Application of ^{15}N-enrichment methodologies to estimate nitrogen fixation in *Casuarina equisetifolia*

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The ^{15}N-enrichment technique for estimating biological nitrogen fixation in *Casuarina equisetifolia* J.R. & G. Forst. was evaluated under field conditions in single-species and mixed-species plantings (with a nonfixing reference species, *Eucalyptus* ×*robusta* J.E. Smith) between ages 6 and 24 months in Puerto Rico. Trenched and untrenched quadrats within the plantations were labelled at 6-month intervals with ^{15}N-enriched ammonium sulfate at a rate of 0.2 g $^{15}N \cdot m^{-2} \cdot year^{-1}$ (2.0 g $N \cdot m^{-2} \cdot year^{-1}$). Analyses of foliar and whole-tree (weighted average) N-isotope ratios, based on periodic nondestructive and whole tree harvests, were used to estimate the proportion of nitrogen derived from biological dinitrogen fixation (PNDFA) and total nitrogen derived from fixation (TNDFA) in *Casuarina*. These results were compared with those obtained using the nitrogen difference method. The ^{15}N-enrichment technique yielded consistent estimates of biological nitrogen fixation in *Casuarina* when either foliar or whole-tree N-isotopic data were used to calculate PNDFA and TNDFA. Estimates of PNDFA in *Casuarina* were similar for trenched monoculture and mixed-species quadrats, and in trenched and untrenched quadrats where the reference species (*Eucalyptus*) was interplanted with the N-fixing species. However, the results indicate that eucalyptus grown in small, untrenched monoculture quadrats is an inappropriate reference for estimating PNDFA in *Casuarina*. During the first 2 years after plantation establishment, *Casuarina* obtained from 48 to 67% of its nitrogen from the atmosphere based on foliar and whole-tree sampling. This amounted to between 82 and 94 $kg \cdot ha^{-1} \cdot year^{-1}$ in the monoculture treatment and between 39 and 62 $kg \cdot ha^{-1} \cdot year^{-1}$ in the mixed stands with *Eucalyptus*. These results also were in close agreement with estimates made using an N-difference method.

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La capacité de fixation de l'azote atmosphérique au champ chez *Casuarina equisetifolia* J.R. & G. Forst. a été évaluée grâce à la technique d'enrichissement au ^{15}N. Ce dispositif, mis en place à Puerto Rico, comprend des plantations âgées de 6 mois contenant une seule espèce et des plantations mixtes (en utilisant une espèce non-fixatrice, *Eucalyptus* ×*robusta* J.E. Smith). Ce dispositif a fait l'objet d'un suivi sur une période de 18 mois. Des parcelles avec et sans tranchées à l'intérieur des plantations ont été marquées, à des intervalles de 6 mois avec du sulfate d'ammonium enrichie de ^{15}N à un taux de 0,2 g $^{15}N \cdot m^{-2} \cdot an^{-1}$ (2,0 g $N \cdot m^{-2} \cdot an^{-1}$). L'analyse périodique non-destructive du rapport d'azote radioactif du feuillage et de l'arbre entier nous a permis de déterminer la proportion d'azote provenant de l'azote fixé biologiquement (PNDFA) et de l'azote total fixé au niveau du *Casuarina* (TNDFA). Les résultats obtenus ont été comparés avec ceux obtenus par la méthode d'azote différentielle. La technique d'enrichissement d'azote nous a donné des estimations similaires sur la fixation biologique de l'azote chez *Casuarina*, en utilisant l'analyse foliaire ou de l'arbre entier, pour calculer le PNDFA ou le TNDFA. L'évaluation de PNDFA chez *Casuarina* est similaire pour les parcelles avec tranchées contenant des cultures simples ou mixtes et pour les parcelles mixtes avec et sans tranchées contenant de l'*Eucalyptus*. Cependat les résultats indiquent que l'*Eucalyptus*, lorsqu'utilisé en parcelles de monoculture sans tranchées, ne peut constituer une référence appropriée du PNDFA chez *Casuarina*. Pendant les 2 premières années il semble, selon l'analyse foliaire et totale, que le *Casuarina* en plantation obtient 48 à 67% de son azote par la fixation d'azote atmosphérique. Ceci correspond, pour les monocultures, à des quantités d'azote variant entre 82 et 94 $kg \cdot ha^{-1} \cdot an^{-1}$ et de 39 à 62 $kg \cdot ha^{-1} \cdot an^{-1}$ dans les parcelles mixtes contenant de l'*Eucalyptus*. Les résultats obtenus sont en accord avec les estimations faites par la méthode d'azote différentielle.

[Traduit par la rédaction]

Introduction

Recent studies on the value of interplanting nitrogen-fixing trees with nonfixing species (Hansen and Dawson 1982; Hu et al. 1985; DeBell et al. 1989; Binkley et al. 1992) have generated interest in quantifying symbiotic nitrogen fixation in trees under field conditions. Available techniques for estimating symbiotic nitrogen fixation in forest ecosystems are limited, however, and all have significant margins of error (Baker 1990).

The ^{15}N-isotopic dilution methodology using enriched ^{15}N holds promise as an effective and relatively straightforward approach for measuring nitrogen fixation in plantations

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under field conditions. This methodology involves the application of a small amount of ^{15}N-enriched fertilizer to the soil. Nitrogen-fixing trees will assimilate nonenriched atmospheric N supplied by nitrogen-fixing root symbionts, in addition to the enriched fertilizer in the soil. Since nonfixing trees do not have access directly to the atmospheric N supply, the resulting $^{15}N/^{14}N$ ratio of tissue N in the two species will differ proportionally to the amount of nitrogen fixed. The essential elements of this methodology have been discussed in several publications, including Fried and Middelboe (1977), Weaver (1986), Rennie (1986), and Danso et al. (1992). The ^{15}N dilution methods have been applied to tree seedlings under laboratory, greenhouse (Gauthier et al. 1985; van Kessel and Nakao 1986) and simulated field conditions (Cornet et al. 1985), and only very recently in field plantations (Sanginga et al. 1989; Mead and Preston 1992).

The widespread acceptance and application of ^{15}N enrichment techniques require that several methodological issues be resolved for tree crops. These pertain to the choice of an apppropriate reference tree species; its location with respect to the nitrogen-fixing species; the relative merits of untrenched and trenched study plots; the rate, timing, and spatial extent of ^{15}N-enriched fertilizer application; and biomass sampling procedures. The present study was designed to experimentally examine several of these issues in young field plantations of a nitrogen-fixing tree species, *Casuarina equisetifolia* using *Eucalyptus* ×*robusta* as a nonfixing reference species. Specifically, we compared estimates of nitrogen fixation for *Casuarina* (*i*) in pure and mixed (with *eucalyptus*) stands; (*ii*) using trenched and untrenched plots; and (*iii*) employing several foliage sampling methods and whole-tree biomass harvests.

Site description

The study area is located at the University of Puerto Rico's Toa Baja experimental farm located on the northern coast of Puerto Rico (18°27′N, 66°10′W, elevation 2 m). Annual precipitation averages 1600 mm, and 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 (isohypothermic Typic Troposamments). Soils and vegetation at the site have been subject to frequent, often severe disturbances in recent decades, including cultivation and large-scale soil perturbations such as sand extractions and levelling. At the time of plantation establishment, the site was dominated by grasses.

Materials and methods

Plot establishment and maintenance

This set of experiments was a subset of a larger biomass production trial of fast-growing tree species. Using a randomized complete block design, three replicate 16 × 16 m plots of each of the following treatments were included in the larger study: (*i*) *Casuarina equisetifolia* monoculture (CM); (*ii*) *Eucalyptus* ×*robusta* monoculture (EM); (*iii*) *Casuarina* + *Eucalyptus* (CE); (*iv*) *Leucaena leucocephala* (Lam.) de Wit monoculture (LM); (*v*) *Casuarina* + *Leucaena* (CL); (*vi*) *Eucalyptus* + *Leucaena* (EL); and (*vii*) unplanted control (UC). Prior to plantation establishment, a trenched quadrat measuring 3 × 3 m (in treatments CM, EM, and LM) or 4 × 4 m (in treatments CE, CL, and EL) located in the center of each plot was prepared. The perimeter of each plot was trenched and a multilayered plastic film barrier installed to a depth of 60–80 cm to prevent lateral flow of water and fertilizer and to restrict root growth into and out of each quadrat. Two additional, untrenched quadrats measuring 4 × 4 m were marked within each plot for treatments CM, EM, and CE. This paper reports the results from the *Casuarina* monoculture (CM), *Eucalyptus* monoculture (EM), and *Casuarina* + *Eucalyptus* (CE) treatments. Evaluation of the remaining treatments including leucaena are reported in Parrotta et al. (1994).

The plantation was established in September–October 1989 using containerized seedlings 30–60 cm in height. *Casuarina* seedlings were treated in the nursery 3 months prior to outplanting with a multistrain inoculum of *Frankia* prepared at Yale University. All *Casuarina* seedlings were well nodulated at the time of outplanting. Trees were planted systematically at a spacing of 1 × 1 m; in the case of the mixed-species treatments, trees were planted in a checkerboard pattern. The two peripheral rows of each plot were considered border rows and were not used for the collection of biomass data. During the first 6 months after planting, plots were manually hoed to control weed competition. Seedling survival during the 1st year of the experiment was 100 and 94% for *Casuarina* and *Eucalyptus*, respectively.

^{15}N labelling

In March 1990 and at 6-month intervals thereafter, ^{15}N-enriched ammonium sulfate was applied to each trenched and untrenched quadrat at a rate of 0.1 g $^{15}N/m^2$ in a total application of 1.0 g N/m^2 (10% ^{15}N atom excess). To the remaining plot area, unenriched ammonium sulfate was applied at the rate of 1.0 g N/m^2. The ammonium sulfate was spread uniformly on the soil by applying as an aqueous solution using garden watering cans.

Biomass sampling and estimation of total nitrogen yields

At 0.5, 1.0, 2.5, and 6.0 months following each ^{15}N-fertilizer application, foliar samples were collected from trees within each of the trenched and untrenched ^{15}N-labelled quadrats. In the untrenched quadrats of treatments CM, EM, and CE, the 4 central trees were sampled. In the trenched quadrats, all trees were sampled, i.e., 9 trees in the CM and EM quadrats and 16 trees (8 per species) in the CE quadrats. Foliage samples were composited for each quadrat at each sampling date. Dry weights of the composited samples were similar across all sampling dates and averaged 70 g (approximately 100 leaves) for *Eucalyptus* and 90 g for *Casuarina*.

During the first 6 months of the study, trees were small enough to permit collection of foliage from lower, middle, and upper portions of the canopy for each tree. As trees grew larger, a pole clipper was required for sample collection, and while all portions of the canopy within a quadrat were sampled, most samples were collected from the most dense, middle canopy zone. For these later sampling dates, twigs (<5 mm diameter) were clipped and all leaves (or photosynthetic branchlets for *Casuarina*) collected. The number of twigs clipped from each tree depended on the number of sampled trees of each species in the quadrat: four twigs/tree in untrenched quadrats with two individuals/species (treatment CE), two twigs/tree in untrenched quadrats with four individuals/species (treatments CM and EM), one twig/tree in trenched quadrats with eight or nine individuals/species (treatments CE, CM, and EM).

On selected sampling dates, additional foliage samples were collected from trees of both species in trenched quadrats to assess potential differences in foliar N-isotopic composition among foliage samples collected from different canopy positions. Canopy strata were defined for the quadrat as a whole. From each tree, single twigs were sampled in each canopy stratum occupied by the tree's canopy. Individual tree samples from each quadrat were composited by species and strata for subsequent chemical analyses.

To determine whether or not foliage sampling provides a reliable indication of whole-tree N-isotopic composition, whole-tree harvests that included selected *Causarina* and *Eucalyptus* from untrenched quadrats were carried out at the end of the second 15 N-labelling period, when trees were 18 months old. In March 1991, a total of 36 trees were harvested (18 per species),

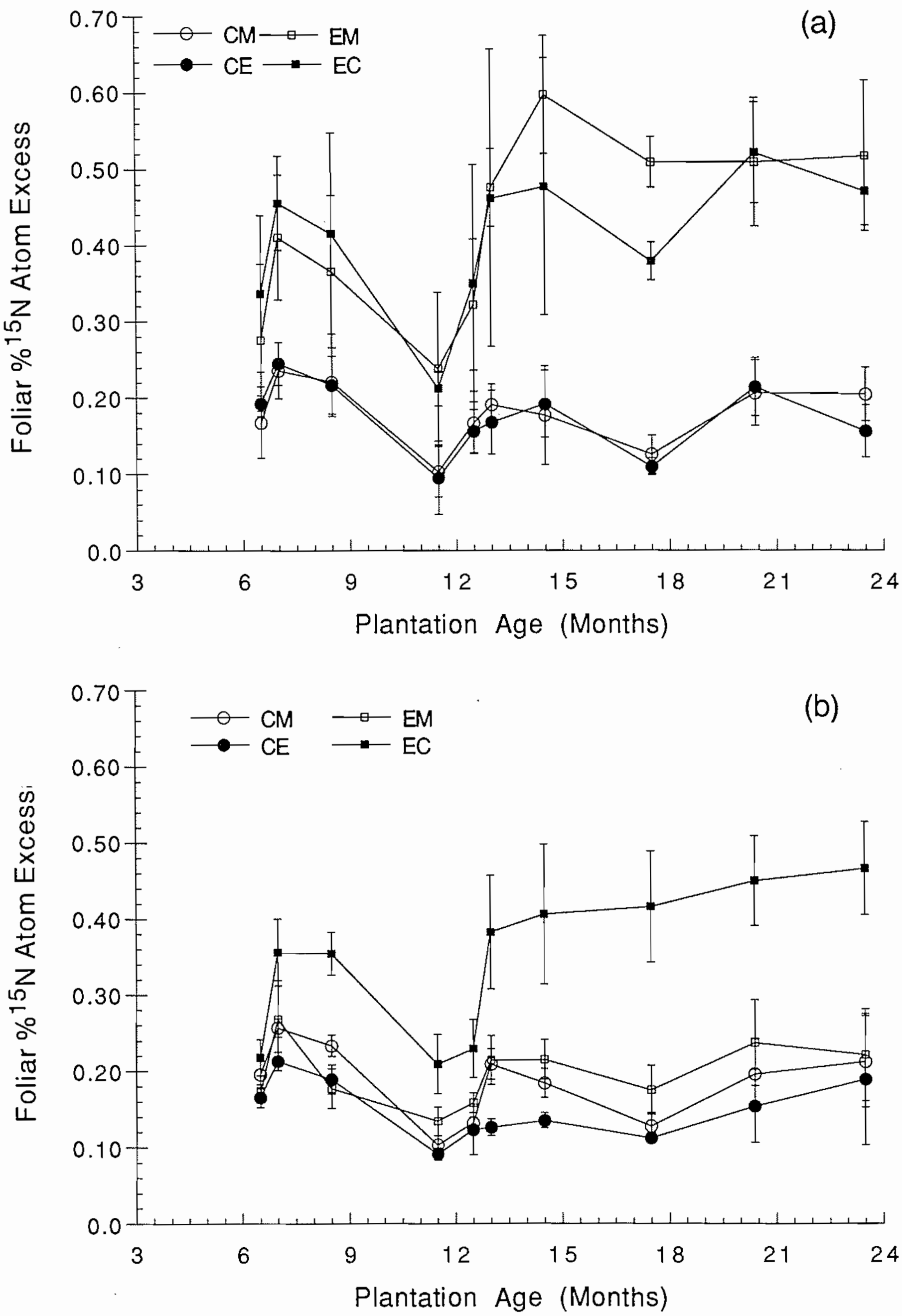

FIG. 1. Foliar percent ^{15}N atom excess in *Casuarina equisetifolia* and *Eucalyptus* ×*robusta* in monoculture (CM and EM) and mixed-species (CE and EC) plots 6 to 24 months after plantation establishment. ^{15}N-enriched fertilizer was applied to quadrats at 6, 12, and 18 months. (*a*) Trenched quadrats. (*b*) Untrenched quadrats. Error bars indicate ±1 SE.

including the root systems of 18 (9 per species). Within each plot, two trees (and one root system) of each species were harvested following measurements of total height and basal diameter. Harvested trees were selected from the central 4-m^2 area of one of the untrenched ^{15}N-labelled quadrats.

Each harvested tree was separated in the field into the following biomass components: leaves (or *Casuarina* branchlets <2 mm diameter), branch or stem segments (BSS) <1 cm diameter, BSS 1–2.5 cm diameter, BSS 2.5–5.0 cm diameter, BSS >5 cm diameter, dead branches, roots <1 cm diameter, and roots (including taproot) >1 cm diameter. Each biomass component was weighed in the field, and a random subsample was taken and dried to constant weight at 70°C to yield conversion factors used to calculate tree biomass on a dry-weight basis, and to develop dimension analysis regressions relating total tree biomass and nitrogen content to stem basal diameter for each species. The dried biomass subsamples were subsequently used for tissue nutrient analyses, including total N and N isotopic composition.

Every 6 months from the beginning of the experiment measurements were made of tree heights and stem basal diameters (5 cm above the base) for all nonborder trees within the experimental plots. These data were used in conjunction with dimension analysis regressions developed from tree harvest data to estimate total biomass and nitrogen accumulation in aboveground and root biomass within the plantation stands.

^{15}N analysis

Leaf and other plant tissue samples were dried at 70°C and ground to fine powder in a Tecator sample mill. Analyses for ^{15}N atom excess and percent N were performed on an ANCA-MS by the Waikato Stable Isotope Unit in Hamilton, N.Z.

TABLE 1. Mean (±1 SE; $n = 3$) percent N and percent ^{15}N atom excess (a.e.) for foliage sampled from different canopy positions within trenched quadrats

Species and treatment	Canopy position	%N	%^{15}N a.e.
Eucalyptus			
EM	Upper	1.76±0.12	0.175±0.029
	Middle	1.41±0.13	0.265±0.025
	Lower	1.29±0.12	0.316±0.007
	Random	1.46±0.23	0.238±0.100
EC	Upper	2.02±0.06	0.217±0.050
	Middle	1.43±0.07	0.325±0.082
	Lower	1.31±0.09	0.311±0.015
	Random	1.58±0.09	0.212±0.023
Casuarina			
CM	Upper	2.15±0.02	0.078±0.019
	Middle	1.76±0.10	0.106±0.025
	Lower	1.67±0.09	0.138±0.020
	Random	1.70±0.07	0.103±0.033
CE	Upper	2.04±0.14	0.081±0.019
	Middle	1.93±0.05	0.120±0.044
	Lower	1.82±0.16	0.173±0.031
	Random	1.97±0.08	0.095±0.048

NOTE: Plantation age of the trees was 12 months, 6 months after initial application of ^{15}N-enriched fertilizer. Composite samples were collected from all canopy positions.

*EM, *Eucalyptus* monoculture; EC, *Eucalyptus* (with *Casuarina*); CM, *Casuarina* monoculture; CE, *Casuarina* (with *Eucalyptus*).

Calculation of total nitrogen pools and whole-tree isotopic ratios

Total nitrogen pools (WTN) and whole-tree (weighted average) percent ^{15}N atom excess (WAE) was estimated on a unit-area basis at 12 and 24 months for *Casuarina* (WTN^{fix}, WAE^{fix}) and *Eucalyptus* (WTN^{ref}, WAE^{ref}) in each of the ^{15}N-labelled quadrats. Using regressions developed from biomass and N concentration data derived from whole-tree harvests (See eqs. 4 and 5), WTN^{fix} and WTN^{ref} were calculated 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. WAE^{fix} and WAE^{ref} were calculated as the weighted averages of percent ^{15}N atom excess for all plant parts, also using regressions developed from whole tree harvest data (Fig. 2).

Calculation of PNDFA and TNDFA

Nitrogen fixation (PNDFA and TNDFA) by each casuarina replicate plot was calculated at 12 and 24 months from foliar atom excess (FAE) and WAE data for the nitrogen-fixing (*Casuarina*) and reference (*Eucalyptus*) species using the formula of Fried and Middelboe (1977) for foliar percent ^{15}N atom excess data:

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

for whole-tree percent ^{15}N atom excess data:

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

Total nitrogen derived from fixation (TNDFA) was calculated as the product of PNDFA and total biomass nitrogen (g/m^2) at 12 and 24 months. For comparison, the difference (ND) in total nitrogen yield of the fixing and references species, sometimes referred to as the "difference" method was also calculated:

$$[3]\quad \mathrm{TNDFA} = \mathrm{WTN}^{\mathrm{fix}} - \mathrm{WTN}^{\mathrm{ref}}$$

Results and discussion

Temporal trends in foliar ^{15}N enrichment

Between each of the first two ^{15}N-enriched fertilizer applications, mean foliar percent ^{15}N atom excess (FAE) fluctuated to a modest degree in both *Casuarina* and *Eucalyptus* in the trenched and untrenched quadrats of both the monoculture (CM, EM) and mixed-species (EC and CE) treatments (Figs. 1*a* and 1*b*). With the exception of *Casuarina* samples at 12 months, however, differences in FAE among sampling dates were not significant in either *Casuarina* or *Eucalyptus*.

For both species in all treatments, peak FAE appears to have occurred at a later date over successive 6-month intervals. This apparent trend may reflect an increasing nitrogen pool size, requiring a longer period for ^{15}N uptake to increase the isotopic ratio of the pool. Comparing foliage samples collected just prior to ^{15}N-enriched fertilizer applications (at 12, 18, and 24 months), there is a consistent trend toward increasing foliar ^{15}N enrichment over time for both species. The similarity between *Casuarina* and *Eucalyptus* with respect to patterns of FAE over time indicates that both species have similar temporal patterns of soil N uptake. These data strongly suggest that under the present experimental conditions, *Eucalyptus* is an appropriate reference species.

Comparing monoculture and mixed-species systems (Fig. 1*a*), no significant differences in FAE were noted for either species across all sampling dates. These data would suggest that the associated species did not significantly affect uptake patterns of soil N nor rates of N fixation during these early phases of plantation growth (<2 years).

Comparison of trenched and untrenched quadrats

For *Casuarina*, there were no significant differences in FAE values between trenched and untrenched quadrats for either CE or CM (Figs. 1*a* and 1*b*). For *Eucalyptus*, there were no significant differences in FAE values between trenched and untrenched quadrats in the EC treatment, although values tended to be lower in the untrenched than trenched quadrats. However, in the *Eucalyptus* monoculture treatment, FAE values were consistently lower in the untrenched than in the trenched quadrats (Fig. 1*b*). Since the N-isotopic ratio of the plant reflects the concentration of available N in the soil, the observed differences in FAE between the untrenched *Eucalyptus* monoculture treatment and other *Eucalyptus* treatments indicate that a greater amount of N was available to these trees, probably owing to root growth beyond the untrenched borders of the ^{15}N-labelled quadrat. These data indicate that *Eucalyptus* grown in single-species plantations may not be an appropriate reference for estimating N fixation in fixing species unless the ^{15}N-labelled area is either (*i*) enclosed by a root-impermeable barrier (i.e., trenched quadrats) or (*ii*) sufficiently large so that trees sampled for N-isotopic composition are surrounded by an adequate buffer area to preclude significant root growth of sampled trees outside of the ^{15}N-labelled area. For this reason, we have not used the EM untrenched data for calculation of PNDFA.

Evaluation of canopy position effects on foliar ^{15}N enrichment

For both species in monoculture and mixed-species treatments, foliar nitrogen concentrations increased consistently from lower to higher canopy positions (Table 1). FAE values, in contrast, generally decreased from lower to higher canopy

TABLE 2. Mean (±1 SE) percent N and percent ^{15}N atom excess (a.e.) for *Casuarina* and *Eucalyptus* biomass components based on whole-tree harvests of trees within untrenched ^{15}N-labelled quadrats (CE treatment (i.e., *Casuarina* (with *Eucalyptus*))) at 18 months after plantation establishment

	Casuarina			*Eucalyptus*		
	n	%N	%^{15}N a.e.	*n*	%N	%^{15}N a.e.
Leaves	12	1.89±0.08	0.133±0.009	11	1.58±0.07	0.271±0.052
BSS						
<1 cm	12	0.60±0.04	0.146±0.012	11	0.35±0.02	0.268±0.050
1.0–2.5 cm	12	0.42±0.02	0.131±0.009	11	0.30±0.02	0.235±0.046
2.5–5.0 cm	12	0.35±0.02	0.140±0.011	10	0.28±0.03	0.219±0.045
>5.0 cm	6	0.31±0.01	0.139±0.009	5	0.22±0.02	0.144±0.024
Dead	12	0.45±0.04	0.141±0.009	11	0.24±0.02	0.244±0.042
Roots						
<1 cm	6	0.70±0.06	0.167±0.021	6	0.47±0.03	0.296±0.056
>1 cm	6	0.39±0.02	0.142±0.015	6	0.31±0.03	0.216±0.077

NOTE: BSS, branch and stemwood plus bark.

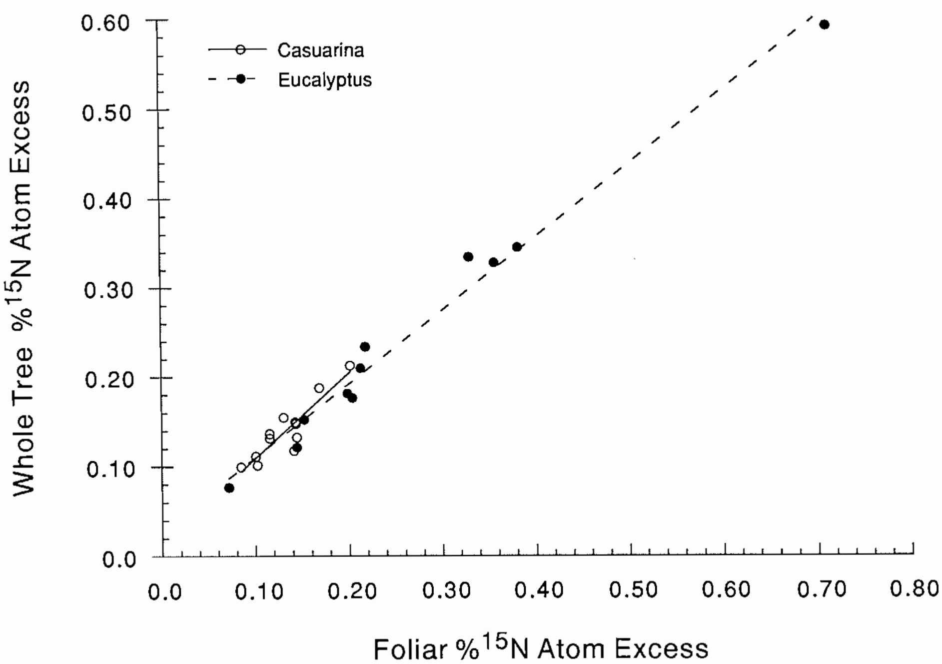

FIG. 2. Relationship between foliar and whole-tree (weighted average) percent ^{15}N atom excess in *Casuarina equisetifolia* and *Eucalyptus* ×*robusta*, based on whole-tree harvest of 18-month-old trees in ^{15}N-labelled quadrats. For *Casuarina*: WAE = 0.014 + 0.953FAE; r^2 = 0.91. For *Eucalyptus*: WAE = 0.028 + 0.819FAE; r^2 = 0.98. For both species: WAE = 0.031 + 0.816FAE; r^2 = 0.98, where WAE and FAE are percent ^{15}N atom excess based on whole-tree biomass sampling and foliar biomass sampling, respectively.

positions, although these differences were not found to be statistically significant. Random sampling from all canopy positions yielded percent N and FAE results that were generally intermediate and similar to those obtained for samples from the middle canopy stratum.

These results indicate that accurate measurements of FAE can be made by using either random or stratified sampling procedures, provided that the same field protocols are followed for both the nitrogen-fixing and reference species. The need for consistency in field sampling appears to be more important for stratified sampling and would thus favor random sampling protocols as a cost-effective and reliable method.

The utility of foliar sampling to estimate WAE and whole-tree N content

The nitrogen concentrations among biomass components of both *Eucalyptus* and *Casuarina* varied considerably, and were highest in foliage and decreased in branch, stem, and root components of increasing diameter. In spite of these variations in N concentrations among biomass components, percent ^{15}N atom excess values were nearly constant across all biomass components for each species (Table 2). While there was considerable variation in both FAE and WAE among trees harvested from the untrenched ^{15}N-labelled quadrats, there was a strong, significant linear relationship between FAE and WAE for both species (Fig. 2). These

TABLE 3. Percent ^{15}N atom excess based on foliar (FAE) and whole-tree (WAE) biomass sampling and total biomass nitrogen in *Casuarina* and *Eucalyptus* grown in trenched and untrenched quadrats in monoculture and mixed-species stands at 12 and 24 months after plantation

Species and treatment*	%^{15}N atom excess				Total biomass N (g/tree)	
	12 months		24 months			
	FAE	WAE	FAE	WAE	12 months	24 months
Casuarina						
CM-u	0.104	0.113	0.212	0.216	19.07	34.40
	(0.011)	(0.011)	(0.060)	(0.057)	(1.51)	(5.48)
CM-t	0.103	0.112	0.204	0.208	17.10	30.47
	(0.019)	(0.018)	(0.035)	(0.034)	(0.29)	(6.51)
CE-u	0.091	0.100	0.189	0.194	18.00	29.06
	(0.008)	(0.008)	(0.086)	(0.082)	(2.12)	(9.56)
CE-t	0.095	0.104	0.157	0.163	22.56	39.80
	(0.028)	(0.027)	(0.034)	(0.032)	(3.78)	(3.46)
Eucalyptus						
EM-u	0.134	0.137	0.221	0.209	9.15	26.37
	(0.019)	(0.016)	(0.060)	(0.049)	(1.86)	(2.90)
EM-t	0.238	0.223	0.517	0.452	8.60	11.87
	(0.058)	(0.047)	(0.098)	(0.080)	(0.10)	(2.02)
EC-u	0.209	0.199	0.466	0.410	9.02	13.94
	(0.039)	(0.032)	(0.061)	(0.050)	(1.70)	(2.00)
EC-t	0.212	0.202	0.472	0.439	9.42	15.06
	(0.013)	(0.011)	(0.045)	(0.040)	(0.30)	(6.62)

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

*CM, *Casuarina* monoculture; CE, *Casuarina* (with *Eucalyptus*); EM, *Eucalyptus* monoculture; EC, *Eucalyptus* (with *Casuarina*); u and t, untrenched and trenched quadrats, respectively.

relationships were used in this study to estimate WAE from isotopic ratios of foliar samples. Further, because the regressions for *Casuarina* and *Eucalyptus* were not significantly different, these data suggest that FAE values can be used to accurately estimate PNDFA and TNDFA in casuarina. Had these regressions for the two species been significantly different, use of FAE data alone would have yielded erroneous results for PNDFA and TNDFA, since differences in FAE between the nitrogen-fixing and reference species would not have accurately reflected differences in WAE.

Estimation of PNDFA and TNDFA

Mean whole-tree percent ^{15}N a.e. (WAE) values estimated for both *Casuarina* and *Eucalyptus* using the equations presented in Fig. 2, are presented in Table 3. Total biomass nitrogen (WTN) for both the nitrogen-fixing and reference species in the ^{15}N-labelled study quadrats (Table 3) were estimated based on whole-tree harvest data for nine trees per species ranging in stem basal diameter from 1.8 to 8.5 cm. For *Casuarina*

$$[4]\quad \mathrm{WTN\ (g/tree)} = 4.075 \times 10^{0.1601D}, \qquad r^2 = 0.97$$

and for *Eucalyptus*

$$[5]\quad \mathrm{WTN\ (g/tree)} = 2.154 \times 10^{0.1678D}, \qquad r^2 = 0.97$$

where WTN is the total N in above- and below-ground biomass and D is stem basal diameter.

These FAE, WAE, and WTN data were used to calculate PNDFA and TNDFA using the formula of Fried and Middelboe (1977; eqs. 1 and 2). Total nitrogen difference (ND) was also calculated (eq. 3). As shown in Table 4, PNDFA estimates were very similar among treatments at 12 and 24 months, and ranged from approximately 48 to 76%. Slightly lower values were obtained using WAE data compared with FAE-based estimates, although these differences were not significant. These results indicate that for this plantation and these experimental conditions, foliar sampling would give as accurate estimates of PNDFA as whole-tree harvesting.

Estimates of TNDFA based on FAE or WAE data were very similar for each treatment at 12 and 24 months. As shown in Table 4, TNDFA did not differ significantly between the estimation methods. These also did not differ significantly from ND estimates, indicating that in this experiment *Eucalyptus* not only took up soil and added ^{15}N-enriched fertilizer N in the same ratio, but also took up the same amount of soil N and labeled N as *Casuarina*. There were no significant differences between the monoculture or mixed culture for the trenched quadrats. Nor were there any significant differences between these values and those of the CE untrenched treatment.

Conclusions

From the results of our study of this young plantation of *Casuarina* and *Eucalyptus*, we have demonstrated that the ^{15}N-enrichment methodology has proven reliable in providing estimates of PNDFA and TNDFA for tree species under field conditions. *Eucalyptus* proved to be an appropriate reference species for *Casuarina* under these experimental conditions. Foliar sampling for determining tissue ^{15}N-enrichment proved to be as accurate as whole tree harvesting, and random sampling of foliage in the canopy was not significantly different from a more systematic sampling strategy. We observed that interplanting the fixing and reference species did not significantly affect the amount of

TABLE 4. Percent nitrogen derived from fixation (PNDFA) and total nitrogen derived from fixation (TNDFA; g/m^2) in *Casuarina* based on foliar (FAE) and whole-tree (WAE) biomass sampling and the N-difference method (ND) in trenched and untrenched quadrats in monoculture and mixed-species stands at 12 and 24 months after plantation

Treatment*		PNDFA				TNDFA				ND	
		12 months		24 months		12 months		24 months			
Fixation	Reference	FAE	WAE	FAE	WAE	FAE	WAE	FAE	WAE	12 months	24 months
CM-t	EM-t	48.7 (16.6)	42.7 (16.8)	54.9 (15.0)	48.1 (16.2)	8.4 (3.0)	7.4 (3.0)	15.4 (3.6)	13.3 (4.1)	8.5 (0.2)	18.6 (4.7)
CE-u	EC-u	50.1 (8.2)	44.2 (7.9)	56.8 (21.4)	50.0 (23.1)	4.4 (0.7)	3.8 (0.7)	10.2 (5.1)	9.4 (4.9)	4.5 (1.3)	7.6 (4.7)
CE-t	EC-t	56.2 (10.1)	49.3 (10.4)	67.1 (6.5)	63.6 (4.6)	6.4 (1.8)	5.7 (1.7)	14.1 (6.0)	13.3 (5.5)	6.6 (1.8)	12.4 (3.6)

NOTE: Values are means with standard errors given in parentheses.
*CM, *Casuarina* monoculture; CE, *Casuarina* (with *Eucalyptus*); EM, *Eucalytpus* monoculture; EC, *Eucalyptus* (with *Casuarina*); u and t, refer to untrenched and trenched quadrats, respectively.

nitrogen fixed per tree, nor the accuracy of the estimates of PNDFA. However, we determined that untrenched quadrats where the reference and fixing species are not mixed are unsuitable for estimating PNDFA. The reference species in this situation does not accurately reflect soil N uptake for the fixing species when the size of the ^{15}N-enrichment plot is small relative to the area exploited by tree roots. We believe that the ^{15}N-enrichment methodology should be utilized more widely for estimating nitrogen fixation of trees and large woody perennial plant species.

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