Nitrogen accumulation in three legumes and two cereals with emphasis on estimation of N_2 fixation in the legumes by the natural ^{15}N-abundance technique*

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Summary. N accumulation and natural ^{15}N abundance in three legumes (groundnuts, cowpeas, and soybeans) and in two cereals (sorghum and maize) were investigated over two seasons in Alfisols with and without N fertilization. Using the N uptake and natural ^{15}N abundance of non-nodulating plants as the indication of N derived from soil and fertilizer, the per cent N derived from atmospheric N_2 was calculated for nodulated plants. In the first experiment, the groundnut genotype contained 85% atmosphere-derived N, but the percentage decreased with N application. Estimates of atmosphere-derived N by the N-difference and ^{15}N-abundance techniques gave identical results. The percentages of atmosphere-derived N estimated by the two methods at different stages of groundnut growth were also similar. In the second experiment, atmosphere-derived N was estimated in plants grown with $0–200\ kg\ ha^{-1}$ applied N. The estimated atmosphere-derived N ranged from 42% to 61% for groundnuts from 33% to 77% for cowpeas, and from 24% to 48% for soybeans, depending on the amount of N applied. Inoculation with a *Bradyrhizobium* strain increased the percentage of atmosphere-derived N in soybean plants grown without any fertilizer N. The natural ^{15}N abundance of sorghum and maize was very close to that of the non-nodulating groundnut, suggesting that these cereals can be used as reference plants in the estimation of atmosphere-derived N by the natural ^{15}N-abundance method.

Key words: N_2 fixation – Natural ^{15}N abundance – N-difference method – Atmosphere-derived N – Non-nodulating genotype

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In general, crop yields are correlated with N accumulation in the plants. In cereals, N is derived from soil and from added fertilizers, but in leguminous crops, it is derived from atmospheric N_2 as well as from soil and added fertilizers. Estimates of the amount of N_2 fixed by legumes will allow a better understanding of the N economy of the cropping system and help in optimizing the use of N fertilizer.

In the semi-arid tropics, some estimates of the amounts of N_2 fixed by leguminous crops have been made by comparing the N accumulated in a given legume with the N accumulated in a non-fixing crop taking up N only from soil and added fertilizer (Selamat and Gardner 1985; Nambiar et al. 1986). The major uncertainty of this difference method is that the N taken up by the reference crop from the soil and fertilizer may not be comparable with the amount of N taken up by the leguminous plant.

Giller et al. (1987) applied ^{15}N-labeled fertilizer (ammonium sulfate) to both nodulating and non-nodulating groundnuts (*Arachis hypogaea* L.), analyzed both groundnuts for ^{15}N dilution, and estimated that 86% – 92% of N in the nodulating groundnut supplied with $10\ kg\ N\ ha^{-1}$ was derived from atmospheric N_2 fixation. Kumar Rao et al. (1987) estimated N_2 fixation by the pigeonpea by the ^{15}N-dilution method, using sorghum as a non-fixing control. Their estimate showed that more than 88% of the N in pigeonpea was derived from N_2 fixation. Although the dilution method is more reliable than the difference method, the application of ^{15}N-labeled fertilizer may affect the level of N_2-fixing activity of the legumes; further, it is not feasible to apply ^{15}N-labeled fertilizer uniformly to the soil profile in large experimental fields.

The natural ^{15}N-abundance method has been applied to estimate the per cent N derived from atmospheric N_2 fixation in several temperate legumes, e.g.,

soybean [*Glycine max* (L.) Merr.] (Amargar et al. 1979; Kohl et al. 1980; Wada et al. 1986; Yoneyama 1987), field bean (*Phaseolus vulgaris* L.) (Rennie 1986), and cowpea [*Vigna unguiculata* (L.) Walo.] (Ofori et al. 1987). The usefulness and limitations of this method were reviewed by Shearer and Kohl (1986). In this paper, we report estimates of atmosphere-derived N in groundnuts, soybeans, and cowpeas grown in the semi-arid tropics, using the natural ^{15}N-abundance method.

Materials and methods

The experiments were conducted in Alfisol fields at the ICRISAT Center, near Hyderabad, India (chemical properties of Alfisols at ICRISAT Center, in general, are pH (H_2O) 6.3, organic C 0.62%, total N 0.06% and total P 140 mg kg^{-1} soil. The plots for groundnuts (*Arachis hypogaea* L.) and cowpeas [*Vigna unguiculata* (L.) Walo.] were not inoculated as they were effectively nodulated by native rhizobia. A set of the plots for soybeans [*Glycine max* (L.) Merr.] was inoculated with *Bradyrhizobium japonicum* TAL 102 obtained from Niftal (University of Hawaii, USA), while another set was not inoculated.

The first experiment was conducted during the 1984–1985 postrainy seasons. The groundnut genotypes tested were Robut 33-1 (nodulating) and a non-nodulating genotype. The sorghum [*Sorghum bicolor* (L.) Moench] genotypes were CSH 8R (an improved hybrid) and M 35-1 (a traditional variety). The plots were fertilized with 17 kg phosphorus ha^{-1} as a basal application. The crops were sown in ridges 60 cm apart, and the plots contained five rows of 5 m. Within the rows the groundnuts were spaced 10 cm apart, and the sorghum plants 15 cm apart. Urea was applied in four equal applications 14, 35, 60, and 80 days after sowing to provide 0, 66.5, 133.5, and 200 kg N ha^{-1}. The sorghum was harvested 120 days after sowing and the groundnut crop was harvested 140 days after sowing.

The second experiment was conducted during the 1985–1986 postrainy season. The seeds of groundnut (nodulating genotype ICGS 11 and the non-nodulating genotype), cowpea (genotype EC 6215), soybean (genotype JS 7244), sorghum (hybrid CSH 8R), and maize (*Zea mays* L., hybrid Deccan 103) were sown on 29 November, 1985, in raised beds, 1.5 cm wide, with three cereal rows and four legume rows per plot. Within the rows, all plants were spaced 20 cm apart. All crops were fertilized with three levels of N, 0, 100, and 200 kg N ha^{-1} as urea. Urea was applied 11, 31, 52, and 73 days after sowing in four equal doses. The samples of whole groundnut plants (ICGS 11 and the non-nodulating genotype) were taken 66, 80, 94, 109, and 129 days after sowing and the final harvest was carried out 137 days after sowing. The soybeans, cowpeas, maize, and sorghum were harvested 119 days after sowing. Acetylene reduction activity was assayed as described by Nambiar and Dart (1983).

Plant parts dried at 70 °C for 3 days were ground to pass through a 1-mm sieve. In the first experiment, the husk and the grain of the sorghum were ground together. Eighty milligrams of the ground sample was digested by the Kjeldahl method, and diluted to 75 ml with distilled water, and 0.33 ml was used to estimate N based on the Berthelot reaction (details in Industrial method no. 218-72 A, 11, Technicon Industrial Systems, Tarrytown, NY 10591, USA). The rest of the sample solution was distilled into 1 *N* sulfuric acid after the addition of 10 *N* sodium hydroxide to obtain ammonium. The resulting ammonium was used to measure the natural abundance of ^{15}N with a Finnigan 250 mass spectrometer (Yoneyama 1987). In the second experiment, 129 days after sowing, the total N accumula-

tion could not be measured in either the nodulating or the non-nodulating groundnut, and the percentage of atmosphere-derived N in the nodulating groundnut was not available.

The natural ^{15}N abundance (δ^{15}N) of the samples was expressed as follows:

$$\delta^{15}N\ (\%_0) = [(R_{sample}/R_{standard}) - 1] \times 1000 \qquad (1)$$

where R is ^{15}N/^{14}N and atmospheric N_2 was used as the standard. The precision of analysis including all procedures was ±0.2‰.

The percentage of atmosphere-derived N (% Ndfa) in an N_2-fixing plant was calculated as follows. By the N-difference method:

$$\%\ Ndfa = [(Ntfp - Ntnp)/Ntfp] \times 100 \qquad (2)$$

where Ntfp is total N in a fixing plant and Ntnp is total N in a non-fixing plant.

By the natural ^{15}N-abundance method:

$$\%\ Ndfa = [(\delta^{15}Nnp - \delta^{15}Nfp)/(\delta^{15}Nnp - \delta^{15}Na)] \times 100 \qquad (3)$$

where ^{15}Nnp is ^{15}N in a non-fixing plant, ^{15}Nfp is ^{15}N in a fixing plant, and ^{15}Na is the ^{15}N value of a given legume grown in solution culture with atmospheric N_2 as the sole source of N.

The value obtained for the nodulating groundnut (Robut 33-1) was −0.7‰±0.3‰, and those for the soybean and cowpea had been obtained previously as −1.6‰ (Yoneyama 1987) and +0.5‰ (Ofori et al. 1987), respectively. The urea used in these experiments was similarly analyzed, and the value obtained was −0.4‰±0.1‰.

Results

Experiment 1

The accumulation of N in non-fixing plants (one groundnut and two sorghum genotypes) increased linearly with the applied N in a very similar fashion, although the apparent efficiency of use of the applied N was very low (10%−15%). However, the accumulation of N by the nodulating groundnut (Robut 33-1) was not affected up to the application of 133.5 kg N ha^{-1}, and tended to decrease with a higher application of N (Fig. 1).

The δ^{15}N values for different parts of both Robut 33-1 and the non-nodulating groundnut genotype at the final harvest (Table 1) show that the variation of δ^{15}N in grain, husk, and shoot at the same fertilizer level was non-significant. Application of fertilizer reduced the δ^{15}N values of the non-nodulating plants.

To estimate the percentage of atmosphere-derived N in Robut 33-1, Eqs. 2 and 3 were applied. The estimates by this δ^{15}N method and by the N-difference method using the non-nodulating groundnut as the reference plant are also shown in Table 1. The atmosphere-derived N calculated by the N-difference and the δ^{15}N methods gave very close values. Without the application of fertilizer N, about 85% of plant N in the nodulating genotype was derived from atmospheric N_2, and the application of fertilizer N reduced the fraction of fixed N_2.

The δ^{15}N values in two genotypes of sorghum grown in the same experiment are shown in Table 2.
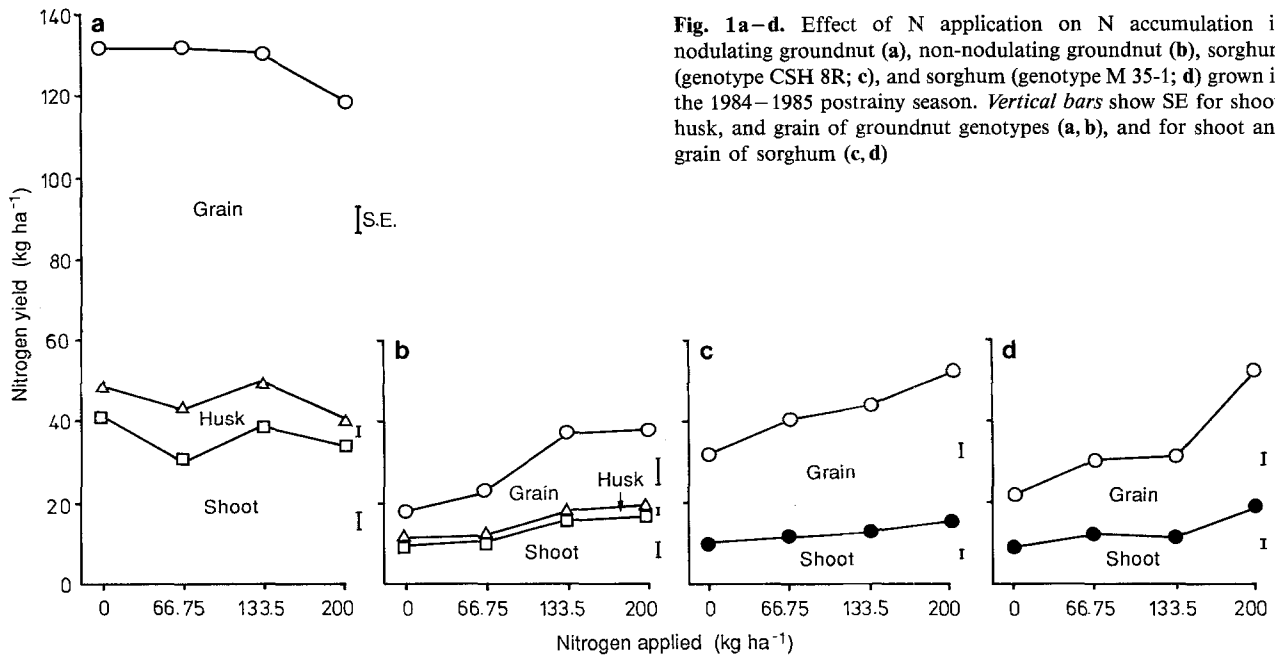
Fig. 1a–d. Effect of N application on N accumulation in nodulating groundnut (**a**), non-nodulating groundnut (**b**), sorghum (genotype CSH 8R; **c**), and sorghum (genotype M 35-1; **d**) grown in the 1984–1985 postrainy season. *Vertical bars* show SE for shoot, husk, and grain of groundnut genotypes (**a, b**), and for shoot and grain of sorghum (**c, d**)

Table 1. Natural abundance of ^{15}N in nodulating (Robut 33-1) and non-nodulating (Nonnod) groundnuts grown in the 1984–1985 postrainy season

N applied (kg ha^{-1})	Plant part	δ^{15}N (‰)[a]		% Ndfa[b] estimated by	
		Robut 33-1	Nonnod	δ^{15}N	N difference
0	Grain	+0.1±0.1	+4.3±0.3		
	Husk	−0.1±0.2	+3.7±0.4		
	Shoot	−0.2±0.4	+3.9±0.3		
	Whole	0.0	+4.1	85	86
66.75	Grain	+0.3±0.4	+3.4±0.5		
	Husk	−0.1±0.2	+2.5±0.5		
	Shoot	−0.3±0.3	+3.3±0.6		
	Whole	+0.3	+3.3	80	83
133.5	Grain	+0.4±0.3	+2.7±0.8		
	Husk	−0.3±0.1	+2.2±0.6		
	Shoot	+0.2±0.4	+2.7±0.9		
	Whole	+0.4	+2.7	71	72
200	Grain	+0.4±0.4	+2.6±0.6		
	Husk	−0.1±0.6	+2.0±0.8		
	Shoot	+0.1±0.4	+2.6±0.6		
	Whole	+0.3	+2.6	70	65
SE (whole)[c]		±0.21**			
Mean (whole)		0.2	+3.1		
SE (whole)[d]		±0.11**			

[a] Means ± SD of four replicates
[b] Ndfa, N derived from the atmosphere
[c] SE for comparing genotypes at different levels of N. **$P<0.01$
[d] SE for comparing genotypes. **$P<0.01$

Both sorghum genotypes had similar δ^{15}N values, although the grain N yields of CSH 8R were higher than those of M35-1. When no fertilizer N was applied, the δ^{15}N of sorghum was similar to those of the non-nodulating groundnut. However, the δ^{15}N of sorghum plants supplied with 200 kg N ha^{-1} was significantly higher than those of the non-nodulating groundnut grown in the same plots.

Experiment 2

Figure 2 shows the ontogenic changes of δ^{15}N values and N contents in the nodulating and non-nodulating

Table 2. Natural abundance of ^{15}N in two genotypes of sorghum grown in the 1984–1985 postrainy season

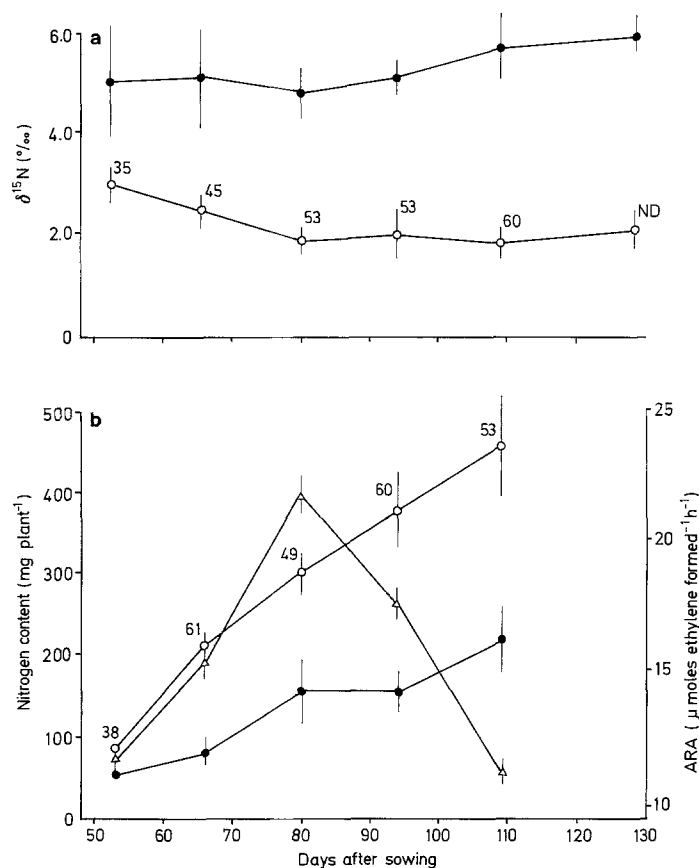| N applied (kg ha^{-1})	Plant part	$\delta^{15}N$ (‰)[a]	
		CSH 8R	M 35-1
0	Grain	+4.0±0.2	+4.0±0.2
	Shoot	+4.1±0.1	+3.7±0.3
	Whole	+4.1	+3.9
200	Grain	+3.9±0.2	+4.1±0.3
	Shoot	+3.1±1.4	+3.3±0.4
	Whole	+3.7	+3.7
SE (whole)[b]		±0.15^{NS}	
Mean (whole)		+3.9	+3.8
SE (whole)[c]		±0.10^{NS}	

[a] Means ± SD of four replicates

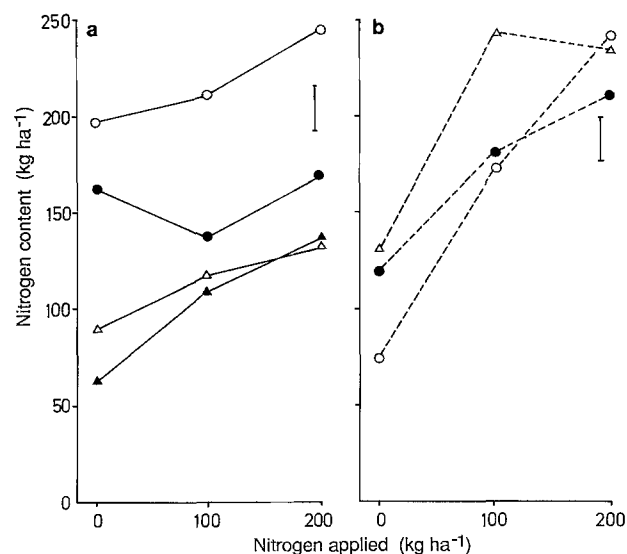[b] SE for comparing genotypes at different levels of N. NS: Nonsignificant

[c] SE for comparing genotypes. NS: Nonsignificant

Fig. 2a, b. Ontogenic changes in $\delta^{15}N$ values (a) and nitrogen contents (b) in nodulating (O———O) and non-nodulating (●———●) groundnut plants and acetylene reduction activity (b, △———△) in nodulating groundnut plants grown without N fertilizer in the 1985–1986 postrainy season. The *numbers* within the figure are the percentages of atmosphere-derived N calculated by the $\delta^{15}N$ method (a) and by the N-difference method (b). Bars show SE. Atmosphere-derived N was not determined (*N. D.*) 129 days after sowing

groundnut plants, and acetylene reduction activity in the nodulating groundnut grown without N fertilizer during the 1985–1986 postrainy season. The $\delta^{15}N$ values of the non-nodulating groundnut did not vary much over the growth stages; the $\delta^{15}N$ values of the nodulating genotype ICGS 11 decreased until 80 days after sowing and subsequently remained almost constant. The contribution from N_2 fixation in the groundnut increased from 35% of the total N assimilated 50 days after sowing to 60% 110 days after sowing. Total N in both the nodulating and the non-nodulating groundnuts increased linearly. The percentage of atmosphere-derived N calculated by the N-difference method was similar to the values calculated by the $\delta^{15}N$ method between 53 and 109 days after sowing, except 66 days after sowing. The acetylene reduction activity of the nodulating groundnut ICGS 11 increased linearly up to 80 days after sowing and thereafter declined rapidly, while N continued to accumulate (Fig. 2).

The N accumulation in the three nodulating and three non-nodulating crop species grown in the 1985–1986 postrainy season is shown in Fig. 3. The N yields in the cowpea and the non-inoculated soybean plants were lower than those of the nodulated groundnut. Inoculation of soybean with a *Bradyrhizobium* strain increased the N yield in plants grown without any applied N, but not in plants grown with fertilizer N.

Fig. 3. Effect of N application on N accumulation in groundnuts, cowpeas, soybeans, maize, and sorghum in the 1985–1986 postrainy season. The soybeans were grown with (*+INO*) and without (*−INO*) inoculation with a *Bradyrhizobium* strain. *Bars* show SE for nodulating (*ICGS 11*) and non-nodulating (*NONNOD*) plants. **a** O, groundnut (ICGS 11); ●, cowpea, △, soybean (+INO); ▲, soybean (−INO). **b** △, maize, ●, sorghum, O, groundnut (NONNOD)

The N uptake by three non-fixing plants grown without any fertilizer N ranged from 75 to 130 kg ha^{-1} (Fig. 3), which is much larger than the values recorded in the previous year (Fig. 1). Sorghum and the non-nodulating groundnut responded linearly to N up to 200 kg ha^{-1}, but maize did not respond well to 200 kg N ha^{-1}. In many of our earlier trials, the growth and yield of the non-nodulating groundnut genotype even at high N levels was much lower than that of nodulating genotypes grown without any N (Nambiar et al. 1986). However, during the 1985–1986 postrainy season, the final N yield of the non-nodulating groundnut with 200 kg N ha^{-1} was almost equal to that of the nodulating groundnut. At present, this cannot be fully explained, but we assume that the available soil N in the 1985–1986 trial was much higher than that in the 1984–1985 trial. During the 1985–1986 trial, considerable leaf fall occurred in the nodulating groundnut ICGS 11 at all N levels due to rust (*Puccinia arachidis*) and late leaf spot (*Phaeoisariopsis personate*), and we observed that the non-nodulating genotype was relatively resistant to these foliar diseases. This might also have contributed to the lower N accumulation in the nodulating groundnut during the 1985–1986 trial.

The δ^{15}N values of the whole plant of three non-nodulating crops and three nodulating crops calculated from the N content and δ^{15}N values of each plant part are shown in Table 3. In all nodulating plants, irrespective of the N-fertilizer levels, the δ^{15}N values were lower than those of the non-nodulating plants (Table 3). The δ^{15}N values of the non-inoculated soybean grown without fertilizer N were higher than those of the inoculated soybean.

The percentage of atmosphere-derived N in the nodulating plants was calculated by both the δ^{15}N method and the N-difference method. For the groundnut, the non-nodulating genotype was used as the reference plant, and for the cowpea and the soybean, the means of δ^{15}N values of three non-fixing plants (+5.0, +3.3, and +2.4 for 0, 100, and 200 N kg ha^{-1}, respectively) were used as the reference values in each treatment. Atmosphere-derived N in the groundnut was calculated as 61% by the δ^{15}N method and 62% by the N-difference method when no fertilizer N was applied, but the estimates by the two methods differed very considerably with increased N fertilizer levels. The atmosphere-derived N in the cowpea plants was 77% by the δ^{15}N method in the non-fertilized plot, and was also decreased by N fertilizer treatments. The atmosphere-derived N in the soybean calculated by the δ^{15}N method was 48% with *Bradyrhizobium* inoculation, and 29% without it, when no N was applied. The percentage decreased with N application in the non-inoculated soybean. For the cowpea and the soybean, no atmosphere-derived N was calculated by the N-difference method since no appropriate non-fixing control was available in this experiment.

Discussion

In the present study, the natural ^{15}N-abundance method was applied to estimate the percentage of atmosphere-derived N in three legumes. About 85% of the total N in the nodulating groundnut Robut 33-1, grown without any added N fertilizer, was derived from N$_2$ fixation, while only 61% was derived from

Table 3. Natural abundance of ^{15}N in non-noduating and nodulating crops grown in the1985–1986 postrainy season

Crop	Genotype	δ^{15}N (‰)[a]			% Ndfa by δ^{15}N			Ndfa[b] by N difference (%)		
		0	100	200	0	100	200	0	100	200
Sorghum	CSH 8R	4.9	3.2	2.5	–	–	–	–	–	–
Maize	Deccan 103	4.8	3.2	2.0	–	–	–	–	–	–
Groundnut	Nonnod	5.2	3.5	2.6	–	–	–	–	–	–
Groundnut	ICGS 11	1.6	1.6	1.2	61	47	42	62	18	0
Cowpea	EC6215	1.4	1.3	1.7	77	67	33	–	–	–
Soybean	JS7244 (+Ino)[c]	1.8	2.2	1.0	48	24	35	–	–	–
	JS7244 (−Ino)[c]	3.1	2.2	0.7	29	26	43	–	–	–
SE[d]			±0.29**							
Mean		3.2	2.4	1.7						
SE[e]			±0.11**							

[a] Data obtained by calculation from δ^{15}N values and N contents of plant parts; N applied in kg ha^{-1}
[b] Ndfa, N derived from the atmosphere
[c] With (+Ino) and without (−Ino) inoculation by *Bradyrhizobium japonicum*
[d] SE for comparing N levels for different genotypes. **$P<0.01$
[e] SE for comparing N levels. **$P<0.01$

N_2 fixation in another nodulating genotype (ICGS 11), apparently due to the higher soil-N availability during the 1985–1986 postrainy season when ICGS 11 was tested. The application of fertilizer N generally decreased N_2 fixation. However, even at 200 kg N ha^{-1}, groundnut Robut 33-1 continues to fix a considerable amount of N. Unlike the groundnuts, N_2 fixation by soybeans was more affected by the application of fertilizer N. We have suggested that the soybean makes a better use of fertilizer N than the groundnut, based on nitrate and nitrate reductase measurements in the leaves (Nambiar et al. 1988).

Dutta et al. (1988) found that in field-grown nodulating groundnuts, the nitrogenase activity assayed by the acetylene reduction method showed a linear relationship with N accumulation during the early growth stage, but during the reproductive stage while the N accumulation continued the acetylene reduction activity rapidly declined. The results shown in Fig. 2 suggest that the continuous accumulation of N in groundnut plants may be due to both absorption of soil N (as observed from the increase in N in the non-nodulating genotype) and N_2 fixation (as observed from the increased N in ICGS 11 without a decrease in the N_2 contribution). This is further evidence that the observed decrease in acetylene reduction activity during the later stages of groundnut growth is not a good reflection of a decrease in N_2-fixing activity during these periods, and that an inference based only on measurements of the acetylene reduction activity could be misleading.

In the present study, the values of atmosphere-derived N obtained by the $\delta^{15}N$ method were close to those obtained by the N-difference method. However, the N-difference method is not universally valid; although straightforward, the difference method assumes that N_2-fixing and non-fixing plants contain the same amount of N derived from soil. Generally, the amounts of N in the above-ground plant parts is compared between N_2-fixing and non-fixing plants. Therefore, the choice of a different-sized reference plant (different N content) or with a different partition of N between the above-ground parts and the roots can lead to different estimates of atmosphere-derived N. In some cases, where the N accumulation in N_2-fixing or non-fixing plant has been affected by insect pests or disease, the N-difference method provides false estimates of atmosphere-derived N. In contrast, the $\delta^{15}N$ method provide good estimates as far as both the N_2-fixing and the non-fixing plants have equal access to natural $^{15}N/^{14}N$ in soil N.

In conclusion, the estimate of the percentage of atmosphere-derived N by the $\delta^{15}N$ method may be useful in estimating N_2 fixation by groundnuts, soybeans, and cowpeas, with sorghum and maize being used as reference plants if no non-nodulating legumes are available and if there are no restrictions on the use of soil and fertilizer N.

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