Fertilizer Research **42:** 77–87, 1995.

Field application of the ^{15}N isotope dilution technique for the reliable quantification of plant-associated biological nitrogen fixation

Robert M. Boddey, Octávio C. de Oliveira, Bruno J.R. Alves & Segundo Urquiaga
EMBRAPA - Centro Nacional de Pesquisa de Agrobiologia, Km 47, Seropédica, Itaguaí, 23851-970, Rio de Janeiro, Brazil

Key words: biological nitrogen fixation, control crops, isotope dilution technique, legumes, ^{15}N slow-release fertilizer

Abstract

To apply the isotope dilution (ID) technique, it is necessary to grow the "N_2-fixing" crop in a soil where the mineral N is labelled with ^{15}N. Normally the "N_2-fixing" crop and a suitable non-N_2-fixing control crop are grown in the same labelled soil and the ^{15}N enrichment of the control crop is assumed to be equal to the ^{15}N enrichment of the nitrogen (N) derived from the soil in the "N_2-fixing" crop. In this case the proportion of unlabelled N being derived from the air via biological N_2 fixation (BNF) in the "N_2-fixing"crop will be proportional to the dilution of the enrichment of the N derived from the labelled soil.

To label the soil, the technique most often used is to add a single addition of ^{15}N-labelled N fertilizer shortly before, at, or shortly after, the planting of the crops. Data in the literature clearly show that this technique results in a rapid fall in the ^{15}N enrichment of soil mineral N with time. Under these conditions, if the control and the "N_2-fixing" crops have different patterns of N uptake from the soil they will inevitably obtain different ^{15}N enrichments in the soil-derived N. In this case the isotope dilution technique cannot be applied, or if it is, there will be an error introduced into, the estimate of the contribution of N derived from BNF.

Several experiments are described which explore different strategies of application of the ID technique to attempt to attenuate the errors involved. The results suggest that it is wise to use slow-release forms of labelled N, or in some cases, multiple additions, to diminish temporal changes in the ^{15}N enrichment of soil mineral N. The use of several control crops produces a range of different estimates of the BNF contributions to the "N_2-fixing" crops, and the extent of this range gives a measure of the accuracy of the estimates. Likewise the use of more than one ^{15}N enrichment technique in the same experiment will also give a range of estimates which can be treated similarly. The potential of other techniques, such as sequential harvesting of both control and test crops, are also discussed.

Introduction

Of the techniques available to quantify the contribution of biological N_2 fixation (BNF) to nodulated legumes and other "N_2-fixing" plants, only techniques which utilize the stable isotope ^{15}N can provide direct estimates of the quantity of biologically-fixed N incorporated into plant tissue. The use of ^{15}N-labelled N_2 gas is usually only feasible for short-term experiments under controlled conditions, but the ^{15}N isotope dilution (ID) technique can be applied to field studies over the whole plant growth cycle.

To apply the ID technique it is necessary to grow the "N_2-fixing" legume in a soil where the mineral N is labelled with ^{15}N. If the ^{15}N enrichment of the N being absorbed from the soil by this plant is known, then the amount of unlabelled N being derived from the air via BNF will be proportional to the dilution of the enrichment of the N derived from the labelled soil.

Normally the "N_2-fixing" crop and a suitable non-N_2-fixing control crop are grown in the same labelled soil and the ^{15}N enrichment of the control crop is regarded as the ^{15}N enrichment of the N in the legume derived from the soil. In fact, if the ^{15}N enrichment of the labelled soil N can be determined by direct analysis of the ^{15}N enrichment of the soil mineral N [12,19,38], then the use of control crops can be dispensed with.

In the case where a non-N_2-fixing control crop is utilized, the basic assumption made in applying the technique is that the ^{15}N enrichment of the N derived from the soil by the "N_2- fixing" crop is equal to the ^{15}N enrichment of the N in the control crop. Alternatively this can be expressed as: The ratio (R) of labelled fertilizer N to unlabelled soil N accumulated by the plants is the same for the "N_2-fixing" crop and the control crop.

Labelling the soil N with ^{15}N

Addition of soluble labelled N fertilizer

The technique most often used is to add a single addition of soluble ^{15}N-labelled N fertilizer (eg. ammonium sulphate or urea) to the surface of the soil shortly before, at, or shortly after, the planting of the crops. This form of ^{15}N addition results in a rapid fall in the ^{15}N enrichment of soil mineral N, as the ^{15}N-labelled N is added to the soil mineral N pool which is continuously being replenished by unlabelled N from the mineralization of soil organic matter [17,38,49]. This is illustrated by data from a recent field experiment where ^{15}N-labelled ammonium sulphate (10 kg N ha^{-1}) was added 1 day after planting of *Phaseolus vulgaris* (Fig. 1 - Boddey *et al.*, unpubl. data). Under these conditions if the control and the legume crops have different patterns of uptake of N from the soil they will inevitably obtain different ^{15}N enrichments in the soil-derived N. As is evident from the basic assumption of the technique this mean that the isotope dilution technique cannot be applied, or if it is, there will be an error introduced into the estimate of the contribution of N derived from BNF [3,11,14,35,45,50,51,52].

The obvious solution to this problem would appear to be to select a non-N_2-fixing control crop which has the same soil-N uptake pattern as the "N_2-fixing" crop. While the uptake of soil N by a non-N_2-fixing crop can be studied by sequential harvests, this is not possible in the case of the "N_2-fixing" crop as there is no way to distinguish between unlabelled N derived from soil and that derived from BNF.

Ledgard *et al.* [28] developed a technique to compare the ratio (R) of fertilizer N to soil N in "N_2-fixing" and control crops which depended on the use of increasing additions of labelled N and the measurement of the natural ^{15}N abundance in both crops in a treatment where no N fertilizer was added. The technique requires great care to be taken to avoid contamination

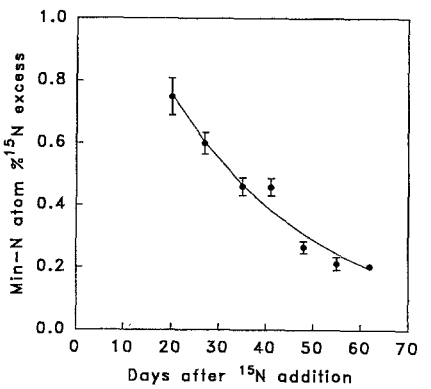

Fig. 1. Decline in ^{15}N enrichment of soil mineral N (KCl extract) in soil amended with 10 kg N ha^{-1} of ^{15}N-labelled ammonium sulphate (10.1 atom % ^{15}N). Vertical bars indicate standard errors of means (20 replicates). Boddey *et al.*, (unpubl. data)

of the unfertilized plots with enriched N and the use of a sensitive (double inlet) mass spectrometer. While the technique can theoretically be used to select appropriate control crops for any particular legume crop (any control crop which attained a ^{15}N enrichment similar to the enrichment of the N derived from the soil by the legume), the extra work involved and its low sensitivity in soils with low natural ^{15}N abundance [3], has not encouraged its application by other workers.

A further technique was explored by Wagner and Zapata [46] based on the interesting idea that if the ratio of added labelled to native unlabelled sulphur taken up from the soil by the two crops is equal, then the ratio of labelled to unlabelled N should also be equal. As both S and N are controlled in the soil by similar microbiological processes the idea was logical. However, in two experiments designed to test this technique, Hamilton *et al.* [20,21] found that there was no fixed relationship between the ratios of labelled to unlabelled S and labelled and unlabelled N among the different control crops and legume crops and thus the ratio of labelled to unlabelled S could not be used as evidence for equal (or unequal) ratios of labelled to unlabelled N derived from soil sources by the different crops.

Rennie and Thomas [36] suggested that it was possible to test if the ^{15}N enrichment of the soil N taken up by the "N_2-fixing" and control was equal, by calculating the soil 'A-value' for each of the crops from the % Ndff and % Ndfs (%N derived from fertilizer and soil, respectively). They quite correctly stated that if the 'A_s-value' was equal for the "N_2- fixing" and control crops then the two crops did remove N from the soil

with the same ^{15}N enrichment. However, to calculate the %Ndfs in the "N_2-fixing" crop it is necessary to apply the ^{15}N ID technique and assume that the basic assumption of equality of (R) for both crops holds [7]. As the technique is based on circular logic, it follows that the ratio R will always be the equal for all crops tested and hence the "test" is no test at all.

It is apparent that testing the basic assumption of the ID technique is extremely difficult and the question arises of the magnitude of possible errors involved in relying on this assumption without testing it. While most investigators appear to be conscious of the risks involved, most studies, even recent ones, generally use a single addition of ^{15}N labelled fertilizer and a single harvest of the "N_2-fixing" and control crops (the single ^{15}N addition/single harvest technique) without testing whether soil N uptake patterns of the two crops were the same.

Magnitude of errors involved in the use of the "single ^{15}N addition single harvest technique"

As it is difficult to discover the ^{15}N enrichment of soil-derived N in a "N_2-fixing" crop, how can it be determined whether there are significant differences between this enrichment and that derived from the soil by the control crop? The answer is by inference: it is logical to assume that if there are large differences in ^{15}N enrichment between different control crops, then it is possible that there will be large differences between the ^{15}N enrichment of the control crop and that of the soil-derived N in the legume.

Only a small proportion of the ID studies to quantify BNF contributions to legumes have used more than one control crop. However, in almost all of them where the soil was amended with a single dose of soluble labelled fertilizer, significant differences between the ^{15}N enrichments of different control crops were recorded [15,19,21,46,50]. This is illustrated by data from a recent field experiment conducted at our Centre, where 4 non-N_2-fixing control crops (non-nod soybean, rice, okra and sorghum) were used in an experiment to quantify the BNF contribution to soybean inoculated with two different *Bradyrhizobium japonicum* strains (Boddey *et al.*, unpubl.).

Figure 2 shows the contrasting uptake patterns of labelled and unlabelled N by the non-nod soybean and the sorghum which resulted at the final harvest (73 days after planting - DAP) of ^{15}N enrichments of 0.1491 and 0.1012 atom % ^{15}N excess in the two controls, respectively. The estimates of the BNF contributions (% N derived from BNF - %Ndfa) to the nodulated soybean ranged from 13.7 to 50.2%, and 31.5 to 59.7%, for the soybean inoculated with the strains 29W and CB 1809 of *B. japonicum*, respectively, depending on which control was used (Table 1).

Table 1. Estimates the contribution of biologically fixed nitrogen to nodulating soybean inoculated with either 29W or CB 1809 strains of *Bradyrhizobium japonicum* using 4 different non-N_2-fixing control crops

Controls	%dfa[b] of nodulating soybean	
	29W	CB 1809
Non nod soybean	47.2	58.3
Sorghum	15.7	32.5
Rice	50.2	59.7
Okra	13.7	31.5
LSD (Student)	30.6	25.0

[a]Soil labelled at planting with a single addition of ^{15}N-labelled ammonium sulphate. Data are means of 4 replicates. Boddey *et al.* (unpubl. data).
[b]% N derived from BNF.

Occasionally differences in N uptake patterns between legume and control crops are so large that application of the ID calculations yields clearly erroneous negative estimates of contributions of BNF [29,33,37,51].

As has been pointed out by several authors when the %Ndfa in the "N_2-fixing" crop is high, the estimate of %Ndfa is relatively insensitive to mismatching of soil N uptake patterns of the different crops [7,22,23]. This is well illustrated. by a simulation of the influence of a 5, 10 or 20% variation in ^{15}N enrichments of different control crops on the estimates of Ndfa of the "N_2-fixing" crop (Fig. 3).

It follows that if it is thought that %Ndfa is low, or could be low, then the application of the ID technique using a single dose of ^{15}N labelled fertilizer at planting with a single harvest at crop maturity, will not yield a reliable estimate of the BNF contribution to the "N_2-fixing" crop.

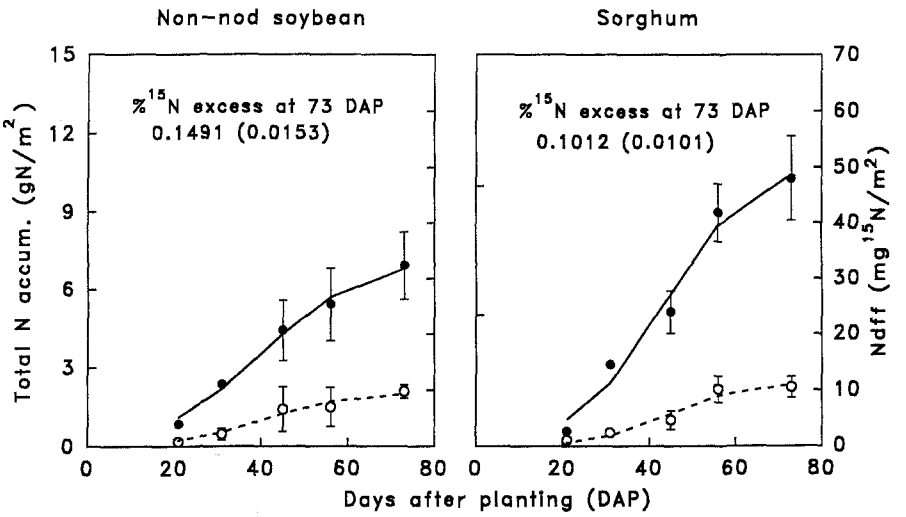

Fig. 2. Accumulation of labelled (- - - 0 - - -) and total N (—•—) by 2 non-N_2-fixing control plants (non-nod soybean and sorghum) in soil amended with 2.0 kg N ha^{-1} of ^{15}N-labelled ammonium sulphate (21.3 atom % ^{15}N) at planting. Vertical bars indicate standard errors of means (4 replicates). Boddey *et al.*, (unpubl. data).

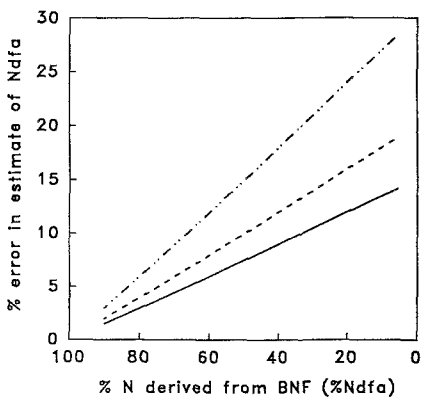

Fig. 3. Simulation of the influence of 5(———), 10 (- - -) or 20% (·····) variation in the ^{15}N enrichment of different control crops on the estimates of N derived from BNF, at decreasing proportions of N derived from BNF (%Ndfa). For the simulation it was assumed that there was a 10% variability in the ^{15}N enrichment of the "N_2-fixing" crop.

Strategies to apply the ID technique to provide reliable estimates of BNF contributions

Labelling the soil to diminish temporal variations of ^{15}N enrichment of soil mineral N

From the above discussion it is apparent that when the soil is labelled with a single dose of ^{15}N-enriched fertilizer at planting, different crops can obtain very different ^{15}N enrichments in the N they accumulate from the soil. An obvious solution to this problem is to attempt to label the soil in such a manner that temporal variations in ^{15}N enrichment of soil mineral N are diminished. Possible techniques were discussed by Chalk [11] and Boddey [3] although just about all techniques have their disadvantages:

a. Pelleting ^{15}N-labelled ammonium sulphate with gypsum was used by Witty [49] and later by others [7,18], but these latter authors showed that it was not very effective in slowing down ^{15}N release.

b. Immobilizing the mineral N from these fertilizers by adding sugars, cellulose, sawdust or straw has also been tried [9,18,30,33,39,40]. The favourable effect of immobilizing the labelled fertilizer was shown in an experiment where 3 control crops were utilized to quantify the BNF contribution to field grown soybean in an experiment performed in the field near Brasília [4]. The data show that in the treatment where sugar was mixed with the ^{15}N-labelled ammonium sulphate fertilizer in a C:N ratio of 10:1, rates of decline of ^{15}N enrichment of the plants with time were considerably reduced (Fig. 4). In the treatment where the labelled fertilizer was immobilized with sugar, the differences between the ^{15}N enrichments of the different control crops were considerably less than when no sugar was added (Table 2).

However, even in cases where such techniques were used to immobilize the added labelled N, significant differences in ^{15}N enrichment between different control crops have been reported [7,46,49].

Table 2. ^{15}N enrichment and total N accumulation of nodulated soybean and 3 non-N_2-fixing control crops grown in soil amended with ^{15}N-labelled ammonium sulphate at planting, with or without the addition of sugar at a C:N ratio of 10:1[a]

Crop	Total N accumulation ($g\ m^{-2}$)		^{15}N enrichment (Atom % ^{15}N excess)	
	-Sugar	+Sugar	-Sugar	+Sugar
Nodulated soybean	17.71	20.36	0.3616	0.3238
Non-nod soybean	15.81	15.96	0.4314	0.3770
Sorghum	6.63	6.18	0.4272	0.3253
Sunflower	16.70	20.93	0.6217	0.4778
LSD (Student)	7.36	11.60	0.1362	0.1481

[a]After Boddey and Urquiaga [4].

The other disadvantage is that adding carbon sources is likely to reduce the availability of soil N to the plants thus affecting (probably positively) the amount of N derived from BNF by the legume. This does not seem to have been the case in the study described above [4] where total N yield of the nodulated soybean and control crops was not significantly affected by the addition of sugar (Table 2).

c. Additions of ^{15}N labelled plant material or compost. This has also been used quite frequently, especially by our group in Rio [5,6,7,24,41]. Again initially ^{15}N enrichment of soil N declines quite quickly, although less quickly than if soluble labelled fertilizer is added. This treatment also immobilises soil N (see b. above) but in our (tropical) conditions after about 18 months soil ^{15}N enrichment becomes virtually stable.

d. Multiple small additions of labelled soluble N fertilizer. This technique has been favoured by many authors [5,6,16,29,42,43], but has been criticized by Rennie [34] on the basis that if test and control crops assimilate different quantities of mineral N between harvests then the residual mineral N in the soil at harvest will be different for the two crops. A further addition of labelled N to this pool will then cause a different initial enrichment in the mineral N for the two crops at the start of the second growth period. However, if the quantity of labelled N added is small compared with plant uptake then it is unlikely that significant amounts of residual mineral N are available at the time of harvest, which as Rennie admits, invalidates this criticism. The evidence suggests that this technique is more effective in reducing temporal changes in ^{15}N enrichment of soil mineral N than the single addition technique, and has the advantage that the ^{15}N enrichment of the soil mineral N is still high towards the end of the crop growth cycle when BNF inputs are usually largest. If small amounts are used this does not significantly alter soil mineral N availability, unlike techniques b. and c. above, so that it is a good technique to use if the objective of the study is to estimate BNF in an actual farming system. The disadvantage comes in the labour involved in the additions and making sure that no significant amounts of ^{15}N salts are absorbed by the leaves of the developing or mature plants. This latter problem can be attenuated by applying the ^{15}N material dried onto sand particles which can be brushed off the leaves and then subsequently washed into the soil by irrigation [2,10].

Use of several control crops

Recently we suggested that a good strategy to apply the ID technique is to use several controls in conjunction with a technique to reduce temporal changes in ^{15}N enrichment of soil mineral N [7]. None of the above techniques will produce a completely stable and uniform ^{15}N enrichment of soil mineral N unless the fertilizer is mixed throughout the whole volume of the soil and then left to equlibriate for many months [41]. As for normal field experiments this is not practical, the idea behind this strategy is that if there are still considerable changes in soil mineral N enrichment with time then each different control crop can give an independent estimate of the BNF contribution to the legume. If the range of these estimates is small, then it is probable that the estimate is fairly accurate, and the range of the different estimates gives an idea of the accuracy. The data in our paper illustrates the use of this approach.

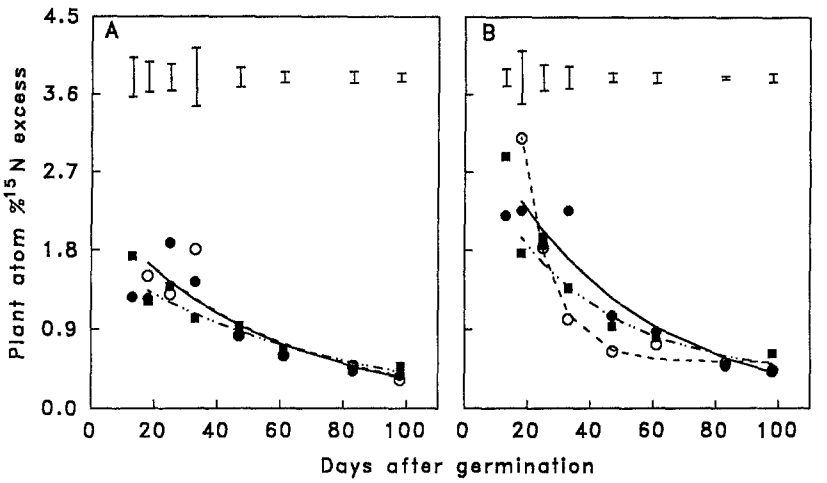

Fig. 4. Decline in ^{15}N enrichment of 3 non-N_2-fixing control plants grown in soil amended with ^{15}N-labelled ammonium sulphate with (A) or without (B) the addition of sugar in a C:N ratio of 10:1. —•— non-nod soybean, - - 0 - - - sorghum, ----■---- sunflower. Vertical bars indicate standard errors of means (4 replicates). After Boddey and Urquiaga [4].

Naturally more work and analyses are involved but a much more reliable estimate of the BNF contribution to the legume is obtained.

Use of two different methods of applying ^{15}N

This technique was suggested by Hamilton *et al.* [20] and its use demonstrated in a recent paper of Viera-Vargas *et al.* [44]. It is recommended that contrasting techniques of application of ^{15}N to the soil are utilized. By contrasting it is meant that they cause different types of changes in ^{15}N enrichment in the soil mineral N with time. Probably this means that either a single addition of soluble labelled fertilizer, or a slow-release form (options b. or c. above) should be used together with the multiple additions technique. The former techniques cause a decrease in ^{15}N enrichment of soil mineral N with time, and the latter can cause an overall increase of enrichment of this N with time. It is preferable also to use several control crops with this technique, although perhaps just 2 may be sufficient instead of 3 or so for the multiple controls technique described above (section: *Use of several control crops*). If the legume and one of the control crops have identical patterns of soil N uptake in the two ^{15}N labelling treatments then the estimates of N derived from BNF by the legume will also be identical. It follows that the control crop which gives the closest agreement in BNF estimate in the two contrasting ^{15}N labelling treatments is that which is giving the estimate closest to the true contribution.

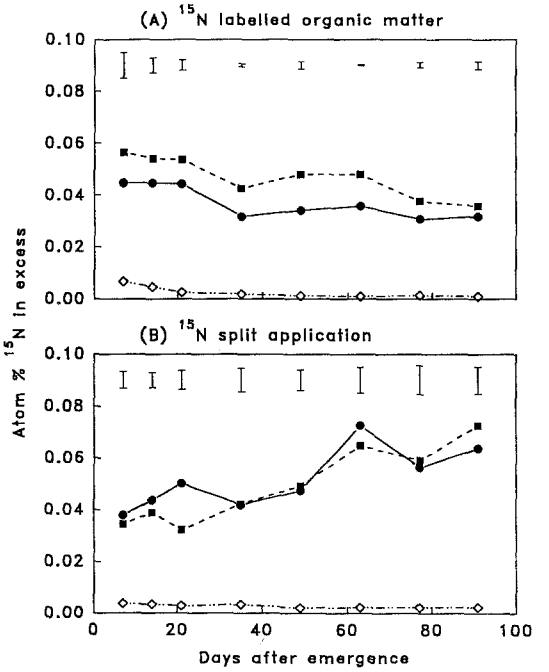

Fig. 5. ^{15}N enrichment of the forage legume *Centrosema* (hybrid Itaguaí ----◊----) and the non-N_2-fixing control crops, *Panicum maximum* (cv IR 442 —•—) and *Sorghum bicolor* (cv BR 301 - - ■ - -), grown in a concrete tank filled with Itaguaí series soil (Typic Hapludulf) amended with either (A) ^{15}N-labelled organic matter or, (B) with unlabelled organic matter and split applications of ^{15}N-labelled ammonium sulphate. Vertical bars indicate standard errors of the means (4 replicates). After Viera Vargas *et al.* [44].

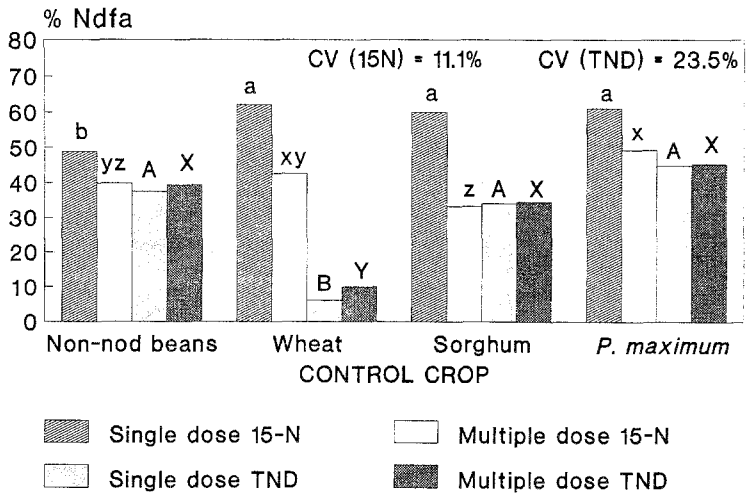

Fig. 6. Estimates of the proportion of plant nitrogen derived from N_2 fixation (% Ndfa) by nodulated common beans (*Phaseolus vulgaris* cv. Negro Argel) using 4 different non-N_2-fixing control crops and either the ^{15}N isotope dilution (ID) or total N difference (TND) techniques. ID estimate of % Ndfa = 100. (1 - (Atom % ^{15}N excess in beans/Atom % ^{15}N excess of control)). TND estimate of % Ndfa = 100. (Total N of beans - Total N of control)/(Total N of beans). CV = Coefficient of variation. Different capital or lower case letters above bars indicate significant differences between means (4 replicates) at $p = 0.05$ (Tukey). After Viera Vargas *et al.* [44].

Typical results showing the contrasting changes in plant ^{15}N enrichment are shown in Figure 5, and when it was applied to quantify the BNF contribution to nodulated *Phaseolus vulgaris* grown in pots, it was found that the non-nodulating mutant of *P. vulgaris* gave similar estimates of N derived from BNF regardless of the labelling technique used (Fig. 6). This technique probably requires even more work, more experimental units and more ^{15}N analyses than the multiple control methods (section: *Use of several control crops*), but it may be worthwhile in some circumstances.

Time course measurements of crop ^{15}N enrichment

This technique can be applied with just one control crop and with a single addition of soluble labelled fertilizer at planting, but better quality results will almost certainly be obtained if a slow-release form of N fertilizer is used and perhaps even more than one control. In this approach several (probably at least 5) complete harvests of each crop (legume + controls) are taken during the season. The harvests should be spaced so that more of them are taken during the time of maximum N_2 fixation by the legume, if this can be predicted. At each complete harvest the material is analysed for total N and ^{15}N enrichment and from these data the curve of the recovery of labelled fertilizer (or excess ^{15}N) can be plotted for each crop. If the curve of the recovery of labelled fertilizer is very similar for the control crop (or one of the control crops) then the estimate of BNF in the legume derived from this control crop should be closest to the true BNF contribution. The equations of growth curve of Hamilton *et al.* [19] can be used to fit the accumulation of labelled fertilizer by the different crops:

$$T\ ^{15}N - c.\ \exp\ (Nu \cdot t)$$

where T ^{15}N is the ^{15}N accumulation by the crop, Nu is the ^{15}N uptake constant and t is the time in days. If the constant Nu and the proportionality constant c are equal the curves can be considered to be of the same shape. Even visually this is usually evident from the graphs. This kind of approach was used by Boller and Nosberger [8], and also by Pareek *et al.* [32], Watanabe *et al.* [48] and Watanabe [47]. Even if the curves are not very similar a good idea of the size of the error in the estimate for the BNF contribution to the legume can be obtained from the data. Again more pots, or plots are required and hence more ^{15}N-labelled material is necessary, as well as a considerable number of extra analyses for N and ^{15}N enrichment.

A modelling technique which theoretically requires no control plant

If the ^{15}N enrichment of the soil mineral N is completely stable with time, space and depth in the

pots/plots then theoretically any truly non-N_2-fixing control plant, or even none at all, can be used to quantify the BNF contribution to a legume. The approach was first suggested by Kohl and Shearer [26] and applied (with a control crop) by our group to quantify BNF contributions to 11 *Panicum maximum* ecotypes [31].

If no control is used then the ^{15}N enrichment of the soil mineral N is estimated from KCl (or similar) extracts of the soil. This was first tried by Chalk *et al.* [12].

By estimating the decline in ^{15}N enrichment of soil mineral N in a soil amended with a single dose of labelled fertilizer using KCl extracts, and evaluating the total N and ^{15}N accumulation in the legume crop, and fitting curves to these parameters, it is theoretically possible to estimate the amount of N and its ^{15}N enrichment taken up by the legume for each day during crop growth, and from this estimate the overall ^{15}N enrichment accumulated by the legume during plant growth. In the two studies published recently [19,38] the authors claim that this approach was successful for both a pot study on soybean and a field study on lupin, respectively. In the first study on soybean one worrying aspect was that the ^{15}N enrichment of the soil mineral N declined much faster under the soybean plants than under two control crops (non-nod soybean and ryegrass). This would suggest that there is an interaction of the crop type with the rate of decline of ^{15}N enrichment in the soil, which if true, would invalidate the whole concept of the ID technique. This was not discussed by the authors, and it may be that it was due to excretion of fixed (unlabelled) N into the soil by the soybean and was an artifact of the very small pots used. In the field study [38] there was a poor fit ($R^2 = 0.60$) of the curve of labelled fertilizer accumulation under the lupin which may have caused some error in the BNF estimate. In both studies control crops were incorporated in the experimental design for verification purposes and BNF estimates derived from these controls and the modelling method were in reasonably good agreement. In neither case did the authors predict the total N accumulation of the control crops using their technique and compare it to the actual N accumulation observed. This seems like a missed opportunity and would have provided the technique with a very good verification procedure.

The technique requires a considerable number of soil samples to be taken from each plot throughout the growth cycle (the above authors sampled at 6 occasions during crop growth) and each must be extracted with KCl and the ^{15}N enrichment of the mineral N from these samples measured by mass or emission spectrometry. In the soils used by these workers mineral N levels were apparently reasonably high throughout plant growth making such measurments of ^{15}N enrichment feasible. However, it is our experience that such analyses on soils of low mineral N content often encountered in tropical soils are extremely difficult, at least for analyses with a mass spectrometer where more than 500 μg N sample^{-1} are usually required. In this case large quantites of soil have to be extracted, the extracts distilled and the large volumes of distillate dried, which means using large quantities of reagents all which may have traces of mineral N in them and cause major errors in the estimates of soil mineral ^{15}N enrichment. These problems have been discussed by Chen *et al.* [13] and should be less acute if an emission spectrometer is used for the ^{15}N analyses or a modern, continuous-flow ANCA-IRMS instrument [1] where less than 50 μg of N are required for analysis.

To summarise, the technique is fairly laborious and difficult to apply in tropical soils of very low mineral N content. It still requires more verification, but it could be a very valuable technique for studies where soil mineral N levels are high and it is not desirable to lower them during the study. As mentioned above (section: *Labelling the soil to diminish temporal variations...*) some of the techniques aimed to slow down temporal changes of ^{15}N enrichment of soil mineral N can immobilize soil N and thus cause changes in the N_2-fixing activity of the legume. If it is desirable that a single dose of labelled fertilizer is used, for example in a study on the effect of fertilizer N on BNF of the legume, then this modelling technique could be much more satisfactory than any other.

Seed nitrogen

If the quantity of N in the seed is a significant proportion of the total N accumulated by the legume, or the control crop, this must be taken into account. This will apply particularly to studies of BNF throughout plant ontogeny where at early harvests seed N constitutes a large proportion of plant total N. Normally this seed N is unlabelled and most of it is incorporated into plant tissue. If the control crop has far less seed N than the legume crop (or vice versa) as may be the case with a grass or cereal being used as a control for soybean (or even worse *Canavalia* spp.) then considerable overestimates of BNF can be recorded. This is apparent in the work of Kucey [27] and has been fully discussed

by Jensen *et al.* [25], Hamilton *et al.* [19] and Smith *et al.* [38].

Conclusion

The main conclusion is that there is no quick and simple method to reliably quantify the contribution of BNF to legume or other "N_2-fixing" crops in the field. As mentioned above (section: *Addition of soluble labelled N fertilizer*) the technique where just one control crop is used with a single addition of soluble ^{15}N labelled fertilizer at, or near planting, with a single harvest at crop maturity, is that most frequently used. This strategy requires few plots, and hence only a small area to be labelled, and only a few samples have to be analysed for ^{15}N enrichment, but as argued above (section: *Magnitude of errors involved...*) the results are of limited value, sometimes useless, especially if BNF contributions are small. All the other techniques described above (sections: *Strategies to apply...* and *A modelling technique...*) require more work, the use of more plots, more ^{15}N enriched material, and all generate more samples to be analysed for ^{15}N. However, these techniques can yield estimates of BNF contributions to plants growing under field conditions for which the limits of accuracy are known with some confidence.

Among the techniques discussed in the sections: *Strategies to apply...* and *A modelling technique...* that requiring least work and expense is the use of a slow-release form of ^{15}N with several non-N_2-fixing control crops, as described by Boddey *et al.* [7]. The major disadvantage of this technique is that such slow-release forms of ^{15}N can often immobilize soil mineral N, sometimes to the point where overall soil N availability is decreased such that the BNF contribution to the legume is increased. If this can prejudice the objectives of the experiment then it is recommended that small frequent additions of labelled fertilizer should be used (section: *Labelling the soil to diminish...* option d.) or the modelling technique described in the section: *A modelling technique...* [19,38].

References

1. Barrie A (1991) New methodologies in stable isotope analysis. In: Stable Isotopes in Plant Nutrition Soil Fertility and Environmental Studies, pp 3–25. Int. Atomic Energy Agency, Vienna
2. Bergersen FJ and Turner GL (1983) An evaluation of ^{15}N methods for estimating nitrogen fixation in a subterranean clover-perennial ryegrass sward. Aust J Agric Res 34: 391–401
3. Boddey RM (1987) Methods for the quantification of nitrogen fixation associated with gramineae. CRC Crit Rev Plant Sci 6: 209–266
4. Boddey RM and Urquiaga S (1990) Quantification of the contribution of BFN to field grown plants: The use of the N isotope dilution technique - problems and some solutions. In: Gueye M, Mulonguy K and Dommergues Y (eds) Maximiser la Fixation Biologique de L'azote pour la Production Agricole et Forestière en Afrique, Vol 2(2), pp 298–316, Collection Actes de l'Inst Sénégalais Recherches Agricoles, Dakar, Senegal
5. Boddey RM, Chalk PM, Victoria RL, Matsui E and Döbereiner J (1983) The use of the ^{15}N dilution technique to estimate the contribution of associated biological nitrogen fixation to the nitrogen nutrition of *Paspalum notatum* cv. batatais. Can J Microbiol 29: 1036–1045
6. Boddey RM, Chalk PM, Victoria RL and Matsui E (1984) Nitrogen fixation by nodulated soybean under tropical field conditions estimated by the ^{15}N isotope dilution technique. Soil Biol Biochem 16: 583–588
7. Boddey RM, Urquiaga S, Neves MCP, Suhet AR and Peres JR (1990) Quantification of the contribution of N_2 fixation to field-grown grain legumes - A strategy for the practical application of the ^{15}N isotope dilution technique. Soil Biol Biochem 22: 649–655
8. Boller BC and Nosberger J (1988) Influence of dissimilarities in temporal and spatial N-uptake patterns on ^{15}N-based estimates of fixation and transfer of N in ryegrass-clover mixtures. Plant and Soil 112: 167–175
9. Broadbent FE, Nakashima T and Chang CY (1982) Estimation of nitrogen fixation by isotope dilution in field and greenhouse experiments. Agron J 74: 625–628
10. Cadisch G, Sylvester-Bradley R and Nosberger J (1989) ^{15}N-based estimates of N_2 fixation of eight tropical forage legumes at two levels of P:K supply. Field Crops Res 22: 181–194
11. Chalk PM (1985) Estimation of N_2 fixation by isotope dilution: An appraisal of techniques involving ^{15}N enrichment and their application. Soil Biol Biochem 17: 389–410
12. Chalk PM, Douglas LA and Buchanan SA (1983) Use of ^{15}N enrichment of soil mineralizable N as a reference for isotope measurements of biologically fixed nitrogen. Can J Microbiol 29: 1046–1052
13. Chen D, Chalk PM and Freney JR (1991) External-source contamination during extraction-distillation in isotope-ratio analysis of soil inorganic nitrogen. Anal Chim Acta 245: 49–55
14. Danso SKA (1988) The use of ^{15}N enriched fertilizers for estimating nitrogen fixation in grain and pasture legumes. In: Beck DP and Materon LA(eds) Nitrogen Fixation by Legumes in Mediterranean Agriculture, pp 345–348. Martinus Nijhoff, Dordrecht, The Netherlands
15. Duque FF, Neves MCP, Franco AA, Victoria RL and Boddey RM (1985) The response of field grown *Phaseolus vulgaris* to Rhizobium inoculation and the quantification of N_2 fixation using ^{15}N. Plant and Soil 83: 333–343
16. Edmeades DC and Goh KM (1978) Symbiotic nitrogen fixation in a sequence of pastures of increasing age measured by a ^{15}N dilution technique. N Z J Agric Res 21: 623–628
17. Fried M, Danso SK and Zapata F (1983) The methodology of measurement of N_2 fixation by non-legumes as inferred from field experiments with legumes. Can J Microbiol 29: 1053–1062

18. Giller KE and Witty JF (1987) Immobilized ^{15}N fertilizer sources improve accuracy of field estimates of N_2-fixation by isotope dilution. Soil Biol Biochem 19: 459–463
19. Hamilton SD, Smith CJ, Chalk PM and Hopmans P (1992) A model based on measurement of soil and plant ^{15}N enrichment to estimate N_2 fixation by soybean (*Glycine max* L. Merril) grown in pots. Soil Biol Biochem 24: 71–78
20. Hamilton SD, Chalk PM, Smith CJ and Hopmans P (1992) Evaluation of reference plants by labelling with ^{35}S in the estimation of N_2 fixation by ^{15}N isotope dilution. Plant and Soil 145: 177–185
21. Hamilton SD, Hopmans P, Chalk PM and Smith CJ (1993) Field estimation of N_2 fixation by *Acacia* spp. using ^{15}N isotope dilution and labelling with ^{35}S. For Ecol Manage 56: 297–313
22. Hardarson G, Danso SKA and Zapata F (1988) Dinitrogen fixation measurements in alfalfa-ryegrass swards using nitrogen-15 and the influence of the reference crop. Crop Sci 28: 101–105
23. Hardarson G, Danso SKA, Zapata F and Reichardt K (1991) Measurements of nitrogen fixation in fababean at different N fertilizer rates using the ^{15}N isotope dilution and 'A value' methods. Plant and Soil 131: 161–168
24. Henzell EF, Martin AE, Ross PJ and Haydock KP (1968) Isotopic studies on the uptake of nitrogen by pasture plants. IV. Uptake of nitrogen from labelled plant material by Rhodes grass and siratro. Aust J Agric Res 19: 65–77
25. Jensen ES, Andersen AJ and Thomsen JD (1985) The influence of seed-borne N in ^{15}N dilution studies with legumes. Acta Agric Scand 35: 438–443
26. Kohl DH and Shearer G (1981) The use of soils lightly enriched in ^{15}N to screen for N_2-fixing activity. Plant and Soil 60: 487–489
27. Kucey RMN (1989) Contribution of N_2 fixation to field bean and pea uptake over the growing season under field conditions in southern Alberta. Can J Soil Sci 69: 695–699
28. Ledgard SF, Morton R, Freney JR, Bergersen FJ and Simpson JR (1985) Assessment of the relative uptake of added and indigenous soil nitrogen by nodulated legumes and reference plants in the ^{15}N dilution measurement of N_2 fixation. I. derivation of method. Soil Biol Biochem 17: 317–321
29. Ledgard SF, Simpson JR, Freney JR and Bergersen FJ (1985) Field evaluation of ^{15}N techniques for estimating nitrogen fixation in legume-grass associations. Aust J Agric Res 36: 247–258
30. Legg JO and Sloger C (1975) A tracer method for determining symbiotic nitrogen fixation in field studies. In: Klein ER and Klein PD (eds) Proc. 2nd Int. Conf. Stable Isotopes, pp 661-666. Oak Brook, USA
31. Miranda CHB and Boddey RM (1987) Estimation of biological nitrogen fixation associated with 11 ecotypes of *Panicum maximum* grown in nitrogen-15-labeled soil. Agron J 79: 558–563
32. Pareek RP, Ladha JK and Watanabe I (1990) Estimating N_2 fixation by *Sesbania rostrata* and *S. cannabina* (syn. *S. aculeta*) in lowland rice soil by the ^{15}N dilution method. Biol Fertil Soils 10: 77–88
33. Patterson TG and LaRue TA (1983) Nitrogen fixation by soybeans: seasonal and cultivar effects, and comparison of estimates. Crop Sci 23: 488–492
34. Rennie RJ (1985) Theoretical errors in sequential N-15 labelling of soil to estimate N_2 fixation by isotope dilution. Crop Sci 25: 891
35. Rennie RJ (1986) Advantages and disadvantages of nitrogen-15 isotope dilution to quantify dinitrogen fixation in field-grown legumes - a critique. In: Field Measurement of Dinitrogen Fixation and Denitrification, Soil Sci Soc Am Spec Publ 18, pp 43–58. Am Soc Agron, Madison, Wisconsin, USA
36. Rennie RJ and Thomas JB (1987) ^{15}N-determined effect of inoculation with N_2-fixing bacteria on nitrogen assimilation in Western Canadian wheats. Plant and Soil 112: 183–193
37. Sanginga N, Danso SKA, Zapata F and Bowmen GD (1990) Influence of reference trees on N_2-fixation estimates in *Leucaena leucocephala* and *Acacia albina* using ^{15}N-labelling techniques. Biol Fertil Soils 9: 341–346
38. Smith CJ, Chalk PM, Hamilton SD and Hopmans P (1992) Estimating N_2 fixation by field-grown lupins (*Lupinus angustifolius* L.) using soil and plant ^{15}N enrichment. Biol Fertil Soils 13: 235–241
39. Talbott HJ, Kenworthy WJ and Legg JC (1982) Field comparison of the nitrogen-15 and difference methods of measuring nitrogen fixation. Agron J 74: 799–804
40. Talbott HJ, Kenworthy WJ, Legg JO and Douglass LW (1985) Soil nitrogen accumulation in nodulated and non-nodulated soybeans: a verification of the difference method by a ^{15}N technique. Field Crops Res 11: 55–67
41. Urquiaga S, Cruz KHS and Boddey RM (1992) Contribution of nitrogen fixation to sugar cane: nitrogen-15 and nitrogen balance estimates. Soil Sci Soc Am J 22: 105–114
42. Vallis I, Haydock KP, Ross PJ and Henzell EF (1967) Isotopic studies on the uptake of nitrogen by pastures. III. The uptake of small additions of ^{15}N-labelled fertilizer by Rhodes grass and Townsville lucerne. Aust J Agric Res 18: 865–877
43. Vallis I, Henzell EF and Evans TR (1977) Uptake of soil nitrogen by legumes in mixed swards. Aust J Agric Res 28: 413–426
44. Viera-Vargas MS, Oliveira OC de, Souto CM, Cadisch G, Urquiaga S and Boddey RM (1994) Use of different ^{15}N techniques quantify the contribution of biological nitrogen fixation to legumes. Soil Biol Biochem (In press)
45. Vose P and Victoria RL (1986) Re-examination of the limitations of nitrogen-15 isotope dilution technique for the field measurement of dinitrogen fixation. In: Field Measurement of Dinitrogen Fixation and Denitrification, Soil Sci Soc Am Spec Publ 18, pp 23–41. Am Soc Agron, Madison, Wisconsin, USA
46. Wagner GH and Zapata F (1982) Field evaluation of reference crops in the study of nitrogen fixation by legumes using isotope tecniques. Agron J 74: 607–612
47. Watanabe I (1991) Errors related to the ^{15}N dilution method for estimating nitrogen fixation. In: Stable Isotopes in Plant Nutrition Soil Fertility and Environmental Studies, pp 83–88. Int Atomic Energy Agency, Vienna
48. Watanabe I, Chui GY and Yoshida T (1990) Estimation of N_2 fixation in soybean and cowpea by using residual ^{15}N. Soil Sci Plant Nutr 36: 375–381
49. Witty JF (1983) Estimating N_2 fixation in the field using ^{15}N-labelled fertilizer: some problems and solutions. Soil Biol Biochem 15: 631–639
50. Witty JF and Giller KE (1991) Evaluation of errors in the measurement of biological nitrogen fixation using ^{15}N fertilizer. In: Stable Isotopes in Plant Nutrition, Soil Fertility and Environmental Studies, pp 59–72. Int Atomic Energy Agency, Vienna
51. Witty JF and Ritz K (1984) Slow release ^{15}N fertilizer formulations to measure N_2-fixation by isotope dilution. Soil Biol Biochem 16: 657–661

52. Witty JF, Rennie RJ and Atkins CA (1988) ^{15}N addition methods for assessing N_2 fixation under field conditions. In: Summerfield RJ (ed) World Crops: Cool Season Food Legumes, pp 715–730. Kluwer, Dordrecht, The Netherlands