Parrotta, J.A., Baker, D.A. & Fried, M. (1994). Estimation of nitrogen fixation in *Leucaena leucocephala* using ^{15}N-enrichment methodologies. In: J.I. Sprent & D. McKey (editors). Advances in Legume Systematics 5: The Nitrogen Factor, pp. 75–82. Royal Botanic Gardens, Kew.

ESTIMATION OF NITROGEN FIXATION IN *LEUCAENA LEUCOCEPHALA* USING ^{15}N-ENRICHMENT METHODOLOGIES

JOHN A. PARROTTA[1], DWIGHT D. BAKER[2] & MAURICE FRIED[3]

[1]Institute of Tropical Forestry, USDA Forest Service, Southern Forest Experiment Station, Call Box 25000, Rio Piedras, PR 00928-2500, USA.
[2]Yale University, School of Forestry & Environmental Studies, New Haven, CT 06511 USA.
[3]National Research Council, 2101 Constitution Ave. NW, Washington DC 20418 USA.

Abstract

An estimation of biological nitrogen fixation by *Leucaena leucocephala* (Lam.) de Wit in monoculture and mixed-species plantations (with *Casuarina equisetifolia* L. ex J.R. & G. Forst., and *Eucalyptus robusta* Sm.) was undertaken over a two-year period in Puerto Rico using the ^{15}N-enrichment methodology. Foliage from *Leucaena* and *Eucalyptus* (the reference species) was sampled at selected intervals between applications of ^{15}N-enriched fertiliser and the N-isotopic composition was used to estimate the proportion of N derived from nitrogen fixation (pNdfa) and total N derived from nitrogen fixation (tNdfa). The influence of different foliage sampling strategies (i.e. leaf age and canopy position) on the estimation of nitrogen fixation by the method was also evaluated.

Leucaena was estimated to obtain approximately 80% of its nitrogen from fixation (pNdfa) at 12 and 18 months, and approximately 70% at 24 months. No consistent differences in pNdfa were noted for *Leucaena* among monoculture and mixed-species treatments. Although differences in isotopic composition were observed among leaf samples from different canopy positions and between older and younger leaves, similar estimates of pNdfa were obtained provided the same canopy positions and leaf ages were sampled for both the nitrogen-fixing and reference species. We estimate that nitrogen fixation (tNdfa) in these two-year-old plantations was 5.1 to 7.1 g N m^{-2} yr^{-1} for *Leucaena* in mixed-species plots and 10.3 g N m^{-2} yr^{-1} in the *Leucaena* monocultures.

Introduction

Nitrogen-fixing trees provide a major source of nitrogen to forested ecosystems and could be a source of nitrogen for non-fixing trees even in short-rotation plantation forests (Hansen & Dawson, 1982; Hu, Juang & Young, 195; Binkley et al., 1992). If nitrogen-fixing trees are to be utilised in the nutrient management of a forest plantation, a realistic estimate of nitrogen fixation must be determined under field conditions. Methods for estimating nitrogen fixation by trees are limited and all have potentially significant sources of error (Baker, 1990; Danso, Bowen & Sanginga, 1992).

The ^{15}N-isotopic dilution technique for estimating nitrogen fixation is the method most likely to be useful for large tree species under field conditions. In this technique, a small amount of ^{15}N-enriched fertiliser is applied to the soil. Nitrogen-fixing trees will assimilate non-labelled atmospheric N supplied by nitrogen-fixing organisms. Since non-fixing trees do not have this supply, the resulting $^{15}N/^{14}N$ ratio of tissue N in the two species will differ in proportion to the amount of nitrogen fixed. This technique has been applied to young trees in very controlled situations (Cornet et al., 1985; Gauthier et al., 1985; Van Kessel & Nakao, 1986) but has not been directly utilized in large plantations.

The infrequency in use of the ^{15}N-enrichment methodology can be attributed to several reasons. Firstly, it is generally regarded to be overly expensive since the cost of ^{15}N-fertilizer is high and the area to be covered for tree species is larger than for studies of agricultural crops. Second and probably more importantly, it is not commonly used because certain methodological issues relating to the large internal N pools of trees (Danso, Bowen & Sanginga, 1992), the location of the non-fixing reference species in relation to the fixing species, and the application of the ^{15}N-fertiliser (Witty, 1983; Chalk, 1985; Danso, Bowen & Sanginga, 1992) complicate the interpretation of N-isotopic data and estimation of nitrogen fixation rates. In this study we undertook an evaluation of the methodological problems associated with biomass sampling and the choice and position of the reference species.

Materials and Methods

A. Site description and experimental design.

The plantation used in this study was established in 1989 at the Toa Baja experimental farm of the University of Puerto Rico, located on the north coast of the island (18°27'N, 66°10'W). The site receives a mean annual precipitation of 1600 mm and mean daily temperatures range from 23.8 to 29.4°C. A full description of the site, the plantation and the experimental design is provided in Parrotta et al. (1993). Briefly, combinations of *Leucaena leucocephala*, *Eucalyptus robusta*, and *Casuarina equisetifolia*, as well as monocultures of the tree species, were planted in 16 × 16 m plots at a spacing of 1 × 1 m. Within each main plot, a trenched mini-plot of 3 × 3 m for monoculture treatments or 4 × 4 m for mixed-species treatments was located at the centre. The purpose of the trenching was to prevent lateral movement of water and enriched fertiliser and to restrict the growth of roots into or out of the mini-plot. Seedlings were 30–60 cm in height at transplanting. ^{15}N-enriched fertiliser was applied within trenched mini-plots at 6, 12, 18 and 24 months after plantation.

B. Biomass sampling and N-isotopic analysis.

Foliage samples were collected at four time intervals following each ^{15}N-fertiliser application: 0.5, 1.0, 2.5 and 6.0 months. A composite sample for each mini-plot consisted of foliage collected from all trees, at randomly selected canopy positions for each tree. Composite samples were similar across all sampling dates in regard to dry weight (mean values: *Leucaena*, 60 g; *Eucalyptus*, 70 g; *Casuarina*, 90 g). Supplementary collections were made on selected dates to evaluate the influence of leaf age or of position within the canopy (i.e. height strata) on foliar N-isotopic ratios and thus pNdfa.

Destructive sampling of trees in unenriched areas of the plantation plots was undertaken at 18 months to assess biomass distribution in above- and below-ground components and to calculate whole-tree nutrient contents. Eighteen trees were harvested for each species and were representative of all tree sizes in the plantation. Litterfall biomass (using four 50 × 50 cm litter baskets per plot) was measured at bi-weekly intervals for all treatments starting at 18 months. Litterfall collected in each plot, sorted by species, was summed over the time interval between ^{15}N-fertiliser applications for analysis of total N. The data obtained from these measurements were used to develop dimension analysis regressions for estimation of total biomass and nitrogen accumulation.

Samples were prepared for N-isotopic analysis by drying at 70°C and ground to fine powder in a Tecator sample mill. Samples were analyzed for $^{15}N/^{14}N$ ratio and percent N on an ANCA-MS at the Waikato Stable Isotope Unit, Hamilton, New Zealand.

C. Estimation of pNdfa and tNdfa.

Proportion of nitrogen derived from atmosphere (pNdfa) by *Leucaena* was calculated from the $^{15}N/^{14}N$ ratios of foliage samples (FAE) using the formula of Fried & Middelboe (1977):

$$[1]\ \text{pNdfa} = [\,1 - \text{FAE}^{fix}/\text{FAE}^{ref}\,] \times 100$$

Eucalyptus robusta was used throughout as reference species. Samples were taken from plants grown in monoculture, with *Leucaena* or with *Casuarina* for the comparisons *Leucaena* monoculture, *Leucaena* and *Eucalyptus* and *Leucaena* and *Casuarina* respectively (see also Table 1).

Total nitrogen derived from fixation (tNdfa) was calculated as the product of the mean cumulative pNdfa and total biomass N (g m^{-2}) at 24 months.

Results and Discussion

A. Influence of canopy position and foliar age on isotopic composition and pNdfa estimates.

While significant differences in foliar isotopic composition (FAE) were observed for both *Leucaena* and *Eucalyptus* among samples collected from different canopy positions, estimated values for pNdfa using data from foliar samples collected from upper, middle and lower canopy positions were quite similar for all treatments (Table 1). These values did not differ significantly from estimates obtained by random sampling from all canopy positions.

TABLE 1. Foliar %^{15}N atom excess in *Leucaena leucocephala* and *Eucalyptus robusta*, and estimated pNdfa based on stratified and random foliar biomass sampling in 1-year-old plantations, 6 months after first ^{15}N-enriched fertilizer application.

Standard errors of means in parentheses (n=3: each replicate composed of samples from 8 trees within ^{15}N-labelled mini-plots).

Treatment*	Reference+	Canopy position	Foliar %^{15}N atom excess Leucaena	Eucalyptus	Estimated pNdfa
LM	EM	upper	0.037 (0.007)	0.175 (0.029)	78.9
		middle	0.066 (0.010)	0.265 (0.025)	75.1
		lower	0.066 (0.014)	0.316 (0.007)	79.1
		random	0.042 (0.005)	0.238 (0.110)	82.4
LE	EL	upper	0.036 (0.014)	0.171 (0.012)	78.9
		middle	0.061 (0.022)	0.238 (0.041)	74.4
		lower	0.072 (0.029)	0.237 (0.014)	69.6
		random	0.043 (0.015)	0.225 (0.033)	80.9
LC	EC	upper	0.044 (0.003)	0.217 (0.050)	79.7
		middle	0.048 (0.013)	0.325 (0.082)	85.2
		lower	0.080 (0.006)	0.311 (0.015)	74.3
		random	0.057 (0.008)	0.212 (0.023)	73.1

*Treatment: LM = *Leucaena* monoculture; LE = *Leucaena* (with *Eucalyptus*); LC = *Leucaena* (with *Casuarina*)

+Reference: EM = *Eucalyptus* monoculture; EL = *Eucalyptus* (with *Leucaena*); EC = *Eucalyptus* (with *Casuarina*)

Leaf age appears to be less important as a factor influencing FAE. Differences between younger and older leaves were not significant regardless of canopy position for either *Leucaena* or *Eucalyptus* (Table 2). These results suggest that consistent results can be obtained either through random sampling of leaves from many canopy positions or by sampling for a limited range of canopy positions, provided that both the *Leucaena* and *Eucalyptus* foliage is sampled using the same protocols.

B. Temporal trends in FAE and influence on estimated pNdfa.

FAE values followed broadly similar temporal patterns between ^{15}N-enriched fertiliser applications for both *Leucaena* and *Eucalyptus* in all treatments (Fig. 1). These data indicate that both *Leucaena* and *Eucalyptus* at this site utilized soil nitrogen and incorporated it in foliar biomass within a short time after its application to the soil.

The pNdfa values calculated from data presented in Fig. 1 were very similar among the *Leucaena* treatments for most sampling dates (Fig. 2). The similarity which we observed among treatments, especially between 6 and 21 months, would suggest that over such periods the reference species and nitrogen-fixing species can be grown in separate plots (monocultures) and be acceptable for pNdfa estimation from a methodological viewpoint. However, by 24 months a deviation may be taking place that may reflect real differences among the monoculture and mixed-species treatments.

The similarity between 6 and 21 months was possible because the mini-plots were trenched to prevent movement of labelled fertiliser and the growth of roots into and out of the plots. However, the use of untrenched labelled plots lead to a very different conclusion (Parrotta, Baker & Fried, 1993). The apparent divergence among treatments in estimated pNdfa after 24 months may reflect actual differences in soil nitrogen levels among treatments and/or differences in nitrogen fixation. Therefore, we feel that some caution should be exercised in designing experiments for this purpose.

It is important to note that estimated pNdfa (a cumulative estimate) ranged from approximately 35% to 80% during the 6-month periods following each application of ^{15}N-enriched fertiliser. The lower values are typically associated with foliage sampling dates immediately following ^{15}N enrichment, while the higher values are associated with foliage sampled toward the end of each 6-month period.

TABLE 2. Foliar %^{15}N atom excess and estimated pNdfa in *Leucaena leucocephala* and *Eucalyptus robusta* as influenced by canopy position and leaf age: LE treatment.

Standard errors of means in parentheses (n=3: each replicate composed of samples from 8 trees within trenched mini-plots collected 12 months after first application of ^{15}N-enriched fertiliser).

Canopy position	Relative leaf age	Foliar ^{15}N atom excess Leucaena	Eucalyptus	Estimated pNdfa
Upper	young	0.080 (0.018)	0.328 (0.084)	75.6
	old	0.089 (0.019)	0.365 (0.059)	75.6
Middle	young	0.087 (0.026)	0.466 (0.019)	81.3
	old	0.098 (0.021)	0.518 (0.140)	81.1
Lower	young	0.103 (0.024)	0.466 (0.123)	77.9
	old	0.109 (0.031)	0.393 (0.117)	72.3
Random sampling		0.075 (0.022)	0.435 (0.064)	82.8

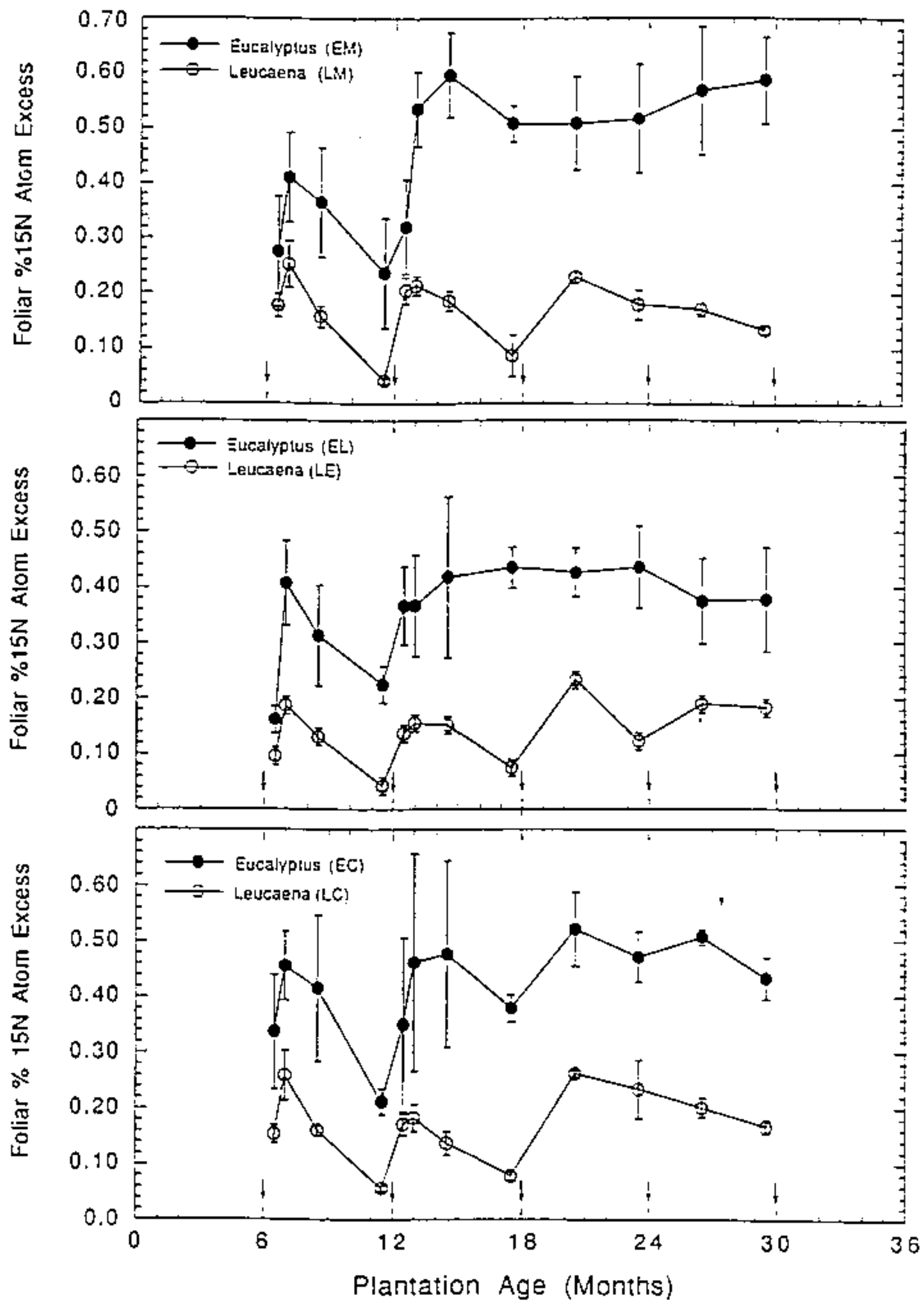

FIG. 1. Foliar % ^{15}N atom excess in *Leucaena leucocephala* and *Eucalyptus robusta* (control species) following ^{15}N enrichment of soils in trenched plantation quadrats between 6 and 30 months after planting. Bars indicate ± SE. Arrows indicate when ^{15}N applied.

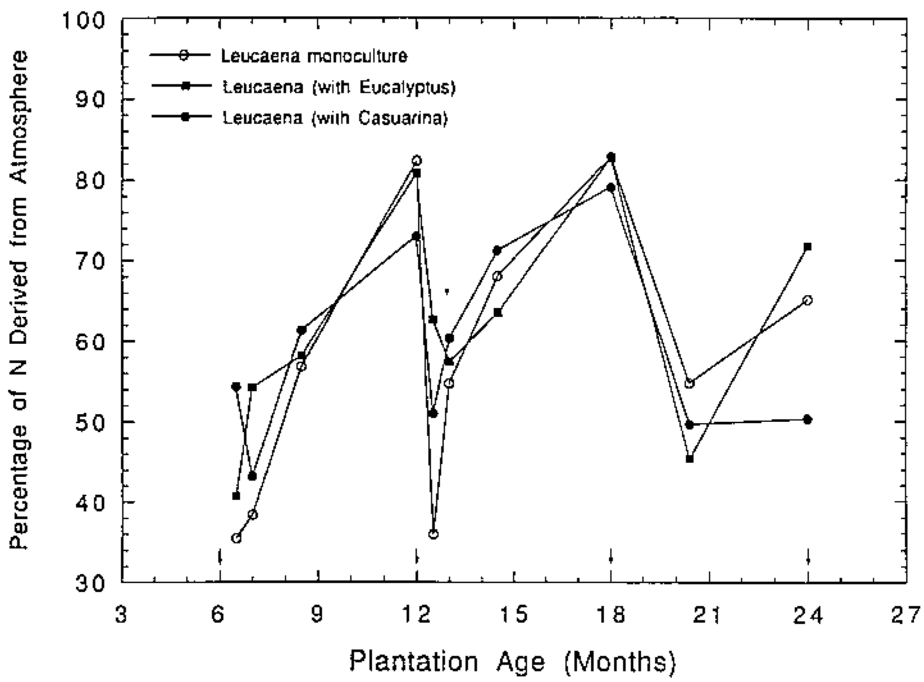

FIG. 2. Estimated pNdfa by *Leucaena leucocephala* in 6–30 month old monoculture and mixed-species plantations. Based on foliar %^{15}N atom excess data, using *Eucalyptus robusta* (in monoculture and mixed-species plantations) as the reference species. Arrows indicate when ^{15}N applied.

Although it is reasonable to suggest that nitrogen fixation in *Leucaena* may fluctuate seasonally, it is extremely unlikely that the cumulative pNdfa estimates presented in Fig. 2 are a reflection of such seasonality. Rather, the almost certain explanation for this change is the rapid uptake into the leaves of the applied ^{15}N by both the fixing and non-fixing species. The proportion of the N in the leaves that comes from nitrogen fixation is thus temporarily lowered drastically in the leaves of the nitrogen-fixing species. However, as nitrogen fixation takes place and this nitrogen reaches the leaves, appreciable dilution of the ^{15}N in the leaves of the nitrogen-fixing species occurs and the actual and calculated values of pNdfa continues to increase until another dose of ^{15}N-labelled fertiliser is added. It is clear that insofar as the whole tree is concerned, the values obtained at the end of the sampling periods just before additional ^{15}N labelled fertiliser is added represents the best estimate of the pNdfa.

Leucaena at 12 and 18 months is obtaining approximately 80% of its nitrogen from fixation. The pNdfa is closer to 70% at 24 months and may be as low as 50% in the mixture with *Casuarina*. This latter values should be viewed with caution. As mentioned earlier, the results of the 24-month sampling date may be confounded by differences in soil nitrogen that may occur when the non-fixing reference species is grown in different mini-plots from the nitrogen-fixing species. In this experiment, only one treatment of the three had identical plots for the nitrogen-fixing and reference species, namely LE/EL. This treatment at 24 months had a pNdfa of 71.8% (Table 3).

C. Preliminary estimates of tNdfa.

Using FAE values for samples collected at 12, 18 and 24 months and estimates of whole-tree nitrogen stores in the ^{15}N-enriched mini-plots, estimates of tNdfa were

TABLE 3. Foliar ^{15}N atom excess, total N pools, and estimated pNdfa and tNdfa in trenched quadrats for treatments LM, LE and LC at 12, 18 and 24 months after plantation establishment.

		Foliar $\%^{15}N$ atom excess			– Total N ($g\ m^{-2}$) –		
Species	Treatment	t12	t18	t24	t12	t18	t24
Leucaena	LM	0.042	0.087	0.180	15.60	20.50	31.60
	LE	0.043	0.075	0.123	12.00	15.30	19.70
	LC	0.057	0.079	0.234	8.25	15.55	20.25
Eucalyptus	EM	0.238	0.509	0.517	7.85	13.10	13.40
	EL	0.225	0.435	0.436	6.60	11.20	13.25
	EC	0.212	0.379	0.472	4.67	7.50	8.22

Treatment		– – pNdfa – –		
FIX	REF	t12	t18	t24
LM	EM	82.4	82.9	65.2
LE	EL	80.9	82.8	71.8
LC	EC	73.1	79.2	50.4

calculated. At 24 months, approximately 5.1, 7.1 and 10.3 g N $m^{-2}\ yr^{-1}$ were derived from nitrogen fixation for *Leucaena* in mixture with *Casuarina*, in mixture with *Eucalyptus*, and in monoculture, respectively. Due to the sampling issue discussed above, the estimates should be regarded as tentative and subject to verification by analysis of whole tree (weighted average) $\%^{15}N$ atom excess values.

Estimated values of tNdfa for *Leucaena* were consistently higher in the LM treatment than in either th LC and LE treatments. This is not surprising given that the density of *Leucaena* stems in the monoculture treatment was twice that in the mixed-species treatments. There was an apparent trend toward higher tNdfa in the LE than the LC treatment which could be attributed to greater competition for soil N by the associated species in the former treatment. Between 12 and 24 months, the *Leucaena* in the LE treatment declined both in total N accumulation and in the pNdfa as compared to the initial 12-month period. Despite our concerns about methodological issues, the values of pNdfa for *Leucaena* which we estimated lie within the range reported for *Leucaena leucocephala* in studies involving other techniques for estimation (Högberg & Kvarnstrom, 1982; Sanginga, Mulongoy & Ayanaba, 1985; Zaharah, Shariffuddin & Subramanian, 1986).

Acknowledgements

We wish to thank the many individual who provided assistance in the field and laboratory, especially Luis Martinez Torres and Carmelo Gonzalez. We also thank the NifTAL Project, University Hawaii, for providing *Rhizobium* inoculum. This work was conducted in cooperation with the University of Puerto Rico and supported by a grant from the United States Department of Agriculture Competitive Grant Office (Grant No. 90-37120-5603). Additional support was provided through the University of Puerto Rico Agricultural Experiment Station by the USDA under the Cooperative Forestry Research (McIntire-Stennis) Act (Project No. PR-MS-0008).

References

Baker, D.D. (1990). Optimizing actinorhizal nitrogen fixation and assessing actual contribution under field conditions. In: D. Werner & P. Mueller (editors). Fast growing trees and nitrogen fixing trees, pp. 291–299. Fisher-Verlag, Stuttgart, Germany.

Binkley, D., Dunkin, K.A., DeBell, D. and Ryan, M.G. (1992). Production and nutrient cycling in mixed plantations of *Eucalyptus* and *Albizia* in Hawaii. *Forest Sci.* **38**: 393–408.

Chalk, P.M. (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.

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

Danso, S.K.A., Bowen, G.D. and Sanginga, N. (1992). Biological nitrogen fixation in trees in agro-ecosystems. *Pl. & Soil* **141**: 177–196.

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

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 Biol. Biochem.* **17**: 375–379.

Hanson, E.A. and Dawson, J.O. (1982). Effect of *Alnus glutinosa* on hybrid *Populus* height growth in a short-rotation intensively cultured plantation. *Forest Sci.* **28**: 49–59.

Högberg, P. and Kvarnstrom, M. (1982). Nitrogen fixation by the woody legume *Leucaena leucocephala* in Tanzania. *Pl. & Soil* **66**: 21–28.

Hu, T.W., Juang, T.C. and Young, C.C. (1985). The role of nitrogen-fixing trees in soil biology. In: J. Burley & J. Stewart (editors). Increasing productivity of multipurpose species, pp. 307–316. IUFOR, Vienna.

Parrotta, J.A., Baker, D.D. and Fried, M. (1993). Application of ^{N}N enrichment methodologies to estimate nitrogen fixation in *Casuarina equisetifolia*. *Canad. J. Forest Res.* (in press).

Sanginga, N., Mulongoy, K. and Ayanaba, A. (1985). Effect of inoculation and mineral nutrients on nodulation and growth of *Leucaena leucocephala*. In: H. Ssali & S.O. Keya (editors). Biological nitrogen fixation in Africa, pp. 419–436. MIRCEN, Nairobi.

Van Kessell, C. and Nakao, P. (1986). The use of nitrogen-15 depleted ammonium sulfate for estimating nitrogen fixation by leguminous trees. *Agron. J.* **78**: 549–551.

Witty, J.F. (1983). Estimating N_2-fixation in the field using ^{15}N-labelled fertilizers: some problems and solutions. *Soil Biol. Biochem.* **15**: 631–639.

Zaharah, A.R., Shariffuddin, H.A.H. and Subramanian, R. (1986). Nitrogen fixation by *Leucaena leucocephala* as measured by the N-15 dilution technique. *Pertanika* **91**: 17–22.