Changes in dinitrogen fixation in maturing stands of *Casuarina equisetifolia* and *Leucaena leucocephala*

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Abstract: Biological dinitrogen fixation in *Casuarina equisetifolia* J.R. & G. Forst. and *Leucaena leucocephala* (Lam.) de Wit was evaluated using the ^{15}N-enrichment technique under field conditions in single-species and mixed-species plantings (with a nonfixing reference species, *Eucalyptus* ×*robusta* J.E. Smith) between 1.0 and 3.5 years of age in Puerto Rico. Following periodic labelling of trenched and untrenched plantation quadrats with ^{15}N-enriched ammonium sulfate, analyses of foliar and whole-tree (weighted average) N-isotopic ratios and total biomass N were used to estimate the proportion of nitrogen derived from biological dinitrogen fixation (PNDFA) and total nitrogen derived from fixation (TNDFA) in *C. equisetifolia* and *L. leucocephala*. The ^{15}N-enrichment technique yielded accurate estimates of dinitrogen fixation in maturing stands of these two tree species provided the reference species (*Eucalyptus*) was grown in close proximity to the N-fixing species in trenched, mixed-species plots. Changes in the $^{15}N/^{14}N$ ratio of soil-available nitrogen in single-species plots of the N-fixing and reference were found to yield inaccurate estimates of dinitrogen fixation in the single-species plots of *C. equisetifolia* and *L. leucocephala* after 2 years of age. The results confirm earlier findings that foliar sampling is a useful nondestructive alternative to whole-tree biomass sampling for the ^{15}N-enrichment protocol. Between 1.0 and 3.5 years after plantation establishment, PNDFA in *C. equisetifolia* remained relatively constant between 50 and 60%, while PNDFA in *L. leucocephala* declined from nearly 100% at 1 year to less than 40% at 3.5 years. The rate of dinitrogen fixation ($kg \cdot ha^{-1} \cdot year^{-1}$) did not decline as the stands matured. Cumulative dinitrogen fixation (TNDFA) estimates at 3.5 years were very similar between species: 73 in *C. equisetifolia* and 74 kg $N \cdot ha^{-1} \cdot year^{-1}$ in *L. leucocephala*.

Résumé : La fixation biologique de l'azote (N_2) par *Casuarina equisetifolia* J.R. & G. Forst. et par *Leucaena leucocephala* (Lam.) de Wit a été évaluée avec la méthode d'enrichissement en ^{15}N au champ dans des plantations pures et mixtes (avec une espèce non fixatrice comme référence, *Eucalyptus* ×*robusta* J.E. Smith) entre l'âge de 1,0 et 3,5 années à Puerto Rico. Suite à un marquage périodique dans des quadrats isolés ou non isolés avec du sulfate d'ammonium enrichi en ^{15}N, les analyses des rapports isotopiques de N du feuillage et de l'arbre au complet (moyenne pondérée) et la biomasse totale de N ont été utilisées pour estimer la proportion de l'azote dérivée de la fixation (PNDFA) et l'azote total dérivé de la fixation (TNDFA) chez *C. equisetifolia* et *L. leucocephala*. La technique d'enrichissement en ^{15}N a donné des estimations précises de la fixation de N_2 dans les peuplements vieillissants de ces deux espèces d'arbres pour autant que l'espèce de référence (*E.* ×*robusta*) croisse à proximité des espèces fixatrices de N_2 dans des parcelles isolées situées dans des plantations mixtes. Les changements dans le rapport $^{15}N/^{14}N$ de l'azote disponible du sol dans des parcelles monospécifiques des espèces fixatrices de N_2 et de l'espèce de référence ont présenté des estimations non précises de la fixation de N_2 dans les parcelles monospécifiques de *C. equisetifolia* et de *L. leucocephala* après l'âge de 2 ans. Ces résultats confirment les observations antérieures montrant que l'échantillonnage foliaire est une alternative intéressante et non destructrice à l'échantillonnage de la biomasse de l'arbre entier dans le protocole d'enrichissement en ^{15}N. De 1,0 à 3,5 ans après l'établissement de la plantation, PNDFA est resté relativement constant, entre 50 et 60%, chez *C. equisetifolia* tandis que PNDFA a diminué de près de 100% à 1 an, à moins de 40% à 3,5 ans chez *L. leucocephala*. Le taux de fixation de N_2 ($kg \cdot ha^{-1} \cdot an^{-1}$) n'a pas diminué avec le vieillissement des peuplements. La fixation cumulative de N_2 (TNDFA) estimée à 3,5 ans était similaire pour les deux espèces : 73 kg $N \cdot ha^{-1} \cdot an^{-1}$ pour *C. equisetifolia* et 74 kg $N \cdot ha^{-1} \cdot an^{-1}$ pour *L. leucocephala*. [Traduit par la Rédaction]

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Introduction

The utilization of nitrogen-fixing trees in forestry and agroforestry applications has been advocated widely (see Werner and Muller 1990) because of the potential for improved soil fertility arising from biological dinitrogen fixation. The actual contribution of nitrogen-fixing species has not been fully documented (Kang and Mulongoy 1992), particularly for more mature stands. This lack of information about actual contribution is the result of methodological problems of estimating dinitrogen fixation (Baker 1990; Danso et al. 1992). Baker et al. (1992) and Parrotta et al. (1994*a*, 1994*b*) demonstrated that dinitrogen fixation may be estimated for trees under field conditions using the ^{15}N-enrichment methodology, if certain precautions are taken to accomodate the special problems of large perennial species. Trenching of labelled plots was shown to be important if species were grown in monoculture (Parrotta et al. 1994*a*). Nondestructive sampling strategies were as accurate in estimating dinitrogen fixation as more destructive strategies (Baker et al. 1992; Parrotta et al. 1994*a*). Because these studies were limited to young stands of trees (<2 years in age), we undertook further studies to investigate the patterns of dinitrogen fixation as trees matured, and to test whether the ^{15}N-enrichment protocols were still valid for older, larger trees. In particular, we were concerned that as nitrogen began to be recycled within a forest plantation, it would influence dinitrogen fixation or our ability to estimate it accurately.

Materials and methods

Experimental plantation design

The study area is located at the University of Puerto Rico's Toa Baja experimental farm, on the northern coast of Puerto Rico (18°27′N, 66°10′W). The site receives an average annual rainfall of 1600 mm that is moderately seasonal in distribution. Average daily temperatures range from 23.8°C in January to 29.4°C in August. The soils are well-drained, alkaline sands (pH 7.9–8.4) of marine origin. Experimental plantations were established in September 1989 using a randomized complete block design that included three replicate 16 × 16 m plots of each of: (*i*) *Casuarina equisetifolia* J.R. & G. Forst. monoculture (CM); (*ii*) *Eucalyptus* ×*robusta* J.E. Smith monoculture (EM); (*iii*) *Leucaena leucocephala* (Lam.) de Wit monoculture (LM); (*iv*) *C. equisetifolia* (with *E.* ×*robusta*) (CE) (1:1); (*v*) *E.* ×*robusta* (with *L. leucocephala*) (EL) (1:1); and (*vi*) *C. equisetifolia* (with *L. leucocephala*) (CL) (1:1). Initial tree spacing within plantations was 1 × 1 m. Prior to plantation establishment, trenched quadrats measuring 3 × 3 m (in monoculture treatments) and 4 × 4 m (in mixed-species treatments), and untrenched quadrats measuring 4 × 4 m (all treatments) were established for subsequent ^{15}N labelling. At 42 months average tree heights ranged from 7.4 to 10.2 m and stem basal diameters from 5.5 to 9.6 cm. Further details on plantation site conditions, experimental design, and establishment are given in Parrotta et al. (1994*a*, 1994*b*).

^{15}N labelling and biomass sampling

^{15}N-enriched ammonium sulfate was applied as an aqueous solution to each trenched and untrenched quadrat at an annual rate of 0.2 g ^{15}N·m^{-2} in a total application of 2.0 g N·m^{-2}·year^{-1}. To the remaining plot area, unenriched ammonium sulfate was applied at the rate of 2.0 g N·m^{-2}·year^{-1}. Fertilizer applications, both ^{15}N enriched and unenriched, were made at 6, 12, 18, 24, 28, and 32 months after plantation establishment. On selected dates following each ^{15}N-fertilizer application, foliar samples were collected from randomly selected canopy positions for all trees within each of the trenched and untrenched ^{15}N-labelled quadrats. Foliage samples were composited by species for each quadrat for each sampling date, dried at 70°C, ground to a fine powder, and analyzed for ^{15}N at.% excess (FAE) and percent N at the Waikato Stable Isotope Unit in Hamilton, New Zealand, using an ANCA-mass spectrometer.

To determine whether or not foliage sampling provides an accurate measure of whole-tree N-isotopic composition, whole-tree harvests (including roots) of 10–15 individuals of each species from both trenched and untrenched ^{15}N-labelled quadrats (all treatments) were carried out at 18 and 42 months after establishment. The harvested trees were representative of the full range of tree sizes present in the plantation at time of harvest. Values for whole-tree ^{15}N at.% excess (WAE) were calculated for each harvested tree as the weighted average of ^{15}N at.% excess values for leaf, branch, stem, and root biomass components as described in Parrotta et al. (1994*a*).

Estimation of total nitrogen pools and litter-fall nitrogen fluxes

Total nitrogen pools (WTN: whole-tree N) were estimated on a unit-area basis at 12, 24, 30, 34, 38, and 42 months for each species in each of the ^{15}N-labelled quadrats. Using regressions developed from whole-tree harvests (see Table 1), WTN values were calculated for each species as the sum of the total nitrogen content of each of the trees in these quadrats from which foliage samples were collected during the study. Estimates of forest floor litter (O-horizon) dry mass and nitrogen content at 36 months were based on collection of all leaf, wood, and bark litter retained by a 1.0-mm sieve from four 0.25-m^2 quadrats randomly located within each plot followed by drying at 65°C to constant weight and analysis for total (Kjeldahl) N.

Litter production and associated nitrogen fluxes to the forest floor were measured in each plantation plot between 18 and 42 months. At biweekly intervals during this period, litter fall was collected from four 0.25-m^2 litter traps (baskets constructed of fine-mesh plastic screen) located in each plot, including one trap in each of the central (trenched) ^{15}N-labelled quadrats. Leaf litter and woody debris collected from each basket was separated by species, and dried at 70°C. Composite samples for each plot and species, consisting of litter collected over 4- to 6-month periods from litter baskets located in the ^{15}N-labelled quadrats, were analyzed for percent N and ^{15}N at.% excess (LAE) as described above for canopy foliage samples. These data were used to estimate the total N content of litter fall and its N-isotopic composition for both the nitrogen-fixing and reference species in each of the experimental treatments.

Table 1. ^{15}N at.% excess based on foliar (FAE) and whole-tree (WAE) sampling and total biomass nitrogen in *C. equisetifolia*, *L. leucocephala*, and *E. ×robusta* grown in trenched quadrats at 3.5 years after plantation.

	^{15}N at.% excess		Total N content ($g \cdot m^{-2}$)		
Treatment[a]	FAE	WAE	Biomass[b]	Litter fall[c]	Total[d]
Casuarina equisetifolia					
CM	0.255	0.230	57.1	16.7	73.8
	(0.038)	(0.041)	(12.9)	(0.4)	(13.2)
CE	0.224	0.209	29.7	10.7	40.4
	(0.059)	(0.041)	(9.4)	(1.3)	(10.2)
CL	0.222	0.207	31.6	11.3	41.9
	(0.044)	(0.031)	(8.5)	(1.2)	(10.7)
Leucaena leucocephala					
LM	0.207	0.172	66.9	39.8	106.7
	(0.034)	(0.034)	(12.9)	(8.3)	(19.8)
LE	0.262	0.228	44.2	27.4	71.6
	(0.044)	(0.045)	(3.5)	(2.2)	(5.6)
LC	0.283	0.250	36.6	21.1	57.7
	(0.037)	(0.037)	(7.8)	(1.2)	(8.7)
Eucalyptus ×robusta					
EM	0.682	0.607	14.9	7.0	21.9
	(0.140)	(0.119)	(2.9)	(0.3)	(2.8)
EC	0.569	0.510	9.0	3.3	12.3
	(0.020)	(0.017)	(1.2)	(0.8)	(1.7)
EL	0.401	0.366	12.9	5.0	18.0
	(0.048)	(0.041)	(2.3)	(1.4)	(2.3)

Note: Values are means with standard errors given in parentheses.

[a]CM, *C. equisetifolia* monoculture; CE, *C. equisetifolia* (with *E. ×robusta*); CL, *C. equisetifolia* (with *L. leucocephala*); LM, *L. leucocephala* monoculture; LE, *L. leucocephala* (with *E. ×robusta*); LC, *L. leucocephala* (with *C. equisetifolia*); EM, *E. ×robusta* monoculture; EC, *E. ×robusta* (with *C. equisetifolia*); EL, *E. ×robusta* (with *L. leucocephala*).

[b]Aboveground + root biomass, based on regressions developed from whole-tree harvest data. For *E. ×robusta*: $WTN = 0.455D^{1.956}$, $r^2 = 0.82$; for *C. equisetifolia*: $WTN = 1.290D^{1.858}$, $r^2 = 0.98$; for *L. leucocephala*: $WTN = 0.605D^{2.498}$, $r^2 = 0.86$; where WTN is whole-tree N content (g/tree) in above- and below-ground biomass, and D is stem basal diameter.

[c]Based on litter-fall biomass and total litter-fall N concentrations, measured between 1.5 and 3.5 years.

[d]Conservative estimates, not including litter-fall N prior to 1.5 years, N fluxes associated with root turnover, or canopy leaching losses.

Calculation of PNDFA and TNDFA

Proportion of nitrogen derived from fixation (PNDFA) by *C. equisetifolia* and *L. leucocephala* in each of the ^{15}N-labelled quadrats was calculated at 12, 24, 30, 34, 38, and 42 months from foliar and whole-tree ^{15}N at.% excess data for the nitrogen-fixing and reference (*E. ×robusta*) species using the formula of Fried and Middelboe (1977):

$$[1] \quad PNDFA = \left(1 - \frac{FAE^{fix}}{FAE^{ref}}\right) \times 100$$

for whole-tree ^{15}N at.% excess data:

$$[2] \quad PNDFA = \left(1 - \frac{WAE^{fix}}{WAE^{ref}}\right) \times 100$$

Total nitrogen derived from fixation (TNDFA) for the same time points was calculated as the product of PNDFA and the total nitrogen contained in biomass and cumulative litter fall ($g \cdot m^{-2}$) at each date:

$$[3] \quad TNDFA = PNDFA \times (WTN^{fix})$$

For comparison, TNDFA was also calculated using the difference method, which compares the total nitrogen yield (N contained in biomass and litter fall) of the N-fixing and reference species:

$$[4] \quad TNDFA = WTN^{fix} - WTN^{ref}$$

Results and discussion

Temporal trends in foliar ^{15}N enrichment

Following an initial 18-month period during which foliar ^{15}N at.% excess values for all species showed considerable fluctuation (discussed in Parrotta et al. 1994*a*, 1994*b*), FAE values in both *C. equisetifolia* and *L. leucocephala*

remained fairly constant and were similar among single- and mixed-species treatments (Fig. 1). In the case of the reference species (*E.* ×*robusta*), foliar ^{15}N enrichment diverged markedly among treatments. Relative to the *E.* ×*robusta* monoculture (EM) treatment, in which FAE values gradually increased over time, FAE values in the mixed-species treatments (EC and especially EL) were markedly lower from approximately 24 months onward.

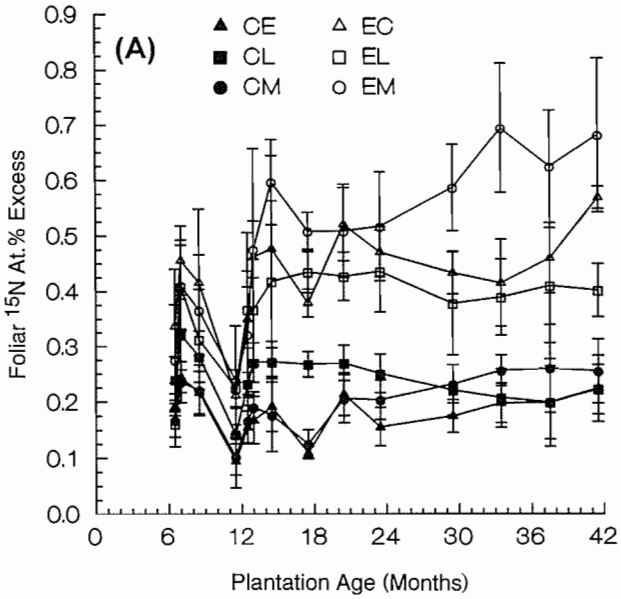
These data suggest that in these mixed-species stands, the N-fixing species influenced the N-isotopic ratios of the plant-available (soil) nitrogen; *Eucalyptus* FAE values in mixtures showed the incorporation of fixed nitrogen derived from decomposition of *C. equisetifolia* and *L. leucocephala* litter. This trend would be more pronounced in the EL than in the EC treatments, probably as a result of higher total N content and more rapid litter decomposition of *L. leucocephala* litter relative to *C. equisetifolia* litter as inferred from indirect measurements (Fig. 2). Higher *E.* ×*robusta* litter-fall N concentrations in the EC and EL treatments relative to the EM treatment (Fig. 3) also demonstrate increases in soil N supply under the influence of the nitrogen-fixing species (particularly *L. leucocephala*).

The divergence in *E.* ×*robusta* FAE values among treatments has important methodological implications. Accurate estimates of biological dinitrogen fixation using the ^{15}N-enrichment technique requires a reference species that obtains soil N from the same pools as the N-fixing species (Danso et al. 1992; Baker et al. 1995). We found that after 24 months, the $^{15}N/^{14}N$ ratio of the soil N pools among *E.* ×*robusta* treatments, as inferred from *E.* ×*robusta* FAE data, diverged as a result of recycling of N fixed by *L. leucocephala* and *C. equisetifolia*. Therefore the *E.* ×*robusta* monoculture treatment would not appear to be a valid reference for estimation of PNDFA in either the *C. equisetifolia* or *L. leucocephala* monoculture treatments after the first 2 years. In older forest stands, reliable estimates of dinitrogen fixation using ^{15}N-enrichment techniques require that the N-fixing and reference species be grown together in mixed-species plots to account for recycling of fixed N. Consequently, accurate estimates of dinitrogen fixation in single-species stands becomes problematic except in very young stands, i.e., before the growth of the N-fixing species begins to significantly influence the isotopic ratio of the soil N pool.

The relative utility of trenched and untrenched ^{15}N-labelled study quadrats

A comparison of FAE data for *C. equisetifolia* and *E.* ×*robusta* in the trenched and untrenched mixed-species quadrats (Fig. 4) indicated that untrenched ^{15}N-labelled quadrats were of limited utility for estimating dinitrogen fixation after 2 years. Beyond this age, FAE values for *E.* ×*robusta* in the untrenched quadrats diverge substantially from those in the trenched quadrats and, by 3.5 years, approach FAE values for *C. equisetifolia*. These data suggest that roots of *E.* ×*robusta* in untrenched quadrats spread laterally from the base of the tree and obtain a larger proportion of their nitrogen from unlabelled soils outside of the ^{15}N-labelled quadrats than do the associated *C. equisetifolia* trees. In theory, untrenched mixed-species quadrats labelled with ^{15}N could be used to estimate dinitrogen fixation,

Fig. 1. Foliar ^{15}N at.% excess in *Casuarina equisetifolia*, *Leucaena leucocephala*, and *Eucalyptus* ×*robusta* in single- and mixed-species trenched quadrats 6–42 months after plantation establishment. ^{15}N-enriched fertilizer was applied to quadrats at 6, 12, 18, 24, 30, 34, and 38 months. (A) *Casuarina equisetifolia* and *E.* ×*robusta* treatments. (B) *Leucaena leucocephala* and *E.* ×*robusta* treatments. Error bars indicate ±SE; $n = 3$. Treatments: CM, *C. equisetifolia* monoculture; CE, *C. equisetifolia* (with *E.* ×*robusta*); CL, *C. equisetifolia* (with *L. leucocephala*); LM, *L. leucocephala* monoculture; LE, *L. leucocephala* (with *E.* ×*robusta*); LC, *L. leucocephala* (with *C. equisetifolia*); EM, *E.* ×*robusta* monoculture; EC, *E.* ×*robusta* (with *C. equisetifolia*); EL, *E.* ×*robusta* (with *L. leucocephala*).



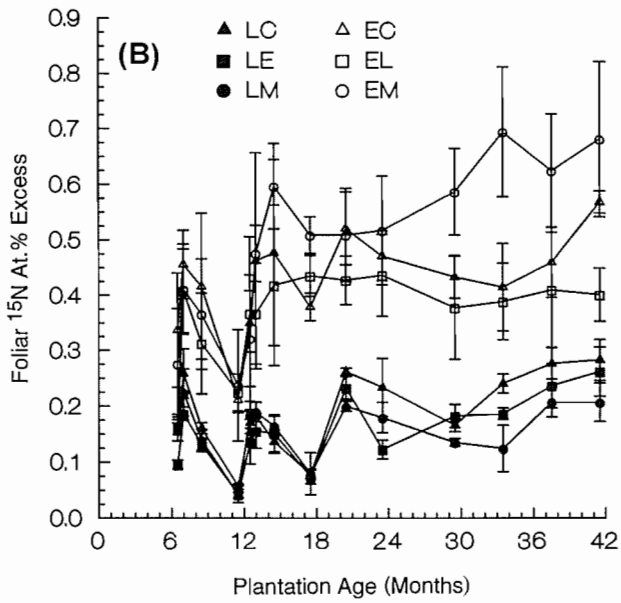


Fig. 2. Mean annual litter-fall nitrogen fluxes and net forest floor (O-horizon) nitrogen accretion, based on litter fall measures between 1.5 and 3.5 years, and nitrogen content of litter standing stocks at 3.0 years, respectively. Error bars indicate ±SE; n = 3. Treatments: EM, *E.* ×*robusta* monoculture; CM, *C. equisetifolia* monoculture; CE, *C. equisetifolia* (with *E.* ×*robusta*); CL, *C. equisetifolia* (with *L. leucocephala*); LM, *L. leucocephala* monoculture; LE, *L. leucocephala* (with *E.* ×*robusta*).

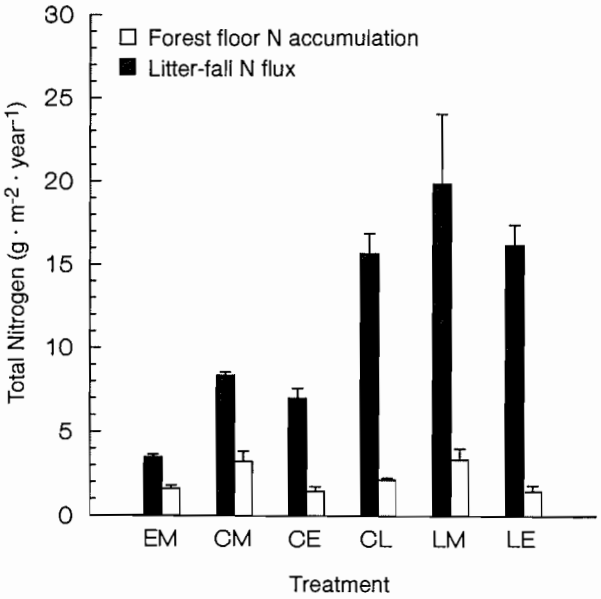
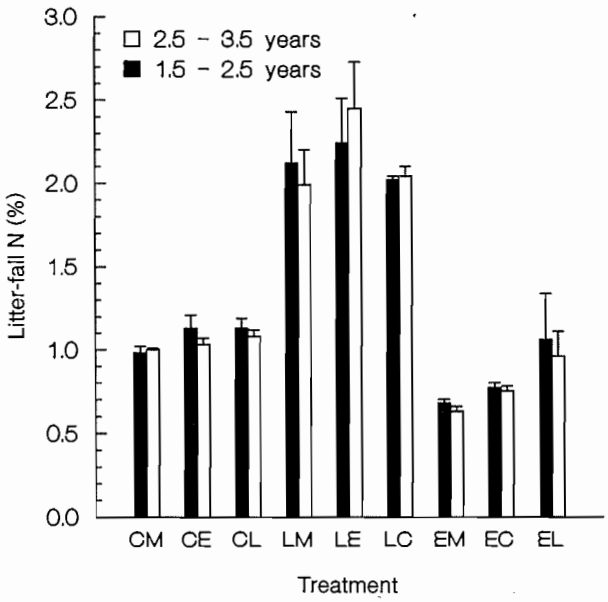


Fig. 3. Weighted average nitrogen concentrations in litter fall collected at biweekly intervals between 1.5 and 3.5 years. Error bars indicate ±SE; n = 3. Treatments: CM, *C. equisetifolia* monoculture; CE, *C. equisetifolia* (with *E.* ×*robusta*); CL, *C. equisetifolia* (with *L. leucocephala*); LM, *L. leucocephala* monoculture; LE, *L. leucocephala* (with *E.* ×*robusta*); LC, *L. leucocephala* (with *C. equisetifolia*); EM, *E.* ×*robusta* monoculture; EC, *E.* ×*robusta* (with *C. equisetifolia*); EL, *E.* ×*robusta* (with *L. leucocephala*).



provided that the ^{15}N-labelled area is at least as extensive as the lateral root spread of the N-fixing and reference trees sampled to obtain FAE values. Except in very young forest stands, this minimum area may be large and the costs of ^{15}N-labelled fertilizer very high. In this study, untrenched plots of 4 × 4 m were too small after 24 months.

Foliar and whole-tree ^{15}N at.% excess values

Do foliage samples adequately represent whole-tree ^{15}N at.% excess? For all species, there was a strong, significant linear relationship between FAE and WAE (Fig. 5). The linear regressions derived from these data for *C. equisetifolia* and *L. leucocephala* were not significantly different from that for *E.* ×*robusta*, and the slopes of the regressions did not differ significantly from 1.0. These results confirm those obtained earlier for *C. equisetifolia* and *E.* ×*robusta* at 18 months (Parrotta et al. 1994*a*), and show that FAE data can be used to estimate PNDFA and TNDFA in *C. equisetifolia* and *L. leucocephala* under the present study conditions.

Effects of stand composition and age on PNDFA

The proportion of nitrogen derived from fixation (PNDFA), estimated using eq. 1, showed different temporal trends between *C. equisetifolia* and *L. leucocephala* (Fig. 6). In *C. equisetifolia* (CE), cumulative PNDFA estimates fluctuated somewhat over time between 43 and 62%, with no consistent trend. These values fall within the broad range of PNDFA estimates reported for *C. equisetifolia* (25–75%) in pot studies using the ^{15}N-enrichment technique (Sougoufara et al. 1990; Sanginga et al. 1990*a*; Mansour and Baker 1994), and in field studies of 3-year-old trees using the natural ^{15}N abundance and total nitrogen difference methods (Mariotti et al. 1992).

For *L. leucocephala* (LE), cumulative PNDFA estimates showed a marked decline from approximately 98% at 1 year to 38% at 3.5 years. The higher PNDFA estimates obtained early in this study fall within the upper range of values reported for *L. leucocephala* up to 12 months old in pot studies (Sanginga et al. 1990*b*; Sanginga 1992; Awonaike et al. 1993; Rao and Giller 1993) and field studies (Sanginga et al. 1989, 1994) using the ^{15}N-enrichment technique. The decline in PNDFA in *L. leucocephala* indicates that over time, relative to *C. equisetifolia*, *L. leucocephala* is relying less on dinitrogen fixation to meet its growth requirements, and more on nitrogen recycled through litter fall and decomposition.

The decline in PNDFA in *L. leucocephala* may be due to the incorporation of comparatively large amounts of fixed N into the soils of these plantations. Cycling of biologically fixed N was demonstrated in young *L. leucocephala* plantations in Hawaii using the natural ^{15}N abundance method (van Kessel et al. 1994). As indicated in

Fig. 4. Foliar ^{15}N at.% excess in *Casuarina equisetifolia* and *Eucalyptus* ×*robusta* in mixed-species trenched (*t*) and untrenched (*u*) quadrats 6–42 months after plantation establishment. ^{15}N-enriched fertilizer was applied to quadrats at 6, 12, 18, 24, 30, 34, and 38 months. Error bars indicate ±SE; $n = 3$. Treatments: CE, *C. equisetifolia* (with *E.* ×*robusta*); EC, *E.* ×*robusta* (with *C. equisetifolia*).

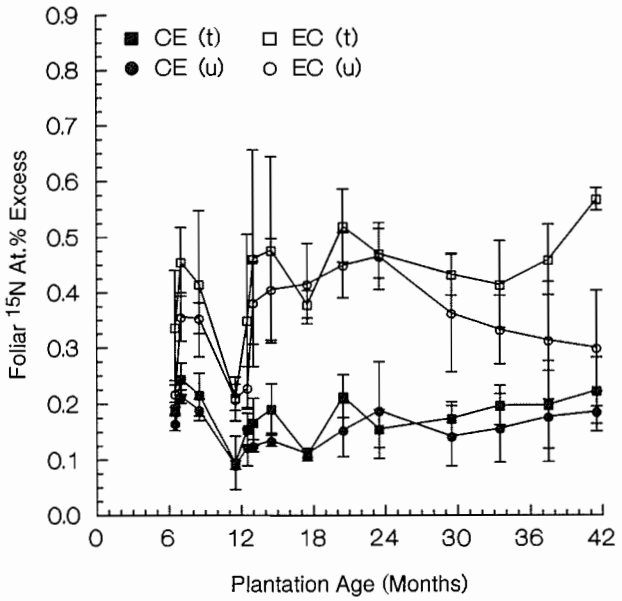


Table 1, the total litter-fall N fluxes measured between 1.5 and 3.5 years, were more than twice as large in *L. leucocephala* than in *C. equisetifolia* treatments and approximately 60% of total biomass N. Although litter decomposition rates were not measured directly in this study, it is clear from a comparison of litter-fall N fluxes to the forest floor and net forest floor (O-horizon) nitrogen accretion that incorporation of nitrogen derived from litter fall into the plantation soils is occuring at a much more rapid rate in treatments with *L. leucocephala* than in those comprised of *C. equisetifolia* and (or) *E.* ×*robusta* (Fig. 2). However, since *L. leucocephala* has a higher biomass N than *C. equisetifolia* and immobilized more N in the tree, there is a real possibility that the decrease in PNDFA over time is characteristic of *L. leucocephala*. Nevertheless since these plantations accumulated larger amounts of nitrogen, lower PNDFA translated into significant TNDFA contributions.

Estimates of TNDFA

Estimated TNDFA in *C. equisetifolia* (CE) and *L. leucocephala* (LE) at 3.5 years were, respectively, 25.4 ± 9.3 $g \cdot m^{-2}$ (73 $kg \cdot ha^{-1} \cdot year^{-1}$) and 26.0 ± 5.6 $g \cdot m^{-2}$ (74 $kg \cdot ha^{-1} \cdot year^{-1}$) (Table 2). In *L. leucocephala*, an estimated 36% of the total nitrogen derived from fixation was recycled within the plantations through litter fall, in contrast with *C. equisetifolia*, in which the litter-fall component was estimated at 26% of TNDFA. Our estimates of TNDFA in *C. equisetifolia* at 3.5 years are similar to those obtained at 2 years at this site (67 $kg \cdot ha^{-1} \cdot year^{-1}$) (Parrotta et al. 1994*a*). The rates are also very similar to those reported for

Fig. 5. Relationship between foliar and whole-tree (weighted average) ^{15}N at.% excess in *Casuarina equisetifolia* (○), *Leucaena leucocephala* (□), and *Eucalyptus* ×*robusta* (●), based on whole-tree harvest of 18- and 42-month-old trees grown in ^{15}N-labelled quadrats. For *C. equisetifolia*: WAE = 0.052 + 0.699 FAE; $r^2 = 0.79$; for *L. leucocephala*: WAE = −0.038 + 1.016 FAE; $r^2 = 0.84$; for *E.* ×*robusta*: WAE = 0.023 + 0.856 FAE; $r^2 = 0.97$. WAE and FAE are ^{15}N at.% excess based on whole-tree biomass sampling and foliar biomass sampling, respectively.

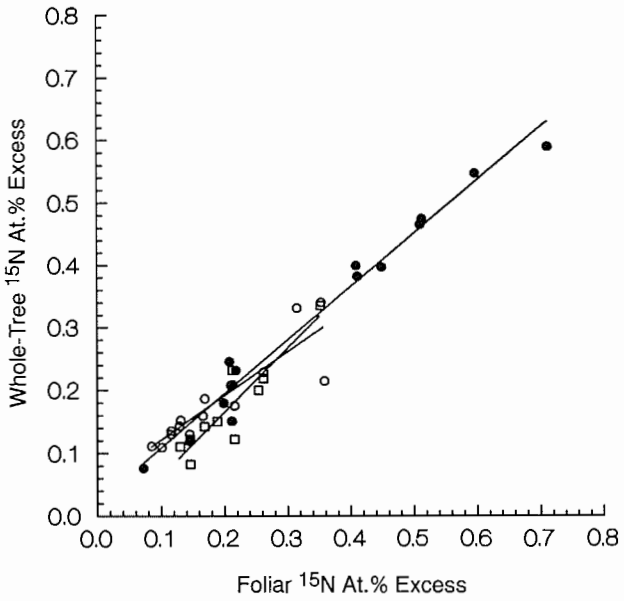


Fig. 6. Percent nitrogen derived from fixation (PNDFA) in *Casuarina equisetifolia* and *Leucaena leucocephala* based whole-tree (weighted average) ^{15}N at.% excess data (WAE) in trenched quadrats in mixed-species plantation stands. Error bars indicate ±SE; $n = 3$. Treatments: LE, *L. leucocephala* (with *E.* ×*robusta*); CE, *C. equisetifolia* (with *E.* ×*robusta*).

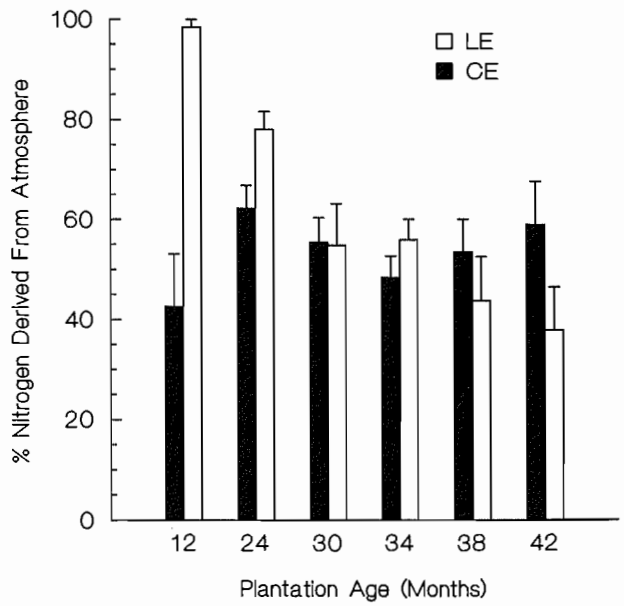


Table 2. Percent nitrogen derived from fixation (PNDFA) based on FAE and WAE data and total nitrogen derived from fixation (TNDFA; $g \cdot m^{-2}$) in *C. equisetifolia* and *L. leucocephala* biomass in 3.5-year-old plantations, based on WAE data and total N content of biomass and litter.

Treatment[a]		PNDFA, %		TNDFA ($g \cdot m^{-2}$)		TNDFA (ND) ($g \cdot m^{-2}$)	
N-fixing sp.	Reference	FAE	WAE	Biomass[b]	Total[c]	Biomass[b]	Total[c]
CE	EC	60.3	59.0	18.8	25.4	20.7	28.1
		(11.0)	(8.7)	(7.5)	(9.3)	(10.6)	(12.3)
LE	EL	34.4	37.8	16.7	26.0	31.3	53.6
		(7.9)	(8.6)	(3.5)	(5.6)	(1.3)	(3.9)

Note: TNDFA estimates based on the N-difference method (ND) are included for comparison purposes.

[a]CE, *C. equisetifolia* (with *E. ×robusta*); LE, *L. leucocephala* (with *E. ×robusta*); EC, *E. ×robusta* (with *C. equisetifolia*); EL, *E. ×robusta* (with *L. leucocephala*).

[b]Estimate based on total standing biomass nitrogen content, excluding litter.

[c]Estimate based on total standing biomass nitrogen content, including litter.

5- to 32-year-old *C. equisetifolia* plantations in India (Srivastava and Ambasht 1994) and Senegal (Domergues 1963; Maily and Margolis 1992) using acetylene reduction and nitrogen difference methodologies (ranging from 60 to 90 $kg \cdot ha^{-1} \cdot year^{-1}$).

TNDFA estimates for *L. leucocephala* stands at 3.5 years were also very similar to those reported for these plantations at 2 years (71 $kg \cdot ha^{-1} \cdot year^{-1}$; Parrotta et al. 1994*b*), although this earlier estimate did not include litter-fall TNDFA contributions. Both of these estimates fall within the range reported for this species in 1- to 4-year-old trees under field conditions using acetylene reduction (Högberg and Kvarnström 1982) and ^{15}N-enrichment techniques (Sanginga et al. 1989, 1994). Our estimates for TNDFA based on FAE data are were very similar to estimates based on the nitrogen difference (ND) method for *C. equisetifolia* but were vastly different from those for *L. leucocephala* (Table 2), indicating the danger of using the total nitrogen difference method in estimating TNDFA. The total nitrogen difference method does not take into account any changes in available soil nitrogen.

Conclusions

The ^{15}N-enrichment methodology proved useful and accurate for estimating dinitrogen fixation in maturing stands of *C. equisetifolia* and *L. leucocephala*, provided that the methodology was applied in a manner to account for N recycling within the stands. In our previous studies of younger trees (Parrotta et al. 1994*a*, 1994*b*), we observed that accurate estimates of dinitrogen fixation were possible in monoculture stands of trees, provided that such plots were trenched. This was no longer possible for older trees because recycling of nitrogen through litter fall changes the pool of soil available N differently in the fixing and reference species plots. In stands where nitrogen has begun to be recycled, the reference species must occur in close proximity to the fixing species to accurately sample the same pool of soil available N over time.

Recycling of fixed nitrogen in tree stands also influences other aspects of the ^{15}N-enrichment methodology and Baker et al. (1995) stated that in such cases a correction for accumulated nitrogen in the standing biomass of the tree would be desirable. It was not necessary in this study because the application of ^{15}N fertilizer was applied at an early age and whole-tree harvests were made at 18 and 42 months.

Foliar sampling was a useful nondestructive alternative for the ^{15}N-enrichment protocol. We observed a high correlation between the ^{15}N at.% excess of foliage samples (FAE) and that of a weighted average of all tree components (WAE). It is possible that for other tree species such a high correlation may not be observed. Therefore, foliar sampling must be tested and validated for each tree species being utilized.

Trenching of the ^{15}N-labelled plot is an important factor in accurately estimating dinitrogen fixation in trees. In this study we observed that unlike the case reported by Parrotta et al. (1994*a*), untrenched plots of 4 × 4 m are not adequate for estimating dinitrogen fixation in these species. This situation appears to result from the growth of the roots of the reference species outside of the ^{15}N-labelled area to a greater extent than that of the fixing species. It is possible that had the labelled area been significantly larger, accurate estimation of dinitrogen fixation might have been possible in the absence of trenching. However, because it is difficult to estimate the probable extent of rooting of tree species at a site prior to establishment of the plantation, it will always be wiser to include trenching in studies of dinitrogen fixation of trees rather than risk obtaining inaccurate data.

Differences in the pattern of dinitrogen fixation over time between the two nitrogen-fixing species studied were observed. *Casuarina equisetifolia* PNDFA remained more or less constant over the 3.5 years of study at a value of approximately 55%. *Leucaena leucocephala* demonstrated a higher PNDFA at the beginning of the study (98%), and a relatively constant decline to less than 40% at the end

of the study. It should be noted that although these plantations were only 3.5 years of age, developmentally they were mature and all of the species were beginning to show mortality related to competition. We believe it unlikely that dinitrogen fixation in *L. leucocephala* was inhibited by an increase in soil-available nitrogen. This leaves uncertain the proportionate (PNDFA) and actual (TNDFA) contributions of *L. leucocephala* to the nitrogen supply in long-term plantations. Longer term studies of *L. leucocephala* at other sites, as well as the effect of coppicing, are needed.

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References

Awonaike, K.O., Danso, S.K.A., and Zapata, F. 1993. The use of a double isotope (^{15}N and ^{34}S) labelling technique to assess the suitability of various reference crops for estimating nitrogen fixation in *Gliricidia sepium* and *Leucaena leucocephala*. Plant Soil, **155/156**: 325–328.

Baker, D.D. 1990. Optimizing actinorhizal nitrogen fixation and assessing actual contribution under field conditions. *In* Fast growing trees and nitrogen fixing trees. *Edited by* D. Werner and P. Mueller. Fischer-Verlag, Stuttgart, Germany. pp. 291–299.

Baker, D.D., Wheeler, R.A., and Fried, M. 1992. Estimation of biological nitrogen fixation by tree legume species using the ^{15}N dilution method. *In* Biological nitrogen fixation and sustainability of tropical agriculture. *Edited by* K. Mulongoy, M. Gueye, and D.S.C. Spencer. John Wiley & Sons, Chichester, U.K. pp. 259–264.

Baker, D.D., Fried, M., and Parrotta, J.A. 1995. Theoretical implications for the estimation of dinitrogen fixation by large perennial plants using isotopic dilution. *In* Nuclear techniques in soil–plant studies for sustainable agriculture and environmental preservation. International Atomic Energy Agency, Vienna. pp. 225–236.

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

Domergues, Y.R. 1963. Évaluation du taux de fixation de l'azote dans un sol dunaire reboisé en filao (*Casuarina equisetifolia*). Agrochemica, **7**: 335–340.

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

Högberg, P., and Kvarnström, M. 1982. Nitrogen fixation by the woody legume *Leucaena leucocephala* in Tanzania. Plant Soil, **66**: 21–28.

Kang, B.T., and Mulongoy, K. 1992. Nitrogen contribution of woody legumes in alley cropping systems. *In* Biological nitrogen fixation and sustainability of tropical agriculture. *Edited by* K. Mulongoy, M. Gueye, and D.S.C. Spencer. John Wiley & Sons, Chichester, U.K. pp. 367–375.

Maily, D., and Margolis, H.A. 1992. Forest floor and mineral soil development in *Casuarina equisetifolia* plantations on the coastal sand dunes of Senegal. For. Ecol. Manage. **55**: 259–278.

Mansour, S.R., and Baker, D.D. 1994. Selection trials for effective N_2-fixing *Casuarina–Frankia* combinations in Egypt. Soil Biol. Biochem. **26**: 655–658.

Mariotti, A., Sougoufara, B., and Dommergues, Y.R. 1992. Estimation de la fixation d'azote atmosphérique par la traçage isotopique naturel dans une plantation de *Casuarina equisetifolia* (Forst.). Soil Biol. Biochem. **24**: 647–653.

Parrotta, J.A., Baker, D.D., and Fried, M. 1994*a*. Application of ^{15}N-enrichment methodologies to estimate nitrogen fixation in *Casuarina equisetifolia*. Can. J. For. Res. **24**: 201–207.

Parrotta, J.A., Baker, D.D., and Fried, M. 1994*b*. Estimation of nitrogen fixation in *Leucaena leucocephala* using ^{15}N-enrichment methodologies. *In* Advances in legume systematics 5: the nitrogen factor. *Edited by* J.I. Sprent and D. McKey. Royal Botanic Gardens, Kew, U.K. pp. 75–82.

Rao, A.V., and Giller, K.E. 1993. Nitrogen fixation and its transfer from *Leucaena* to grass using ^{15}N. For. Ecol. Manage. **61**: 221–227.

Sanginga, N. 1992. Early growth and N_2-fixation of *Leucaena* and *Gliricidia* at different levels of phosphorus addition. Fertil. Res. **31**: 165–173.

Sanginga, N., Mulongoy, K., and Ayanaba, A. 1989. Nitrogen fixation of field-inoculated *Leucaena leucocephala* (Lam.) de Wit estimated by the ^{15}N and the difference methods. Plant Soil, **117**: 269–274.

Sanginga, N., Bowen, G.D., and Danso, S.K.A. 1990*a*. Genetic variability in symbiotic nitrogen fixation within and between provenances of two *Casuarina* species using the ^{15}N-labelling methods. Soil Biol. Biochem. **22**: 539–547.

Sanginga, N., Bowen, G.D., and Danso, S.K.A. 1990*b*. Assessment of genetic variability for N_2 fixation between and within provenances of *Leucaena leucocephala* and *Acacia albida* estimated by ^{15}N labelling techniques. Plant Soil, **127**: 169–178.

Sanginga, N., Danso, S.K.A., Mulongoy, K., and Ojeifo, A.A. 1994. Persistence and recovery of *Rhizobium* ten years after inoculation on *Leucaena leucocephala* grown on an Alfisol in southwestern Nigeria. Plant Soil, **159**: 199–204.

Sougoufara, B., Danso, S.K.A., Diem, H.G., and Dommergues, Y.R. 1990. Estimating N_2 fixation and N derived from soil by *Casuarina equisetifolia* using labelled ^{15}N fertilizer: some problems and solutions. Soil Biol. Biochem. **22**: 695–701.

Srivastava, A.K., and Ambasht, R.S. 1994. Soil moisture control of nitrogen fixation activity in dry tropical *Casuarina* plantation forest. J. Environ. Manage. **42**: 49–54.

van Kessel, C., Farrell, R.E., Roskoski, J.P., and Keane, K.M. 1994. Recycling of the naturally occurring ^{15}N in an established stand of *Leucaena leucocephala*. Soil Biol. Biochem. **26**: 757–762.

Werner, D., and Muller, P. 1990. Fast growing trees and nitrogen fixing trees. Gustav Fischer-Verlag, Stuttgart.