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N_2 FIXATION BY *SESBANIA ROSTRATA* AND *SESBANIA SESBAN* ESTIMATED USING ^{15}N AND TOTAL N DIFFERENCE METHODS

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Summary—N_2 fixation by the stem-nodulated legume *Sesbania rostrata* was compared in waterlogged and non-waterlogged conditions with that of a non-stem-nodulated *Sesbania* species, *Sesbania sesban*. To estimate N_2 fixation, two methods were used, the ^{15}N direct isotope dilution method and the difference method.

Due to stem nodulation *S. rostrata* fixed much more N_2 in 60 days (0.78–0.68 g N_2 per plant in waterlogged soils or 0.62–0.59 g N_2 in drained soils) than *S. sesban* (0.06–0.05 g N_2 per plant or 0.13–0.12 g N_2). There was a satisfactory agreement between the isotopic and difference method estimates. From these values it was calculated that about 83–109 kg N_2 and 7–18 kg N_2 ha^{-1} would be fixed in 60 days by *S. rostrata* and *S. sesban* respectively. The results of this experiment confirm the high N_2-fixing potential of the stem-nodulated legume *S. rostrata* as compared to that of a non-stem-nodulated species, *S. sesban*.

INTRODUCTION

Bacteria of the genus *Rhizobium* usually form N_2-fixing nodules on the roots of most leguminous plants. Only a few legume species are also able to form nodules on their stem. Dreyfus and Dommergues (1981) reported profuse stem-nodulation in *Sesbania rostrata*, an annual sahelian legume which grows in waterlogged soils during the rainy season. Both stem and root nodules of *S. rostrata* are induced by a specific strain of *Rhizobium* sp. The stem nodules fix N_2 actively. In a first evaluation of N_2 fixation by field grown *S. rostrata*, the amount of N_2 fixed was estimated to be about 200 kg ha^{-1} during growth for 7 wk (Rinaudo *et al.*, 1982). This trait explains why *S. rostrata* has been successfully used as green manure in paddy fields (Dreyfus *et al.*, 1985). Up to now, N_2 fixation by *S. rostrata* has been assessed only by the difference method which compares the total N contents of N_2-fixation and non-N_2-fixing plants. Since this method of assessment is liable to criticism, it was found necessary to compare these estimates with estimates obtained through the ^{15}N direct isotope dilution method. The ^{15}N method determined in the N_2-fixing plants the dilution of soil-derived ^{15}N by symbiotically-fixed N. In an attempt to evaluate the advantage of stem-over-root-nodulation, we compared nitrogen fixation by *S. rostrata* with N_2 fixation by a non stem-nodulated *Sesbania* species, *Sesbania sesban*. The latter species is valued as a fodder, green manure and shade plant in Asia. A further treatment to be evaluated was the effect of waterlogging on N_2 fixation by either type of legume.

MATERIALS AND METHODS

Experimental design

The experiment was conducted in the greenhouse of the ORSTOM station in Dakar Senegal during the cool season (December 1985 to February 1986). At that time of the year, the day–night temperature is 20–24°C and the photoperiod is only 12 h. Seeds were treated and surface sterilized with concentrated H_2SO_4. The times of treatment in H_2SO_4 were as follows: *S. rostrata*, 45 min; *S. sesban*, 30 min. The seeds were then washed several times with sterile water and germinated for 24 h on sterile soft agar (0.8% or 8%) in Petri dishes. Seedlings were then transferred to 30 cm dia buckets filled with 20 kg of soil. To avoid nodulation by indigenous rhizobia this soil had been previously sterilized with methylbromide.

The soil used was a typical sandy and neutral soil (psamment; vernacular name: Dior) with 93% sand, 0.3% and 0.025% of C and N, respectively, pH 7.0. After emergence the plants were thinned to one per bucket and each bucket received 1 g of K_2HPO_4 as PK fertilizer which is the equivalent of 140 kg ha^{-1}. Plants were grown for 2 months in the greenhouse. Since *S. rostrata* is photoperiod-dependent species whose development is hindered during short-day periods, plants had to be kept under additional artificial light for 14 h.

Inoculation procedure

As we did not isolate any *Rhizobium* strain able to effectively nodulate both stems of *S. rostrata* and roots of *S. sesban*, we selected, out of 36 strains, the best N_2-fixing strain for each of the host-plants: strain ORS 571 for nodulation of roots and stems of *S. rostrata* and strain ORS 502 for nodulation of *S. sesban*. Strain ORS 571 was grown in YL medium (Dreyfus *et al.*, 1983) and strain ORS 502 in YMA medium (Vincent, 1970).

Root inoculation of both *Sesbania* species was achieved by adding 10 ml of a liquid culture of *Rhizobium* to the soil at sowing and stems of *S. rostrata* were inoculated 3 wk after sowing by spraying the stems with a liquid culture of strain ORS 571. Uninoculated treatments remained uncontaminated throughout the experiment.

Treatments

Four treatments with four replications each were used in waterlogged or drained conditions.

Treatments 1 and 5: Uninoculated *S. rostrata* plants.
Treatments 2 and 6; Uninoculated *S. sesban* plants.
Treatments 3 and 7: Root-and stem-inoculated *S. rostrata* plants.
Treatments 4 and 8: Root-inoculated *S. sesban* plants.

Plants submitted to treatments 1, 2, 3 and 4 were grown under waterlogged conditions. After 1 wk of growth, soil was kept constantly flooded and a 2 cm-deep surface layer of water was maintained in the buckets.

Plants submitted to treatments 5, 6, 7 and 8 were grown in drained conditions. Soil was hand-watered frequently so that the soil moisture was kept close to the field capacity. ^{15}N labelled fertilizer was applied to all treatments immediately after the plants have been thinned at the rate of 0.2 g N per bucket, equivalent on an area basis to 28.3 kg N ha^{-1} as a solution of $(^{15}NH_4)_2SO_4$ with 4.973 atom % ^{15}N excess. The effect of induced N deficiency on symbiotic N_2 fixation of *S. rostrata* and *S. sesban* was determined using $(^{15}NH_4)_2SO_4$ treated soil with 5 g of glucose added per bucket to immobilize inorganic N (Witty, 1984). Glucose and $(^{15}NH_4)_2SO_4$ were mixed to give a solution with a C:N ratio of 10:1.

Plants were harvested 60 days after sowing. At that time *S. rostrata* plants were 1.6 m high and *S. sesban* 1.1 m high. Due to the 14 h photoperiod, no flowering was observed.

Estimation of N_2 fixation

Leaves and stems were sampled separately, dried to a constant weight at 60–70°C. Then, each fraction was completely ground into 100 μm powder. Samples of each powdered fraction were analyzed for total N content according to the Kjeldahl method (Bremmer, 1965). ^{15}N analyses were carried out at the Seibersdorf laboratory (IAEA) using Dumas' method (the combustion performed in this technique converts total N directly to N_2) and mass spectrometry (Fiedler and Proksch, 1975). For each individual plant N and ^{15}N values were calculated taking into account the weight, N and ^{15}N contents of leaves and stems.

The direct isotope dilution method

The procedure involves the incorporation of ^{15}N labelled fertilizer directly into soil in the presence of both inoculated (N_2-fixing) and uninoculated (non-N_2-fixing) plants. This method is based on differential dilution in the plant of ^{15}N labelled fertilizer by soil and fixed nitrogen. It is possible to assess the portion of a legume's N that is derived from N_2 fixation, distinct from all other sources of N (Fried and Broeshart, 1975; Legg and Sloger, 1975; Fried and Middleboe, 1977; Bremmer, 1977; Wagner and Zapata, 1982; Vose *et al.*, 1982; Rennie and Rennie, 1983; Fried *et al.*, 1983).

The % plant N derived from the atmosphere (% N dfa), soil (% N dfs) and fertilizer (% N dff) were calculated as follows:

$$\%\text{N dfa} = \left[\frac{1 - \text{atom \% }^{15}\text{N ex. (fs)}}{\text{atom \% }^{15}\text{N ex. (nfs)}}\right] \times 100,$$

where fs and nfs are fixing and non-fixing plants, respectively.

$$\%\text{ N dff} = \left[\frac{\text{atom \% }^{15}\text{N ex. (plant)}}{\text{atom \%}^{15}\text{N ex. (fertilizer)}}\right] \times 100$$

N dfs = 100 − (% N dfa + % N dff).

The individual % N dfa values for each N_2-fixing plant were calculated taking into account each of the atom % ^{15}N ex. (fs) and atom % ^{15}N ex. (nfs) values.

If N was the total N content of each N_2-fixing plant, the N_2 fixed per plant was calculated as follows:

$$N_2 \text{ fixed (g plant}^{-1}) = \frac{\%\text{ N dfa}}{100} \times N$$

For the N_2-fixing and non-N_2-fixing plants we calculated FUE = "Fertilizer Use Efficiency".

It is commonly expressed as percentage:

$$\text{FUE} = \frac{\text{Total N uptake} + \text{N dff (fraction)}}{\text{Rate fertilizer N applied}} \times 100.$$

The difference method

The quantity of N_2-fixed was measured by the difference between the total N content of the shoots of inoculated (N_2-fixing) plants (Treatment 3, 4, 7 and 8) and uninoculated (non-N_2-fixing) plants (Treatment 1, 2, 5 and 6) growing with the same application of fertilizer. For each individual N_2-fixing plant, N_2 fixation was estimated to be the difference between the total N content of the shoots of each of these plants and the average total N content of the shoots of uninoculated plants (Williams *et al.*, 1977; Talbot *et al.*, 1982).

RESULTS

Influence of the different treatments on the dry weight and N content of Sesbania rostrata and Sesbania sesban

Results are shown in Table 1. Inoculating *S. rostrata* with *Rhizobium* strain ORS 571 increased its dry wt and total N content respectively by 28% and 81% in waterlogged soil (Treatment 3) and by 24% and 50% in drained soil (Treatment 7). Inoculating *S. sesban* with *Rhizobium* strain ORS 502 increased its dry wt and total N content by 17% and 20% in drained soils (Treatment 8) but had no significant effect in waterlogged soils (Treatment 4).

Effect of waterlogging on nodulation (Table 1)

In waterlogged soils (Treatment 3), dry wt of root-nodules from *S. rostrata* was found to be negligible (0.03 g $plant^{-1}$) as compared to that of stem-nodules (1.4 ± 0.5 g $plant^{-1}$). In drained soils (Treatment 7), root-nodules of *S. rostrata* weighed 0.3 ± 0.08 g $plant^{-1}$ and stem-nodules 1.0 ± 0.3 g.

The dry wt of root-nodules from *S. sesban* was 0.45 ± 0.1 g $plant^{-1}$ in drained conditions (Treatment 8), and only 0.1 ± 0.5 g in waterlogged conditions (Treatment 4).

Table 1. Influence of the different treatments on nodule weight, dry weight and N content of 2-month-old *Sesbania rostrata* and *Sesbania sesban* grown in waterlogged (W) or drained (D) soil[1]

	Treatments				Dry wt (g $plant^{-1}$)			N content (%)		N total
Species	Ref.	Inoc.[2]	Water regime	Nodule	Stem	Leaves	Total	Stem	Leaves	(g $plant^{-1}$)
S. rostrata	1	0	W	0	16.8 ± 3.9^{c}	8.0 ± 1.4^{b}	24.9 ± 3.6^{c}	0.71 ± 0.07^{c}	3.80 ± 0.4^{c}	0.42 ± 0.12^{d}
	3	+	W	Root 0.03 Stem 1.4 ± 0.3	20.5 ± 6.0^{b}	11.5 ± 3.4^{a}	32.0 ± 9.5^{b}	1.14 ± 0.04^{a}	4.48 ± 0.07^{b}	0.76 ± 0.21^{b}
	5	0	D	0	21.7 ± 1.6^{b}	11.7 ± 1.2^{a}	33.4 ± 0.5^{b}	0.82 ± 0.31^{bc}	3.54 ± 0.61^{c}	0.58 ± 0.03^{c}
	7	+	D	Root 0.3 ± 0.08 Stem 1.0 ± 0.3	29.6 ± 4.7^{a}	11.7 ± 1.5^{a}	41.3 ± 6.0^{a}	0.98 ± 0.12^{abc}	4.94 ± 0.31^{a}	0.87 ± 0.12^{a}
S. sesban	2	0	W	0	5.9 ± 2.7^{d}	7.9 ± 1.5^{b}	13.9 ± 4.0^{d}	1.01 ± 0.21^{abc}	4.31 ± 0.31^{b}	0.40 ± 0.06^{d}
	4	+	W	0.1 ± 0.05	5.9 ± 1.0^{d}	8.3 ± 1.2^{b}	14.2 ± 1.7^{d}	1.15 ± 0.32^{a}	4.61 ± 0.12^{ab}	0.45 ± 0.04^{d}
	6	0	D	0	13.5 ± 2.6^{c}	11.5 ± 0.6^{a}	24.9 ± 3.0^{c}	0.97 ± 0.11^{abc}	3.84 ± 0.72^{c}	0.59 ± 0.12^{c}
	8	+	D	0.45 ± 0.11	16.7 ± 1.7^{c}	12.5 ± 0.8^{a}	29.2 ± 2.2^{bc}	1.12 ± 0.22^{ab}	4.29 ± 0.11^{b}	0.71 ± 0.07^{b}
CV[3]					12.6	10.0	10.9	14.6	5.8	10.6

[1]Mean values ± confidence interval ($P = 0.05$).
[2]Inoculation: 0, uninoculated plants; +, inoculated plants.
[3]Coefficient of variation (%).
For each experiment, numbers in column with same letters (a, b, c or d) do not differ significantly, $P = 0.05$ (Newman and Keuls, 1957—Snedecor and Cochran, 1957).

Effect on the different treatments on atom % ^{15}N excess and utilization of applied fertilizer (Table 2)

Inoculation of *S. rostrata* by strain ORS 571 significantly decreased atom % ^{15}N excess of both stem and leaves of *S. rostrata* in waterlogged and drained soils. Waterlogging reinforced this effect. On the other hand, inoculation of *S. sesban* by strain ORS 502 slightly decreased the atom % ^{15}N excess. FUE was always decreased by waterlogging, but it was not affected by inoculation.

Effect of the treatments on N_2 fixation (Table 3)

Nitrogen fixation (% N dfa) was significantly higher in *S. rostrata* than in *S. sesban*. Waterlogging significantly increased % N dfa in *S. rostrata*. The amount of N_2 fixed followed a similar trend.

DISCUSSION

Nodulation and N_2 fixation

Stem-nodulation enabled *S. rostrata* to produce two to three times more nodules than *S. sesban* during the same growth period in waterlogged or drained soils (Table 1). The high N_2-fixing potential of *S. rostrata* is probably directly associated with this profuse stem-nodulation (Dreyfus, 1982). As shown in Table 3, each *S. rostrata* plant fixed 0.68–0.78 g N_2 in waterlogged soil or 0.59–0.62 g N_2 in drained soils. In the field, normal planting densities vary from 120,000 to 160,000 plants ha^{-1}. Therefore one can calculate that a field of *S. rostrata* with 140,000 plants ha^{-1} fixes 95–109 kg N_2 or 83 to 87 kg N_2 according to the water regime. These values must be interpreted with caution and are probably over estimated because ^{15}N was incorporated into the soil by adding the labelled fertilizer together with glucose. The resulting rapid microbial build up, followed by a slow release of mineral ^{15}N, probably induced a relative N deficiency which allowed a more active N_2-fixing activity (Fried *et al.*, 1983; Witty, 1984). Consequently the actual N_2 fixation by *S. rostrata* was probably higher than would have occurred in normal field conditions.

By comparison each *S. sesban* plant fixed 0.05–0.06 g N_2 in waterlogged soil or 0.12–0.13 g N_2 in drained soils. In these conditions the estimated N_2 fixation by a field of *S. sesban* with 140,000 plants ha^{-1} is only 7–8 kg N_2 or 17–18 kg N_2 depending on the water regime.

Effect of waterlogging

Waterlogging drastically affect root-nodulation of both species but do not affect stem-nodulation of *S. rostrata* (Table 1). Total nodule weight of *S. rostrata* does not vary with waterlogging, but the presence of root nodules in drained soils (Table 1, Treatment 7) seems to slightly reduce the stem-nodules mass.

As shown in Table 3, nitrogen fixation by *S. rostrata* does not suffer from the effects of waterlogging, by contrast, N_2 fixation by *S. sesban* was markedly reduced by waterlogging. The difference in the behaviour of both *Sesbania* species can be explained, (1) by the fact that root nodules, but not roots, of both *Sesbania* species decay rapidly with waterlogging which impedes oxygen diffusion in the

Table 2. Atomic % ^{15}N excess and fertilizer use efficiency (FUE) of 2-month-old *Sesbania rostrata* and *Sesbania sesban* grown in waterlogged (W) or drained (D) soil

	Treatments			Atom % ^{15}N excess[1]		
Species	Ref.	Inoc.[3]	Water regime	Stem	Leaves	FUE[2]
S. rostrata	1	0	W	0.511[a]	0.450[a]	28.8 ± 14.8
	3	+	W	0.239[c]	0.219[b]	24.7 ± 10.7
	5	0	D	0.404[abc]	0.353[ab]	31.1 ± 3.6
	7	+	D	0.249[c]	0.235[b]	30.1 ± 5.2
S. sesban	2	0	W	0.447[ab]	0.372[ab]	23.4 ± 9.8
	4	+	W	0.397[abc]	0.309[ab]	22.6 ± 6.4
	6	0	D	0.371[abc]	0.319[ab]	29.1 ± 4.9
	8	+	D	0.318[bc]	0.250[b]	28.7 ± 1.6
CV[4]				21.6	21.9	

[1]For each experiment, numbers in column with same letter (a, b or c) do not differ significantly, $P = 0.05$ (Newman and Keuls, 1957—see Snedecor and Cochran, 1957).
[2]Fertilizer use efficiency; mean values ± confidence inteval ($P = 0.05$).
[3]Inoculation: 0, uninoculated plants; +, inoculated plants.
[4]Coefficient of variation (%).

Table 3. N_2 fixation by 2-month old *Sesbania rostrata* and *Sesbania sesban* as grown in waterlogged (W) or drained (D) soil estimated by isotopic or difference methods[1]

				N_2 fixation	
Treatment	Species	Water regime	Method of assessment	% N dfa	N_2 fixed (g plant^{-1})
3	*S. rostrata*	W	Isotopic	51 + 10.1	0.78 + 0.31
			Difference	45 ± 13.1	0.68 ± 0.21
7	*S. rostrata*	D	Isotopic	36 ± 5.7	0.62 ± 0.06
			Difference	35 ± 12.4	0.59 ± 0.31
4	*S. sesban*	W	Isotopic	13 ± 8.0	0.06 ± 0.03
			Difference	11 + 5.2	0.05 ± 0.03
8	*S. sesban*	D	Isotopic	18 ± 2.5	0.13 ± 0.03
			Difference	18 ± 2.3	0.12 ± 0.11

[1]Mean values ± confidence interval ($P = 0.05$).

soil and (2) by the fact that stem nodules do not suffer from the same limitations.

Methodology of assessment of N_2 fixation

There was a satisfactory agreement between the difference and isotopic method estimates, a fact already mentioned by a number of authors (Talbot *et al.*, 1982; Witty, 1983; Cornet *et al.*, 1985).

Fertilizer use efficiency (FUE)

In drained soils FUE was higher than that in waterlogged soils, probably because, in the second case, some denitrification occurred. However with both water regimes, FUE remained at a low level (23–31%), a situation comparable to that reported for *Casuarina equisetifolia* and *Acacia holosericea* in the same soil as the one we used (Gautheir *et al.*, 1985; Cornet *et al.*, 1985). Because of the low FUE in most soils of West Africa, more fertilizer is required to get an adequate response to N fertilizer. This contributes to the increase in the cost of using nitrogen fertilizer in West African agriculture.

In conclusion, our results confirm the high N_2-fixing potential of the stem-nodulated legume *S. rostrata* which, in waterlogged or drained soils, produce higher dry matter and nitrogen yield than *S. sesban*. The difference between the two species is comparable to that observed in the field where *S. rostrata* produced 25 tons and *S. sesban* only 12 tons of biomass (fresh wt) ha^{-1} for a growth period of 45 days (I. Ndoye, unpublished data). In Asia several non-stem-nodulated species of *Sesbania*, such as *Sesbania aculeata*, *S. sesban*, *S. cannabina*, are traditionally used as green manure in paddy fields where waterlogging conditions are likely to occur during the growth of these legumes species. Stem-nodulation, which is probably an important factor of adaptation of this legume to waterlogging, enables *S. rostrata* to fix large amounts of N_2 in such conditions. Therefore the use of *S. rostrata* as green manure in paddy fields appears to be a substitute for chemical N fertilization and should be recommended. Furthermore, attempting to transfer the stem-nodulation ability to other species of *Sesbania* used as green manure would probably be a fruitful approach to increasing rice yield in the tropics.

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REFERENCES

Bremmer J. M. (1965) Total nitrogen. In *Methods of Soil Analysis* (A. Black *et al.*, Eds), pp. 1149–1178. American Society of Agronomy, Madison, Bremmer J. M. (1977) Use of nitrogen-tracer techniques for research on nitrogen fixation. In *Biological Nitrogen Fixation in Farming Systems of the Tropics* (A. Ayanaba and P. J. Dart, Eds), pp. 335–352. Wiley, Chichester.

Cornet F., Otto C., Rinaudo G., Diem H. G. and Dommergues Y. R. (1985) Nitrogen fixation by *Acacia holosericea* grown in field-simulating conditions. *Oecologia Plantarum* **6,** 211–218.

Dryfus B. and Dommergues Y. R. (1981) Nitrogen fixing nodules induced by *Rhizobium* on the stem of the tropical legume *Sesbania rostrata. FEMS Microbiology Letters* **10,** 313–317.

Dreyfus B. (1982) La symbiose entre *Rhizobium* et *Sesbania rostrata,* legumineuse à nodules caulinaires. Thèse de Doctorat d'Etat, Université Paris VII.

Dreyfus B., Elmerich G. and Dommergues Y. R. (1983) Free living *Rhizobium* strain able to grow on N_2 as the sole nitrogen source. *Applied Environmental Microbiology* **45,** 711–713.

Dreyfus B., Rinaudo G. and Dommergues Y. R. (1985) Observation on the use of *Sesbania rostrata* as green manure in paddy fields. *Mircen Journal* **1,** 111–121.

Fiedler R. and Proksch P. (1975) The determination of nitrogen-15 by emission and mass spectrometry in biochemical analysis: a review *Analytica Chimica Acta* **78,** 1–62.

Fried M. and Broeshart H. (1975) An independent measurement of the amount of nitrogen fixed by a legume crop. *Plant and Soil.* **43,** 707–711.

Fried M. and Middelboe V. (1977) Measurement of amount of nitrogen fixed by a legume crop. *Plant and Soil* **47,** 713–715.

Fried M., Danso S. K. A. and Zapata F. (1983) The methodology of measurement of N_2 fixation by non legumes as inferred from field experiments with legumes. *Canadian Journal of Microbiology* **29,** 1053–1062.

Gauthier D., Diem H. G., Dommergues Y. R. and Ganry F. (1985) Assessment of N_2 fixation by *Casuarina equisetifolia* inoculated with *Frankia* ORS 021001 using ^{15}N methods. *Soil Biology & Biochemistry* **17,** 375–379.

Legg J. O. and Sloger C. (1975) A tracer method for determining symbiotic nitrogen fixation in field studies. In *Proceedings of the Second International Conference on Stable Isotopes* (E. R. Klein and P. D. Klein, Eds), pp. 661–666. Oak Brook, Illinois.

Rennie R. J. and Rennie D. A. (1983) Techniques for quantifying N_2 fixation in association with non legumes under field and greenhouse conditions. *Canadian Journal of Microbiology* **29,** 1022–1035.

Rinaudo G., Dreyfus B. and Dommergues Y. R. (1982) Influence of *Sesbania rostrata* green manure on the nitrogen content or rice crop and soil. *Soil Biology & Biochemistry* **15,** 111–113.

Snedecor G. W. and Cochran W. G. (1957) *Méthodes Statistiques*: Association de Coordination Technique Agricole.

Talbot H. J., Kenworthy W. J. and Legg J. O. (1982) Field comparison of nitrogen-15 and difference methods of measuring nitrogen fixation. *Agronomy Journal* **74,** 799–804.

Vincent J. M. (1970) *A Manual for the Practical Study of Root-Nodule Bacteria.* Blackwell, Oxford.

Vose P. B., Ruschel A. P., Victoria R. L., Saito S. M. T. and Matsui E. (1982) ^{15}N as a tool in biological nitrogen fixation research. In *Biological Nitrogen Fixation Technological for Tropical Agriculture* (P. H. Graham and S. C. Harris, Eds), pp. 575–592. Centro International de Agriculture Tropical, Calif.

Wagner G. H. and Zapata F. (1982) Field evaluation of reference crops in the study of nitrogen fixation by legumes using isotope techniques. *Agronomy Journal* **74,** 607–612.

Williams W. A., Jones M. B. and Delwiche C. C. (1977) N_2 fixation measurement by total N differences and ^{15}N A-values in lysimeters. *Agronomy Journal* **69,** 1023–1024.

Witty J. F. (1983) Estimating N_2 fixation in the field using ^{15}N labelled fertilizer: some problems and solutions. *Soil Biology & Biochemsity* **15,** 631–639.

Witty J. F. (1984) The use of slow release ^{15}N fertilizer formulations in measuring N_2 fixation by isotope dilution. *Soil Biology & Biochemistry* **16,** 657–661.