – No discrimination between ^{15}N and ^{14}N in soil
   – Control does not fix N_2
   – No isotope discrimination in N assimilation and metabolism
   – Plant parts sampled are representative of whole plant
   – In small plants seed N has no effect, or is allowed for

2. Soil N pool of reference = soil N pool of legume
   True if legume and reference:
   – are grown for same duration
   – are grown on the same soil
   – derive their N from the same soil N pool
   (a) have similar N uptake patterns, or
   (b) ^{15}N distribution in soil is uniform, and
   (c) soil enrichment is stable with time.

3. Legume takes up soil N and fertiliser N in the proportions present in the soil
   True if – No discrimination between ^{15}N and ^{14}N
   – no discrimination between different forms of N

4. That the process of measurement does not affect N_2 fixation
   True if – Added N fertiliser has no effect
   – Soil mineral N availability is not affected if a carbon source is used to immobilise ^{15}N, or when ^{15}N-labelled organic matter is incorporated.

5. That the method measures the amount of N_2 fixed
   True if – All fixed N is recovered in the crop.

[a] Adapted from Witty et al. (1988)

subplots saves on area and so encourages replication. Yield estimates for total crop N, from which amounts of N_2 fixed are calculated, should be taken from the much larger surrounding plot (section 3.1.1). If only one harvest is to be taken to provide an estimate of seasonal N_2 fixation it is desirable that plants are harvested at physiological maturity rather than at full maturity after complete senescence. Non-uniform distribution of ^{15}N in plant organs has been noted in both ^{15}N-enrichment and natural abundance experiments due to (a) changes in soil ^{15}N enrichment or the relative contributions of soil N uptake and N_2 fixation during periods of growth when the various plant tissues are laid down, or (b) isotopic discrimination between plant metabolic processes (Bergersen et al. 1988, 1989; Chalk 1985). It is essential, therefore, to harvest the entire plant before there is any significant leaf fall or tissue loss for accurate estimates of N_2 fixation. For this same reason, plant parts should not be separated and any subsampling should be done on a whole plant basis. This is not practical, however, in perennial woody legume investigations. Subsampling from mature trees for instance could be restricted to regrowth tissue if they are pruned at regular intervals for animal fodder or as a green manure mulch (Nair 1988). While some tree legume studies have utilised ^{15}N (e.g. Cornet et al. 1985), the specific problems of sampling and choice of appropriate reference plants have yet to be fully addressed.

If natural ^{15}N abundance and ^{15}N-enrichment investigations are being conducted on the same field experiment, it is essential that natural abundance plots are always sampled and handled before proceeding to the ^{15}N enriched plots. To ensure there is no transfer of traces of ^{15}N-enriched material to natural abundance samples separate implements (e.g. secateurs) should be used to harvest and collect plant tissue or soil from each plot where possible. Alternatively all items used for harvesting should be washed and scrubbed before re-use (see Bergersen 1988).

6.3.4 Preparation of samples and analysis

Preparation of samples for analysis will be dependent upon the instrumentation used for measuring ^{15}N.

Mass spectrometers resolve ionised molecules of N_2 into those of mass 28 ($^{14}N_2$), 29 (^{14}N:^{15}N) and 30 ($^{15}N_2$) and electrically measure the relative concentrations of the ions. Usually the N_2 is generated from NH_4^+ (derived from Kjeldahl digestion and distillation; Section 3) by oxidation with a reagent such as Na- or Li-hypobromite. This reaction is done in an evacuated container and the N_2 produced is introduced into the mass spectrometer. Some systems can directly oxidise plant material to produce N_2 for analysis (for details see Bergersen 1980; Fiedler 1984).

Optical emission spectrometers utilise a different principle. In these, N_2 is generated by heating ground plant material with copper oxide in a sealed glass tube. When excited by an electrical discharge through the tube, the various N_2 species emit UV radiation at different wavelengths and the relative intensity of the emission bands is measured (for details see Bergersen 1980; Fiedler 1984). The measurement of the band intensities alone does not always result in the calculation of the true ^{15}N abundance. When measurements are carried out on the NO 1–4 or NO 1–5 model emission spectrometers the use of calibration curves prepared from a set of

calibration tubes is necessary to determine true band intensities (Fiedler 1984). Emission spectrometers are cheaper than mass spectrometers and can use small samples of N (< 10 μg) but are much less sensitive and precise in measuring the ^{15}N content of samples. The apparatus is really suitable only where a standard deviation of 3% for replicate samples is acceptable and where the ^{15}N values of the samples are higher than 0.05-0.10 atom % excess (Bergersen 1980). Great care is also needed with sample preparation when analysing such small amounts of N since contamination potentially becomes very important. While mass spectrometry is the oldest, most accurate and widely used method of determining ^{15}N, most instruments require sample sizes of 0.5-1 mgN. However, some newer mass spectrometers can analyse samples of <100 μgN.

6.3.5 Relationships between isotope ratios, atoms % ^{15}N and $\delta^{15}N$

The mass spectrometer measures the isotope ratio R, in a sample of N_2.

$$R = \frac{\text{mass } 29}{\text{mass } 28} = \frac{b}{a} \qquad (1)$$

where a is the signal produced by ions of mass 28 ($^{14}N_2$) and b that produced by ions of mass 29 ($^{14}N:^{15}N$). These signals are proportional to the relative concentrations of molecules of mass 28 and 29 in a sample of N_2.

The ratio $^{15}N/^{14}N$ is sometimes used for calculation of δ

$$^{15}N/^{14}N = \frac{b/2}{a + b/2} = \frac{b}{2a + b} = \frac{R}{2 + R} \qquad (2)$$

But this ignores the presence of undetected amounts of $^{15}N_2$ (mass 30) in mass-equilibrated samples of N_2.

The term atoms %^{15}N (i.e. the percentage of N atoms which are ^{15}N) is usually used to describe the abundance of ^{15}N in a sample of N_2 generated from any N-containing compound.

$$\text{Atoms \% } ^{15}N = \frac{100\,(b/2+c)}{a + b + c} \qquad (3)$$

where c is the signal produced by ions of mass 30 ($^{15}N_2$).

At equilibrium at room temperature, in the reaction:

$$^{14}N_2 + {}^{15}N_2 \rightleftarrows 2\,^{14}N:^{15}N^*$$

$K = b^2/ac = 4$, i.e. $c = b^2/4a$

Substituting in (3):

---

* This equilibrium is not attained at room temperature unless the N_2 is generated from a monatomic source such as NH_4^+, because the N_2 molecule is very unreactive. Consequently, non-equilibrium proportions exist when samples of N_2 generated from highly ^{15}N-enriched sources are mixed with air N_2 for example.

$$\text{Atoms \% } {}^{15}N = \frac{100(b/2 + b^2/4a)}{(a + b + b^2/4a)}$$

$$= \frac{100(2ab + b^2)}{(4a^2 + 4ab + b^2)}$$

$$= \frac{100(b/a)}{(2 + b/a)} \quad \text{from (1)}$$

$$= \frac{100\ R}{2 + R} \qquad (4)$$

However, at high values of atoms %, the above calculation becomes inaccurate because K is not exactly equal to 4. Therefore in these cases, all 3 peaks at mass 28, 29 and 30 should be measured and atoms % calculated as in (3). This should also be done for samples of N_2 in which molecules of mass 28, 29 and 30 are not in equilibrium.

The term $\delta^{15}N$ (‰) can be calculated in three ways to give similar but not identical values.

$$\text{(i)} \quad \delta = \frac{1000\ (R_{sample} - R_{standard})}{R_{standard}} \qquad (5)$$

in which the standard is usually atmospheric N_2 (0.3663 atom %) or another standard of known relationship to atmospheric N_2. By definition the $\delta\ ^{15}N$ of air–N_2 therefore is zero.

$$\text{(ii)} \quad \delta = \frac{1000[{}^{15}N/{}^{14}N_{sample} - {}^{15}N/{}^{14}N_{standard}]}{{}^{15}N/{}^{14}N_{standard}} \qquad (6)$$

or

$$\delta = \frac{1000[(\text{atoms \% } {}^{15}N)\text{sample} - (\text{atoms \% } {}^{15}N)\text{standard}]}{(\text{atoms \% } {}^{15}N)\text{standard}} \qquad (7)$$

It is obvious from (1) to (4) above, that slightly different values for δ will be calculated, according to the expression used and the enrichment of the sample. These effects are shown in the following examples at 4 levels of enrichment.:

R	0.00735293*	0.00736764	0.00749999	0.00882352	0.0220588
$^{15}N/^{14}N$	0.003663*	0.003670	0.003736	0.004392	0.010909
atoms % ^{15}N	0.3663*	0.3670	0.3736	0.4392	1.0909
$\delta^{15}N$(i)	0*	2.00	20.0	200	2000
$\delta^{15}N$(ii)	0*	1.91	19.9	199	1978

* Values for air N_2 as standard.

To convert a δ value to atoms $\%^{15}N$

(a) where δ is calculated as in (5) above:

$$R_{sample} = R_{standard}\,(1 + \delta/1000)$$

and Atoms $\%\ ^{15}N$ sample $= (100\ R_{sample})/(2 + R_{sample})$

(b) where δ is calculated as in (6) above:

$$\text{Atoms } \%\ ^{15}N_{sample} = (\text{Atoms } \%\ ^{15}N_{standard})(1 + \delta/1000).$$

6.3.6 Evaluation of analytical data and estimation of N_2 fixation

6.3.6.1 ^{15}N-enrichment

The percentage of legume N fixed from atmospheric N_2 (P) is estimated from the following equation:

$$P = 100 \times \left\{1 - \frac{(\text{atoms } \%\ ^{15}N \text{ excess legume N})}{(\text{atoms } \%\ ^{15}N \text{ excess soil derived N})}\right\} \qquad (8)$$

where atoms $\%\ ^{15}N$ excess = (atoms $\%\ ^{15}N_{sample}$ – (atoms $\%\ ^{15}N_{air N_2}$) where the atom $\%\ ^{15}N$ in air N_2 is 0.3663. The atoms $\%\ ^{15}N$ of soil-derived N is generally estimated from the atoms $\%\ ^{15}N$ of a non-N_2-fixing reference plant grown in the same soil over the same period as the legume.

An example of the calculation of P and estimates of N fixed (kg/ha) using a ^{15}N enrichment method is presented in Tables 6.3 and 6.6 respectively (see also discussion at end of Section 6.3.6.2). The data are taken from a field experiment in which a single application of 0.4 g of $K^{15}NO_3$ (ca. 95 atoms $\%\ ^{15}N$) dried onto sand (to ensure uniform application to soil) was made to both the test-legume (lupin) and reference (wheat) in 0.9 × 0.9m microplots (^{15}N-fertiliser application equivalent to 0.7 kg N/ha) confined by metal shields inserted 0.12 m into the soil and spanning 5 rows (Evans et al. 1987). Six plants were sampled from each of 4 replicate microplots 128 days after sowing, and 0.44 m^2 of each microplot was harvested on day 193.

6.3.6.2 Natural ^{15}N abundance

An estimate of P is obtained using the following equation analagous to equation (8):

$$P = 100 \times \frac{\delta^{15}N \text{ (soil N)} - \delta^{15}N \text{ (legume N)}}{(\delta^{15}N \text{ (soil N)} - B)} \qquad (9)$$

where $\delta^{15}N$ (soil N) is commonly obtained from a non-N_2-fixing reference plant grown in the same soil as the legume, and B is the $\delta^{15}N$ of the same N_2-fixing plants when grown with N_2 as the sole source of N. Isotopic fractionation during N_2 fixation is minimal but not zero and should be taken into account. The value of B is

Table 6.3 Content of ^{15}N in shoots of lupin and a non-N_2-fixing reference plant (wheat) from ^{15}N enriched microplots, and calculation of the proportion of plant N derived from N_2 fixation (P)[a].

Species	Days from sowing					
	128			193		
	atom % ^{15}N	atom % ^{15}N excess[b]	P[c]	atom % ^{15}N	atom % ^{15}N excess[b]	P[c]
Lupin	0.4344	0.0681	78.6	0.4112	0.0449	84.5
Wheat	0.6852	0.3189	–	0.6561	0.2898	–

[a] Derived from the data of Evans et al. (1987). Mean of 4 replicates.
[b] Calculated as atom % ^{15}Nexcess = (atom % ^{15}N sample) – (atom % ^{15}N airN_2). Where atom % ^{15}N airN_2 = 0.3663.
[c] Calculated from equation (8).

Table 6.4 Examples of the $\delta^{15}N$ of the total N of effectively nodulated legumes grown on media free of combined N.

Species	$\delta^{15}N$ of plant parts (‰)	
	shoots only	whole plant
Lupinus spp.	– 0.55[a]	– 0.05[b]
Vigna umbellata	– 0.91	0.04[c]
Glycine max	– 1.30	– 0.79[d]
Cajanus cajan	– 0.90	0.29[e]
Arachis hypogea Spanish type	0.86	
	0.65[f]	0.70[e]
Virginia type	0.67[f]	0.73[e]
Lablab purpureus	– 1.36	– 0.14[e]
Desmodium uncinatum	– 0.56	0.06[e]
Centrosema spp.	– 1.08	0.03[e]
Stylosanthes hamata	– 0.26	– 0.23[e]
Calopogonium spp.	– 0.95	0.05[e]
Pueraria spp.	– 1.22	0.10[e]
Medicago sativa	– 0.92[g]	–[h]
Trifolium pratense	– 0.88[g]	–
Phaseolus vulgaris	– 1.97[g]	–
Vicia faba	– 0.63[g]	–
Pisum sativum	– 1.00[g]	–

[a] Evans et al. (1987) with *R. lupini* strain WU425
[b] Bergersen et al. (1986) — mean of 12 *R. lupini* strains
[c] Rerkasem et al. (1988)
[d] Bergersen et al. (1988)
[e] M.B. Peoples and D.M. Hebb, unpublished data
[f] Including pegs and nuts
[g] Mariotti et al. (1983)
[h] Not reported

determined by analysis of $\delta^{15}N$ of total N accumulated by a nodulated legume grown in N-free media. A 'B-value' should ideally be prepared for each new legume species studied. Examples of values of B for a number of different legumes are presented in Table 6.4. The appropriate value used in equation (9) will depend upon the tissue sampled for analysis (i.e. shoot only, or shoot plus excavated roots).

An example of the determination of *P* using the natural ^{15}N abundance procedure is presented in Table 6.5. Lupin plants were harvested twice during the crop's growing season (at 128 and 193 days after sowing) for crop N and ^{15}N. The ^{15}N abundance of the legume and non-N_2-fixing reference plant (wheat) at each sampling provided

Table 6.5 Natural abundance of ^{15}N in shoots of lupin and a non-N_2-fixing reference plant (wheat), and calculation of the proportion of plant N derived from N_2 fixation (*P*)[a].

Species	Days from sowing					
	128			193		
	atom % ^{15}N	$\delta^{15}N$[b]	*P*[c]	^{15}N	$\delta^{15}N$[b]	*P*[c]
Lupin	0.36629	-0.03	88.4	0.36642	0.33	81.3
Wheat	0.36774	3.93	–	0.36782	4.15	–

[a] Derived from the data of Evans et al. (1987). Mean of 6 replicates.
[b] Calculated from equation (7) Section 6.3.5, where atom % $^{15}N_{standard}$ = 0.3663 atom % ^{15}N.
[c] Calculated from equation (9), where B = -0.55 (Table 6.4)

Table 6.6 Calculation of the seasonal pattern of N_2 fixation in a field crop of lupin using ^{15}N-enrichment and natural ^{15}N abundance techniques

Period (days)	Crop N (kg/ha)[a]	Estimate of *P* (Proportion of plant N from N_2 fixation)			Estimate of fixed N (kg/ha)[d]	
		^{15}N-enriched[b]		Natural abundance[c]		
		I	(%)	II	I	II
(A) 0–128	136	78.6		88.4	106.9	120.2
(B) 0–193	306	84.5		81.3	258.6	248.8
128–193	170	89.4[f]		75.9[f]	151.7[e]	128.6[e]

[a] Derived from the data of Evans et al. (1987)
[b] From Table 6.3
[c] From Table 6.5
[d] Calculated as Crop N × *P*/100
Estimates of N fixed by ^{15}N-enrichment (I) or natural ^{15}N abundance (II) techniques were within 12% of each other.
[e] Incremental values between harvests at 128 and 193 days after sowing were calculated by difference, i.e. (B) – (A)
[f] (Incremental estimate of fixed N/Incremental change in crop N) × 100.

estimates of the average P for the lupin crop from sowing to the day of harvest. Calculations of N_2 fixation inputs in Table 6.6 (ie. Crop N × P/100) also represented quantities of N fixed over the period from sowing to time of harvest. The amounts of N fixed in the interval between harvests therefore, were derived by difference. Procedures to calculate standard errors of estimates of P and N_2 fixation are detailed in the appendix to Bergersen et al. (1989).

6.3.6.3 Adjustment for seed N

When small plants are harvested for N_2 fixation determinations by isotope-dilution, seed N can represent a significant amount of the total legume N and/or influence the ^{15}N enrichment of dry matter (particularly in natural ^{15}N abundance studies). Samples of the original seed stock sown in an experiment should always be retained for analysis, and calculations adjusted to account for the contribution of seed N if necessary. A similar error can also result from seed N in the reference crop leading to an underestimation of the ^{15}N enrichment of the soil plant-available N pool. This in turn would result in an underestimation of N_2 fixation when used in equations (8) or (9). Such errors will be dependent on the ratio of reference seed N : total reference N at harvest and can be corrected using the following:

$$^{15}\text{N enrichment (soil N pool)} = \frac{^{15}\text{N enrichment (reference N)} \times \text{Total reference N}}{(\text{Total reference N} - \text{reference seed N})} \qquad (10)$$

7
Conclusion

There have been considerable advances in the methodology of measuring N_2 fixation in recent years, particularly in establishing a better knowledge of the limitations of the various techniques in current use (summarised in Table 7.1; overleaf). In order to obtain accurate estimates of N_2 fixation in the field, these limitations must be recognised and procedures used to either reduce their effect or to check on their relative importance in the calculation of symbiotic activity. This is especially true when the choice of method is restricted by technical, environmental or plant cultural factors. Ideally, N_2 fixation measurements should be related to soil mineral-N content and plant nodulation. Studies should also be associated with research on N transfer or effects on subsequent crops if an overall estimate of the value of fixed N_2 is to be obtained. Finally, methodology should be chosen in light of the overall objectives of the study. Sophisticated and elaborate techniques may not be appropriate when only qualitative or comparative determinations are required.

Table 7.1 Summary of the advantages and limitations of methods for estimating N_2 fixation by legumes in the field[a]

Advantages	Limitations
N–Solute method	
• inexpensive • simple • analyses done by simple colorimetric assays • need not be totally destructive • may be done on an individual plant basis • assesses plant dependence on atmospheric N_2 and soil N	• indirect (must establish calibration with plant N_2 fixing status) • short–term estimate • cannot be used for interspecific comparisons without calibrating each species • calibrations may be influenced by developmental stage in some species • cannot be used on some amide–producers
N Difference method	
• direct • relatively simple • adjusts for soil–derived N	• requires suitable non N_2 fixing reference plant • legume and reference plant must absorb the same amount of soil N
Isotope dilution techniques	
• direct • give time–integrated estimate of % N fixed • assesses plant dependence on atmospheric N_2 and soil N	• requires suitable non–N_2 fixing reference
(a) ^{15}N enrichment method	
• potentially accurate	• instrumentation and ^{15}N enriched materials expensive • requires addition of ^{15}N–labelled compound • legume and reference plant must absorb the same relative amounts of N from the soil and added ^{15}N • ^{15}N enrichment of plant–available soil N can change with depth and time
(b) Natural ^{15}N abundance method	
• No ^{15}N addition required • $\delta^{15}N$ of plant–available soil N can be relatively constant with depth and time • choice of reference plant may be less important than ^{15}N enrichment studies	• requires a precise mass spectrometer and meticulous analytical procedures • insensitive if $\delta^{15}N$ (soil) nears $\delta^{15}N$(air) • field variability may be large in some cases • may have to allow for isotopic fractionation during N_2 fixation

[a]Adapted from Ledgard and Peoples (1988)

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10
Appendix

10.1 Sources of *Rhizobium* strains

The NifTAL *Rhizobium* Germplasm Resource is a comprehensive collection of *Rhizobium* for numerous legumes (tropical and temperate) and is maintained at the NifTAL Project. Strains are available on written request to: Curator, *Rhizobium* Germplasm Resource, University of Hawaii, NifTAL Project and MIRCEN, P.O. Box 0, Paia, Hawaii 96779, USA.

There are also other laboratories/institutions which maintain collections of *Rhizobium*:

Dr Carlos Batthyany Nitrosoil Florida 622 4 Piso Buenos Aires Argentina	Rhizobia for tropical legumes
Dr R.J. Roughley Australian Inoculants Research and Control Service Horticultural Research Station Gosford, NSW 2250 Australia	AIRCS strains
Dr R.A. Date CSIRO Division of Tropical Crops and Pastures Mill Road St Lucia, Queensland 4067 Australia	Rhizobia for tropical legumes
Mr J. Brockwell Microbiology Section CSIRO Division of Plant Industry Canberra, ACT 2600	Rhizobia for clovers, medics, and other temperate species

Professor J.R. Jardim Friere Rhizobium MIRCEN IPAGRO Caixa Postal 776 90000 Porto Alegre Rio Grande Do Sul Brazil	Rhizobia for tropical legumes
Dr D.J. Hume Crop Science Department University of Guelph Guelph, Ontario N1G 2W1 Canada	Rhizobia for pea, lupin, alfalfa, and soybean
Dr J. Day Soil Microbiology Department Rothamsted Experimental Station Harpenden, Herts A15 2JQ UK.	Rhizobia for clovers, alfalfa, peas, beans, and other temperate legumes
Plant Diseases Division DSIR Private Bag Auckland New Zealand	Rhizobia for clovers, alfalfa, and lupin
Dr D.F. Weber ISDA CCNFL Building 001, Room 309 BARC–W Beltsville, MD 20705 USA	Rhizobia for temperate legumes

More addresses of institutions which have *Rhizobium* collections can be found in: Skinner, F.A., Hamatova, E., and McGowan, V.F. 1983. In: World catalogue of *Rhizobium* collections Skerman, V.B.D., ed., World data centre for microorganisms at the University of Queensland, Brisbane, Australia..

10.2 Australian inoculant producers

All three manufacturers listed below can supply inoculants for most legumes.

Australian Laboratories Pty Ltd
PO Box 8
Regents Park, NSW 2143

Root-nodule Pty Ltd
84 Rawson Road
Woy Woy, NSW 2256

Inoculant Services
'Teangi'
Bethanga, Vic. 3691.