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Applicability of the natural ^{15}N abundance technique to measure N_2 fixation in *Arachis hypogaea* grown on an Ultisol

G. CADISCH[1]*, K. HAIRIAH[2] AND K.E. GILLER[1]

[1] Department of Biological Sciences, Wye College, University of London, Wye, Kent TN25 5AH, UK
[2] Department of Soil Science, Faculty of Agriculture, Brawijaya University, Malang, Indonesia
* Corresponding author (fax: +44-1233-813140; e-mail: g.cadisch@wye.ac.uk)

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Abstract

Measurements of N_2 fixation by *Arachis hypogaea* grown on an Ultisol (Grossarenic Kandiudult) in North Lampung, Sumatra were obtained by i) the ^{15}N dilution method by applying a small dose of ^{15}N in solution mixed with a carbon source and ii) by the ^{15}N natural abundance method ($\delta^{15}N$). For both methods non-nodulating groundnuts and maize were used as reference plants. While the ^{15}N dilution method led to a large spatial variation (both in depth and time) in plant available ^{15}N, spatial variations of the natural ^{15}N abundance with soil depth (6–9 ‰), time (9–12 ‰ over one year) and space were comparatively small. The $\delta^{15}N$ of the mineralizable N pool was greater than that of the total soil N which was reflected in high $\delta^{15}N$ values of the reference plants.

Above ground plant parts of groundnuts grown in a N free media were negatively enriched in ^{15}N while nodules were not enriched (0 ‰). Isotopic discrimination occurred both during N_2 fixation (–1.8 / –1.0 ‰ for soil inoculum and *Bradyrhizobium* WYE 899 respectively) and transport of fixed N into different plant tissues.

The proportion of N derived from N_2 fixation varied from 45–54 % using the natural abundance method and non-nodulating groundnut and maize as references respectively in 1995 but fixation dropped significantly in the second year of evaluation (21–16 %). There was a good agreement in the amount of N_2 fixed on average of the two years (21–24 kg N ha^{-1}) between the natural ^{15}N abundance method and ^{15}N dilution method where an adequate reference plant was available. However the ^{15}N dilution method was much more sensitive to a matching planting time between the reference and fixing plant compared to the $\delta^{15}N$ method. Although the ^{15}N natural abundance method was less prone to temporal and spatial alterations in $\delta^{15}N$ it is nevertheless advocated to use the same precautions as for the ^{15}N dilution method with regard to a careful matching of the legume and the reference plant and accounting for ^{15}N variation within the plant. It is concluded that under the relatively high plant available ^{15}N conditions in this soil the ^{15}N natural abundance method is a viable alternative method to measure N_2 fixation of groundnut under field conditions.

Keywords: Arachis hypogaea, biological N_2 fixation, natural ^{15}N abundance, ^{15}N dilution, spatial and temporal variability

Introduction

To sustain soil fertility in agricultural systems, nutrients exported in agricultural products or lost to the environment need to be replaced. The *Rhizobium*-legume symbiosis provides potentially an alternative to N fertilizers to balance N losses through its ability to fix atmospheric N_2. Hence there is a need to develop accurate and cost-effective methods to measure inputs from biological N_2 fixation under field conditions.

The simplest way to obtain an estimate of biological N_2 fixation under field conditions is by comparing the N yields of legume based systems with that of a non-fixing control (N difference method). However the method depends on plant yield and is therefore often not reliable. Direct measurements of nitrogenase activities (acetylene reduction assay) have not reliably proven to yield integrated quantitative measurements of N_2 fixation under field conditions (Giller & Wilson, 1991). An estimate of the different N sources in plants can be obtained with the ^{15}N dilution method. The method requires the application of a small dose of ^{15}N enriched fertilizer to the soil prior to planting. Based on the assumption that a non-fixing reference plant takes up a similar proportion of soil-N:fertilizer-^{15}N as the fixing plant, the proportion of N derived from the atmosphere can be calculated (McAuliffe *et al.*, 1958). Drawbacks of the ^{15}N dilution method are the high ^{15}N fertilizer costs, the decline in ^{15}N enrichment of plant available soil-N with time and the non-uniform ^{15}N distribution with soil depth (Witty, 1983). These effects lead to substantial errors if the temporal and spatial N uptake of the non-fixing reference plants differs from that of the legume (Ledgard *et al.*, 1985).

With the wider availability of high precision mass-spectrometers it is now possible to routinely measure small enrichments of ^{15}N in soil and plant systems and thus to make use of the occurrence of natural enrichment of ecological pools. The ^{15}N enrichment of soil organic matter (SOM) should represent the enrichment of atmospheric N_2 (which is constant at 0.3663 atom % ^{15}N (Mariotti, 1983)) as N in SOM has originally been derived from inputs of residues from N_2 fixing plants or microorganisms. However, most soils are slightly enriched in ^{15}N compared to the atmosphere (Yoneyama *et al.*, 1993) although exceptions occur (Vitousek *et al.*, 1989). Sources for natural ^{15}N enrichment of soils are ammonia volatilization, nitrification, denitrification, fire, (i.e. particularly processes which involve a change of phase, e.g. liquid to gas) and physical processes, e.g. leaching (Nadelhoffer & Fry, 1994; Pate *et al.*, 1993; Shearer & Kohl, 1986). The resulting slight enrichment in ^{15}N can be used as a natural soil labelling (Shearer & Kohl, 1986; Delwiche & Steyn, 1970). Plants dependent on soil nitrogen often have natural ^{15}N abundance values close to soil ^{15}N enrichments, although in both temperate and tropical conditions apparently non-N_2 fixing plants can have ^{15}N enrichments below those of the SOM (Högberg & Alexander, 1995; Domenach *et al.*, 1989). In N_2 fixing plant tissues N becomes diluted by the lower natural ^{15}N abundance of fixed atmospheric N_2. Plants completely dependent on N_2 fixation have values close to the atmospheric N_2 or are even slightly depleted (Yoneyama *et al.*, 1986; Steele *et al.*, 1983). Although the use of the natural ^{15}N abundance has been proposed to be a useful method for estimating N_2 fixation in crops (Bremer & van Kessel, 1990; Kohl *et al.*, 1980) and pasture legumes (Un-

kovich *et al.*, 1994; Cadisch *et al.*, 1993) a careful interpretation of the results is necessary as the $\delta^{15}N$ is affected by a number of factors such as life form, forms of N available to plants (Bremner & Tabatabai, 1973; Bremner & Keeney, 1966), mycorrhizal status (Högberg & Alexander, 1995; Pate *et al.*, 1993; Högberg, 1990) and discrimination processes occurring during N_2 fixation (Cadisch *et al.*, 1993; Shearer & Kohl, 1986).

We tested the hypothesis that the ^{15}N natural abundance technique is a more reliable and cheaper alternative than the ^{15}N dilution method on an Ultisol in southern Lampung, Indonesia. We hypothesized that i) the natural ^{15}N enrichment of this weathered soil is sufficiently high for use of the ^{15}N abundance method, ii) the more uniform natural ^{15}N enrichment of the soil makes the choice of the reference plant less important and iii) isotopic discrimination during N_2 fixation can be accounted for.

Materials and methods

Site

The experiment was carried out at the BMSF (Biological Management for Soil Fertility) project site of Brawijaya University at North Lampung, Sumatra, Indonesia (4°30'S, 104°98'E) which is described in detail by van der Heide *et al.* (1992) and Hairiah *et al.* (2000). The soil at the study site is a Grossarenic Kandiudult with 65% sand, 17% silt and 18% clay. The soil is well drained and the topsoil organic N and natural ^{15}N abundance values are given in Table 1. Other soil fertility characteristics (0–20 cm) were: pH (H_2O) 5.4; 2.2 % organic C by Walkley-Black method; 11 mg kg^{-1} P (Bray II), cation exchange capacity 5.02 $cmol_{(+)}$ kg^{-1}; 0.34, 0.16, 2.29 and 1.10 $cmol_{(+)}$ kg^{-1} of Na^+, K^+, Ca^{2+} and Mg^{2+}, respectively in ammonium acetate pH 7 according to Hairiah *et al.* (2000). The area has an average annual temperature of 26.3 °C, humidity of 96% and rainfall of 2580 mm. Rainfall during the experimental period (April to July 1995) was on average 181 mm per month.

Crop management

Groundnut was established in a comparative multi-species rotation system experiment described by Hairiah *et al.* (2000). Systems included: i) *Gliricidia sepium*/*Peltophorum dasyrrachis* alley cropping with mixed rice+maize followed by groundnut and cowpea, ii) *Flemingia* alley cropping with mixed rice+maize followed by groundnut and cowpea, iii) mixed rice+maize crop followed by groundnut and subsequently by mucuna (*Mucuna pruriens var utilis*) and iv) mixed rice+maize crop followed by groundnut and subsequently by cowpea. A basal fertilizer rate of 60 kg ha^{-1} P_2O_5 and 60 kg ha^{-1} K_2O was applied to the rice+maize crop. Groundnut (*Arachis hypogaea*, local variety Mahesa) was planted in April 1995 and 1996 after harvesting rice+maize into these systems with a spacing of 0.5 × 0.25 m, 2 plants per hole and plot size of 12 × 13 m. Groundnuts were harvested in July 1995 and 1996 respectively. At harvest plants were separated into roots, shoots, shell and grain, dried (at 50 °C

for 3 h followed by 2 hours at 75 °C), weighed and ground to <1 mm. The experimental design was a randomized block with four replicates.

^{15}N dilution method

A week before planting groundnut in 1995, ^{15}N was applied as ammonium sulphate at a rate of 10 kg N ha^{-1} (10.2 atom %^{15}N) in solution mixed with sugar at a C:N ratio of 10:1 in order to immobilize the ^{15}N more rapidly into the soil microbial biomass (Giller & Witty, 1987). The ^{15}N application area was 4 × 4 m in the crop rotation systems and 5 × 3 m in the hedgerow system and was performed in three replicates. Within the ^{15}N application area one macroplot of 3 × 1m for the fixing groundnut and three microplots of each 1 × 1 m were established. Non-nodulating groundnuts were planted in microplot one in 1995 and in microplot 2 in 1996 whereas the others were planted with fixing groundnuts. This rotational system for the non-nodulating groundnuts was chosen in order to avoid ^{15}N memory effects by the pre-crop. The non-nodulating groundnut (obtained originally from ICRISAT, Hyderabad, India) served as a reference plant for the ^{15}N method and plants were checked for occurrence of nodules at harvest and nodulating plants eliminated. In 1995 after emergence the non-nodulating groundnut plants developed disease/nutritional disorder symptoms (which later disappeared) and a row of maize plants was planted (10 days after groundnut) next to the non-nodulating plants to act as an alternative non-fixing plant to estimate N_2 fixation. In 1996 maize was again included as a reference plant but planted at the same time as groundnut. Maize plants, including roots to 20 cm depth, were harvested at the same time as groundnut plants. A 0.5 m border strip was maintained for all plots.

Based on the assumption that the non-fixing reference plant takes up a similar proportion of soil-N:fertilizer-^{15}N as the fixing plant the proportion of N derived from the atmosphere can be calculated as (McAuliffe *et al.*, 1958):

$$\%\, N_2 fixation = [1 - \frac{atom\ \%^{15}N\ Nexcess\ fixing\ legume}{atom\ \%^{15}N\ Nexcess\ non\ fixing\ reference}]\ x100 \qquad (1)$$

where atom % ^{15}N excess = atom % ^{15}N – 0.3663 (natural ^{15}N abundance of atmospheric N_2)

Harvest area was 3 × 1 m for the fixing plant and 1 × 1 m for the reference plants. All biological materials were analyzed for ^{15}N enrichment using an automated CN analyzer (Roboprep) coupled to a mass-spectrometer (Model 20–20, Europa Scientific, Crewe).

Natural ^{15}N abundance method

In every cropping system there was one replicate out of the four which did not receive ^{15}N enriched fertilizer and was thus used for measurements of natural abundance of ^{15}N in plants and soils. The distance to the ^{15}N enriched plots ensured that no field contamination problems occurred. Planting and harvesting procedures were as above however, harvested material was handled separately from enriched samples

to avoid contamination. Calculations for N_2 fixation for the natural abundance method were as follows (Amarger *et al.*, 1979):

$$\%\, N_2 fixation = [\frac{\delta^{15}N\ non\ fixing\ reference - \delta^{15}N\ fixing\ legume}{\delta^{15}N\ non\ fixing\ reference - B}]\ x100 \qquad (2)$$

where $\delta^{15}N$ ‰ = [($^{15}N/^{14}N$ sample / $^{15}N/^{14}N$ standard) – 1] × 1000 and where standard is atmospheric N_2. *B* is the $\delta^{15}N$ value of the same N_2 fixing plant when grown with N_2 as the sole source of N and accounts for the discrimination which occurs during N_2 fixation (see below).

Natural abundance of ^{15}N of soil samples was obtained after direct combustion of air-dried and ground samples. To measure the ^{15}N enrichment of plant available mineral N soil samples were incubated at field capacity for 24 days at 28 °C in the dark before extracting mineral N with 2 *M* KCl and analysis of $\delta^{15}N$ after diffusion. Correction for background ^{15}N in diffused samples was done as proposed by Kelley *et al.* (1991).

Discrimination experiment

To evaluate ^{15}N discrimination during N_2 fixation and subsequent distribution of fixed N within the plant, a glasshouse experiment at Wye College was set up in 1996/97. Groundnut (local variety Mahesa as above) seeds were surface sterilized and planted in pots in quartz sand and supplied twice daily with a N free nutrient solution (modified from Hammer *et al.* (1978)), made up using deionised water. Plants were inoculated four times with a soil suspension from the field site or strain WYE 899 at two replicates each. At harvest plants were separated into nodules, roots, shoots, shell and grain, dried at 40 °C and analyzed for $\delta^{15}N$.

Results

Soil ^{15}N enrichments

The total N content of the soil decreased gradually with increasing soil depth (Table 1). In the microplots where ^{15}N labelled fertilizer had been surface applied some of the applied ^{15}N had moved downwards. However the majority of the ^{15}N was still found in the topsoil at crop harvest creating a decreasing ^{15}N gradient with increasing soil depth. In contrast, the natural ^{15}N abundance of total soil N of unenriched plots increased with soil depth. The ^{15}N signature of the mineral N in the natural abundance plots was not significantly affected by soil depth but was much greater than that of total soil N.

^{15}N discrimination during N_2 fixation

Plants depending completely on N_2 fixation showed ^{15}N discrimination during N_2 fixation as suggested by the negative weighted average $\delta^{15}N$ values (Table 2). The

Table 1. Total N and natural ^{15}N abundance of soil samples from ^{15}N labelled or unlabelled plots taken at the end of the groundnut cycle in July, 1995.

Soil depth	Total N	^{15}N labelled plots	Natural abundance plots	
(cm)	(mg N/g soil)	Total N ($\delta^{15}N$, ‰)	Total N ($\delta^{15}N$, ‰)	Mineral N[1] ($\delta^{15}N$, ‰)
0–10	1.7	99.9	5.7	13.1
10–20	1.1	31.1	7.0	11.8
20–30	0.7	19.3	9.2	12.1
F test	0.0001	0.001	0.0001	ns
SED	0.01	17.65	0.25	0.94

[1] after incubation for 24 days.

degree of ^{15}N fractionation varied among different plant parts. Stover was highly depleted in ^{15}N. In contrast, nodules showed no significant ^{15}N discrimination. Inoculation had no significant effect on ^{15}N discrimination. Seeds used in this experiment had a $\delta^{15}N$ value of 0.54. Corrections for the ^{15}N content in the initial seed material resulted in slightly lower weighted whole plant mean $\delta^{15}N$ values of –2.5 and –1.4 for soil and WYE 899 inoculum respectively.

^{15}N partitioning among tissues in field grown plants

Stover materials from the unlabelled field plots in 1995 had a lower ^{15}N signature than roots for both fixing and non-fixing plants (Table 3). The largest variation in $\delta^{15}N$ however occurred when comparing stover and pod materials. Particularly in the

Table 2. ^{15}N discrimination during N_2 fixation and translocation of ^{15}N from nodules to shoots ('B' values) of groundnut grown in N-free sand culture in the glasshouse.

Plant part	Inoculum ($\delta^{15}N$, ‰)	
	Soil suspension[1]	WYE 899
Grain	–0.8	–1.2
Shell	–1.4	–1.5
Stover	–2.6	–2.1
Root	–0.4	0.0
Nodules	0.0	0.0
Weighted plant mean	–1.8	–1.0
Weighted plant mean[2]	–2.5	–1.4
F test[3]	ns	
SED	0.16	

[1)] soil suspension obtained from soil from Lampung field site.
[2)] whole plant weighted mean corrected for seed ^{15}N.
[3)] for weighted mean only; ns = not significant (P>0.05).

Table 3. Translocation of ^{15}N into different plant parts of nodulating groundnuts or non-nodulating groundnuts and maize reference plants grown in unlabelled natural ^{15}N abundance plots Lampung, 1995 and related estimates of N_2 fixation using individual plant parts.

	Nodulating Groundnut ($\delta^{15}N$)	Reference plants ($\delta^{15}N$)		Proportion of N derived from N_2 fixation (%)[1]	
		Non-nod GN	Maize	Non-nod GN	Maize
Shoot	3.8	9.9	9.7	49	48
Roots	4.6	12.9	11.6	63	58
Shell	5.6	16.0	na	59	na
Grain	4.9	16.6	na	67	na
F test	ns	0.02	ns	ns	ns
SED	0.8	1.7	0.8	10.7	10.8
Weighted mean	4.6	12.1	9.8	53	44

[1] using B value according to Table 2.
na = not available; ns = not significant ($P>0.05$).

non-nod groundnut the grain was significantly enriched in ^{15}N. Maize showed similar though not significant different $\delta^{15}N$ patterns between shoots and roots as groundnut. For calculating N_2 fixation we used weighted means values to account for this variation as measurements based on $\delta^{15}N$ values of single plant parts may be misleading.

N_2-fixation

Measurements of $\delta^{15}N$ values in non-fixing reference plants (non-nod groundnut and maize) and fixing groundnut grown at Lampung were taken during the 1995 and 1996 groundnut cropping season (Table 4). In 1995 the fixing groundnut had substantially lower $\delta^{15}N$ values (4.6 ‰) than the non-fixing reference plants (12.1/9.8 ‰). Calculations of the proportion of N derived from atmosphere in groundnut using the natural ^{15}N abundance method suggested approximately 50% N_2 fixed in 1995 with little influence of the reference plant used (44–53 %). Measurements using the ^{15}N dilution method and non-nod groundnut as a reference plant resulted in similar N_2 fixation estimations (46 %) in 1995. However, when using late-sown maize as a reference plant the ^{15}N dilution method seriously underestimated the proportion of N derived from N_2 fixation (23 %) in 1995.

The proportion of N derived from N_2 fixation in 1996 was strongly reduced compared with 1995 as suggested by the natural ^{15}N abundance method. This was partly due to lower ^{15}N abundance of the plant available mineral N as suggested by the reference plants but also directly due to a reduced uptake of non-enriched atmospheric N_2 as suggested by the increased $\delta^{15}N$ values of the fixing groundnut. The reduction in N_2 fixation of about 30 % was apparent with both reference plants. The results of the ^{15}N dilution method similarly suggested a lower N_2 fixation potential in 1996 al-

Table 4. Comparison of estimations of the proportion of N derived from N_2 fixation of groundnut at Lampung, Sumatra using either the natural ^{15}N abundance method (n=4) or the ^{15}N dilution method (n=12) and contrasting reference plants. Values in brackets are standard errors of means.

Method	Weighted mean ^{15}N enrichment ($\delta^{15}N$, atom % ^{15}N excess)			Proportion of N derived from N_2 fixation (%)	
	Fixing groundnut	Reference plants		Reference plants	
		Non-nod groundnut	Maize	Non-nod groundnut	Maize
1995					
^{15}N natural abundance	4.6 (0.4)	12.1 (0.6)	9.8 (0.4)	53 (4.1)[1]	44 (5.4)[1]
^{15}N dilution	0.2211 (0.0161)	0.413 (0.0253)	0.304 (0.0150)	46 (3.5)	23 (5.6)[2]
1996					
^{15}N natural abundance	6.7 (0.4)	9.1 (0.2)	8.6 (0.6)	21 (3.8)[1]	16 (8.7)[1]
^{15}N dilution	0.0441 (0.0016)	0.0671 (0.0022)	0.0664 (0.0026)	33 (3.2)	32 (4.4)

[1] using a B value of −1.8‰

[2] maize sown two weeks after crop in 1995

Table 5. Average (1995 and 1996) yields and N_2 fixation estimates in the natural ^{15}N abundance (n=4) or the ^{15}N dilution (n=12) plots using non-nodulating groundnut as a reference plant. Values in brackets are standard errors of means.

	Method	
	^{15}N natural abundance	^{15}N dilution
Grain yield (kg ha^{-1})	631 (208)	626 (76)
Total N yield (kg N ha^{-1})	53 (13)	58 (6)
Amount of N_2 fixed (kg N ha^{-1})	21 (6)	24 (3)

though the reduction was less pronounced (9–13 %). Both reference plants led to a similar estimate of the proportion of N derived from N_2 fixation in the second year.

Plant growth and total N accumulation were similar in both ^{15}N natural abundance and ^{15}N enriched plots (Table 5). The estimates of the amount of N_2 fixed on average of 1995 and 1996 using non-nodulating groundnut as a reference plant amounted to 21–24 kg N ha^{-1} yr^{-1} and were not different for the two methods.

Discussion

Spatial variations in soil ^{15}N enrichments

The hypothesis that vertical variations in soil natural ^{15}N abundances are smaller than when using the ^{15}N dilution method has been confirmed by the present data. Strongly decreasing ^{15}N enrichments with increasing soil depths are commonly observed where ^{15}N had been surface applied (Peoples *et al.*, 1996) due to the cation retention capacity of soils and incorporation of ^{15}N into soil organic matter. In contrast, although the ^{15}N natural abundance of total soil N increased with soil depth the plant available ^{15}N pool did not (Table 1). Ledgard *et al.* (1984) also reported that soil $\delta^{15}N$ values increased with soil depth and that the extractable soil mineral $\delta^{15}N$ signature was more uniform with depth. They found that the $\delta^{15}N$ values of non-N_2-fixing plants were lower than the natural ^{15}N abundance of total soil nitrogen. These findings contrast with our results and emphasize the need to establish the natural abundance of ^{15}N in plant available soil N pools rather than relying on total soil $\delta^{15}N$ signatures. Indeed in our experiment the natural abundance of ^{15}N of the readily mineralizable N (aerobic incubation) was higher than that of total soil N in agreement with the higher ^{15}N enrichment found in non-fixing reference plants. In contrast, Ledgard *et al.* (1984) found that the natural abundance of ^{15}N in extractable mineral N was lower than that of total soil N and so were his reference plants. As shown by Turner *et al.* (1987) the natural ^{15}N abundance of plant available N is strongly influenced by management practices and crop history. This is also evident in our experiment where recycling of groundnut stover with low ^{15}N natural abundance in 1995 led to reduced $\delta^{15}N$ values in the non-fixing reference plants in the subsequent year.

Spatial variability in ^{15}N has sometimes been reported to be a problem in the ap-

plication of the ^{15}N natural abundance method to measure N_2 fixation. Bremer & van Kessel (1990) reported differences in $\delta^{15}N$ values as large as 6.5 ‰ within a transect of 42 m. However, the variation in $\delta^{15}N$ values of total soil N at this site was relatively small (standard error of mean for total soil N = 0.1–0.2 ‰ for the three soil depths (n=6)) and was thus not likely a major source of errors in the estimation of N_2 fixation.

Temporal variations in soil ^{15}N enrichments

Although there were apparent differences in the $\delta^{15}N$ enrichment of reference plants between years, the hypothesis that temporal variations in soil natural ^{15}N abundances are smaller than when using the ^{15}N dilution method has been confirmed. Where ^{15}N had been added rapid changes in the soil available ^{15}N pools occurred which followed a double exponential decay function of $y = 1492 \exp(-0.3594\ t) + 99 \exp(-0.0205\ t)$ where y is soil $\delta^{15}N$ and t is months after ^{15}N application (unpublished result). These large changes in the plant available soil ^{15}N enrichment were also evident from the large decrease in ^{15}N enrichments of reference plants between 1995 and 1996 (Table 4). In 1995 maize was sown 10 days after the leguminous crop and hence maize encountered an already lower ^{15}N enrichment of plant-available soil N than the fixing and non-fxing groundnuts in the plots were ^{15}N was applied. This led to a proportionally small ^{15}N enrichment in the maize and hence a low estimate of N_2 fixation. Thus the basic assumption of the ^{15}N dilution method that the non-fixing reference plant takes up a similar proportion of soil-N to fertilizer-^{15}N as the fixing plant was violated and led to an erroneous estimation of N_2 fixation. The results also indicated that the precautions to reduce rapid changes in plant available ^{15}N by applying the enriched N with a carbon source had limited success and could not prevent an erroneous estimate. Ledgard *et al.* (1985) and Witty (1983) also observed erroneous N_2 fixation estimates when using the ^{15}N dilution technique with some reference plants. They attributed it to the change in the ^{15}N enrichment of plant-available soil N with time interacting with differences in the pattern of N assimilation between the fixing and non-fixing reference plant.

^{15}N discrimination during N_2 fixation

In order to use the natural ^{15}N abundance method to estimate N_2 fixation estimates of the potential isotopic discrimination occurring in fixing plants must be accounted for. Our results showed a significant discrimination against ^{15}N during N_2 fixation (weighted mean data, Table 2). Isotopic fractionation during N_2 fixation is species-specific and is little affected by environmental conditions (Peoples *et al.*, 1991; Kohl *et al.*, 1983). Above ground parts of plants depending on N_2 fixation are often found to have negative $\delta^{15}N$ values as also observed in this study although some species or species-*Rhizobium* combinations can induce positive shoot $\delta^{15}N$ values (Steele *et al.*, 1983). On the other hand, nodules of species which transport fixed N as ureides are commonly enriched in ^{15}N (Cadisch *et al.*, 1993; Shearer *et al.*, 1982) and the degree of ^{15}N enrichment of nodules has been associated with its N_2 fixing efficiency (Kohl

et al., 1983). While we observed nodules of young plants to have positive $\delta^{15}N$ values (data not presented) nodules of mature plants were not significantly enriched (Table 2). This confirms results of Shearer *et al.* (1982) who found a $\delta^{15}N$ of 0.5 ‰ for nodules of *Arachis hypogaea*.

The choice of rhizobial strains strongly influences isotopic fractionation (Cadisch *et al.*, 1993; Steele *et al.*, 1983). The effect of different strains on isotopic fractionation appears to be greater than that of variation between different varieties (Unkovich *et al.*, 1994). Variations in natural abundance of ^{15}N due to different strains can amount to as much as 2 ‰ (Steele *et al.*, 1983). In our experiment the two tested inoculum sources did not significantly differ in their isotopic fractionation but our values were substantial different from the B value of 0.7 ‰ found by Peoples *et al.* (1992) with a mixture of three strains. In the field a range of rhizobial strains are likely to be involved in the legume-*Rhizobium* symbiosis. Thus fractionation values obtained from the site specific soil suspension are likely to be the most appropriate for promiscuous legumes and were used in the calculations for N_2 fixation in this study.

^{15}N enrichments of plants depending on N_2 fixation may change during crop growth (Unkovich *et al.*, 1994) presumably because of ^{15}N supplied in the seed and the further discrimination during grain filling. In our case mature plants were used for both the measurement of isotopic discrimination during N_2 fixation and for measuring N_2 fixation in the field to minimise errors due to ontogenetic drift in B values.

Variation of ^{15}N within the plant

Variations in $\delta^{15}N$ values between different leguminous plant parts can origin from i) ^{15}N discrimination during transport of ^{15}N within the plant, ii) changes in the N_2 fixation ability during the development of the plant and iii) changes in the ^{15}N signature of the plant available N pool. A strong isotopic fractionation was associated with transport and synthesis of organic N compounds within the plant. This was reflected in the variation of $\delta^{15}N$ among different plant parts of groundnut grown in N-free media (Table 2). There was also some variation of N_2 fixation or transport of fixed N within the plant as depicted by the N_2 fixation results using individual plant parts in field grown plants (Table 3). Bergersen *et al.* (1988) also observed dynamic changes in $\delta^{15}N$ values during organ development of soybean and Peoples *et al.* (1991) reported different $\delta^{15}N$ values in different leaf strata of soybeans. Thus although temporal variations in the natural ^{15}N abundance in soils appear to be smaller than those associated with the ^{15}N dilution method, N_2 fixation estimates based on individual plant parts led to similar errors with both methods. It is thus desirable to use whole plant $\delta^{15}N$ estimates for N_2 fixation evaluations rather than subsamples of single leaf or individual organs (Peoples *et al.*, 1991). This principle is relatively easy to apply to crops (at least to the combined above-ground parts) but may be difficult for large tree species.

N_2 fixation estimates using the ^{15}N natural abundance method

N_2 fixing groundnut plants had significantly lower $\delta^{15}N$ values than both non-fixing control plants thus following the initial hypothesis that assimilation of atmospheric

N_2 leads to a ^{15}N dilution effect in fixing plants. The proportion of N derived from N_2 fixation averaged 53 % and 44 % in 1995 for non-nodulating groundnut and maize reference plants respectively. McDonagh *et al.* (1993) and Peoples *et al.* (1992) found similar estimates of the proportion of N derived from N_2 fixation by groundnut in NE Thailand and S Queensland, Australia respectively. The drop in N_2 fixation in 1996 was associated with a reduced plant growth indicating effects of environmental stress most probably water shortages during dry spells. The two reference plants, non-nodulating groundnut and maize, led to similar estimations of N_2 fixation. They appear thus both suitable reference plants for this experiment although maize had lower $\delta^{15}N$ values than non-nod groundnut. The reason that the differences in $\delta^{15}N$ values between non-nodulating groundnut and maize had relatively small effects on N_2 fixation was due to the relatively high ^{15}N natural abundance of the plant available ^{15}N. A $\delta^{15}N$ value of at least 6–10 ‰ has been quoted to be required for reliable estimates of N_2 fixation (Ledgard & Peoples, 1988; Shearer & Kohl, 1986; Mariotti *et al.*, 1983). However, Unkovich *et al.* (1994) suggested that sites with reference plant $\delta^{15}N$ values greater than 2 ‰ would already permit assessments of N_2 fixation when employing a vigorous sampling scheme. Given the variability of field plant samples of both fixing and non-fixing plants and the uncertainties associated with the estimates of the *B* value the former range is probably more adequate for reliable N_2 fixation estimations using the ^{15}N natural abundance method.

Natural ^{15}N abundance vs ^{15}N dilution method

Both methods resulted in a similar estimate of the amount of N_2-fixed on average over the two years when using non-nodulating groundnut as a reference plant (Table 5). Also both methods predicted a substantial reduction of N_2-fixation in the second year. Thus the closeness of the estimates and trends predicted by the two ^{15}N methods suggests that given a good matching reference plant both methods are suitable for the soil-crop combination under investigation. Although a good agreement in N_2 fixation estimations between two methods does not always automatically imply that both methods provide a correct estimate. There appeared temporal over- or underestimations of the proportion of N derived from N_2-fixation of 10 % between the methods that were not attributable to a known cause.

In 1995 using late sown maize as a reference plant led to greatly erroneous estimations of N_2 fixation with the ^{15}N dilution method but not with the natural ^{15}N abundance method. The lower susceptibility of the natural abundance method to the timing of planting was because the temporal variation in plant available ^{15}N was lower and hence the need for a synchronized N uptake was less important. However, results of Turner *et al.* (1987) and Bremer & van Kessel (1990) showed substantial variation of $\delta^{15}N$ values with time can occur in certain systems.

Although the ^{15}N natural abundance method was less prone to temporal and spatial alterations in $\delta^{15}N$ it is nevertheless necessary to advocate the use of the same precautions as for the ^{15}N dilution method (Witty, 1983) with regard to a careful matching of the legume and the reference plant. Variations in $\delta^{15}N$ within the plant, either

due to ^{15}N discrimination during N_2 fixation or during translocation, appear to be the factors that most strongly affected estimates of N_2 fixation when using the ^{15}N natural abundance method in this experiment. Despite the early pessimism about the possible scope of the ^{15}N natural abundance method to measure N_2 fixation under field conditions (Hauck *et al.*, 1972) this current study and others have found that in soils of sufficient ^{15}N enrichment (6–10 ‰) and with a careful assessment of ^{15}N discrimination in plants the method can be used to estimate N_2 fixation in legume crops.

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