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THEORETICAL IMPLICATIONS FOR THE ESTIMATION OF DINITROGEN FIXATION BY LARGE PERENNIAL PLANT SPECIES USING ISOTOPE DILUTION

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Abstract

THEORETICAL IMPLICATIONS FOR THE ESTIMATION OF DINITROGEN FIXATION BY LARGE PERENNIAL PLANT SPECIES USING ISOTOPE DILUTION.

Estimation of symbiotic N_2 fixation associated with large perennial plant species, especially trees, poses special problems because the process must be followed over a potentially long period of time to integrate the total amount of fixation. Estimations using isotope dilution methodology have begun to be used for trees in field studies. Because of the special problems of trees, certain precautions should be taken in the experimental design of isotope dilution studies. The fixing and non-fixing species should be interplanted so that each is exposed to the same changes in soil environment over the duration of the study. Under most circumstances, ^{15}N enriched fertilizer should be applied to the soil in a miniplot around which there has been placed a plastic film barrier to prevent the tree roots from gaining access to unlabelled N outside of the treated area differentially. A concurrent study of biomass accumulation and tissue N content should be undertaken to ensure that both the fixing and non-fixing species are physiologically similar and that N recycling is not influencing the accuracy of estimates of N_2 fixation. Formulas for estimation are presented that take into account the special needs of tree studies.

1. INTRODUCTION

The isotope enrichment method for estimating dinitrogen fixation, based on the dilution in the plant of an applied isotopically enriched fertilizer, has been utilized recently for tree crops under field conditions [1–3]. This method works very well for young trees in plantations because they are essentially equivalent to annual crop species for which the methodology was developed originally [4]. However, additional considerations must be made for more mature trees or those in natural stands. Nonetheless, stable isotope techniques do have the potential for providing modestly accurate estimations. A review is made of the difficulties of applying the isotope dilution technique to woody perennial species, and examples are provided from our own field studies of tree species.

1.1. Definition of the case study

A plantation of *Casuarina equisetifolia*, *Leucaena leucocephala* and *Eucalyptus robusta* was established on an experimental farm at the University of Puerto Rico in 1989, as decribed by Parrotta et al. [3]. The trees were planted in different patterns within the plantation to determine whether the experimental design influenced the estimation of N_2 fixation. There were six treatments and each treatment was replicated three times. Each treatment plot was 16 m $\times$ 16 m, with trees planted at a spacing of 1 m. Some plots were planted with equal mixtures of two species (*Eucalyptus*, plus one of the fixing species), whereas others were planted as monocultures of each. Smaller plots ('miniplots' of 4 m $\times$ 4 m for mixed species treatments and 3 m $\times$ 3 m for single species treatments) within the main treatment plot were labelled with ^{15}N enriched ammonium sulphate at a rate of 0.2 $g \cdot m^{-2} \cdot a^{-1}$ in a total application of 2 $g \cdot m^{-2} \cdot a^{-1}$, divided into several equal applications. In some of these labelled plots, a trench was dug around the area to be labelled with ^{15}N enriched fertilizer and a plastic barrier installed to prevent root access to soil outside of the ^{15}N treatment area. Total harvests of some trees from each treatment were made at 12 and 42 months and regressions calculated to predict biomass from linear measurements made at other sampling times.

2. TREE GROWTH AND N ACCUMULATION

Trees grow and accumulate N in their stems and roots through assimilation or dinitrogen fixation and lose N through litterfall and root turnover (Fig. 1). Growth in terms of N accumulation can be defined by the equation

$$\Delta N = N^{WT}_{(t_1)} + N^{lr}_{(t_0-t_1)} - N^{WT}_{(t_0)} \tag{1}$$

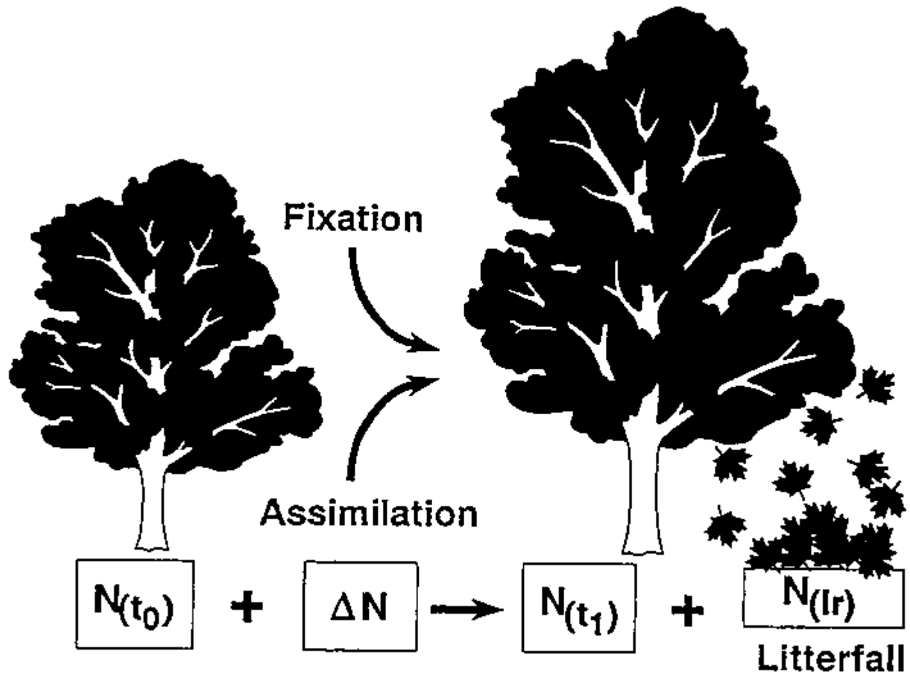

FIG. 1. Schematic representation of the process associated with growth and N accumulation and loss in trees from one timepoint (t_0) to another (t_1).

where $N^{WT}_{(t_1)}$ is the amount of N in the standing biomass of the tree at time t_1, $N^{WT}_{(t_0)}$ is the amount of N in the standing biomass at time t_0, and $N^{lr}_{(t_0-t_1)}$ is the amount of N lost in the form of litterfall and root turnover between t_0 and t_1. The goal of stable isotope methodology is to distinguish whether the *accumulated* N (ΔN) is derived from assimilation of N from the soil environment or from fixation of N_2 from the atmosphere. Actively growing trees obtain some N for current growth from internal reserves and lose some N through litterfall and root turnover as they age. For accurate estimations of dinitrogen fixation of large perennial species, the experimental design should include consideration of changes in overall biomass and the stored N in that biomass.

2.1. Estimation of biomass

The proportion of N derived from fixation by a symbiotic plant is yield *independent*. In simple terms, this means that both a large and a small plant may obtain the same percentage of N from fixation. However, the total amount of N fixed over a given period of time is yield *dependent*. This can be understood if we refer to the two parameters of N_2 fixation by a symbiotic plant. First is the proportion of N derived from atmosphere, abbreviated pNdfa. Second is the total mass of N derived from atmosphere (tNdfa), which is the total amount of N fixed. A large tree and a small shrub may have the same pNdfa, but if the tree is producing five times as much biomass per year containing the same %N then the tNdfa of the tree is five times greater than that of the shrub.

Determination of biomass in standing woody plants can be done through destructive harvesting, after which either the total harvested material, or some sample of it, is dried and weighed. Biomass estimation can be made using linear measurements (height, basal diameter, etc.) from which estimates of biomass are derived via formulas. To use measurements of height, diameter or other non-destructive measurements, regressions must exist that predict mass from them. Such regressions have been developed for only a few species and even these may not be sufficient for local growth conditions. Litterfall, which is also a part of the plant biomass and plant N, can be determined by sampling. Root turnover should also be included in ΔN, but it is not readily measured or estimated. In our studies in Puerto Rico, biomass was estimated from linear measurements based on two destructive harvests at 12 and 42 months. Variance among the replicates was considerable. Therefore, for the purpose of estimating pNdfa of ΔN (described below) we were required to make our calculations using the mean values of biomass N.

3. ENRICHMENT OF SOIL N WITH LABELLED FERTILIZER

Because tree species are large and require greater areas for growth, application of ^{15}N enriched fertilizers in studies of trees is much more problematic than in studies of small annual crops. Uniform fertilizer applications over the experimental plots should be made in quantities small enough so as not to appreciably affect the N_2 fixation, but not so small that the residence time in the soil is very short compared with the duration of growth of the trees. In general practice, application of label is made at a rate of approximately 0.2 g $^{15}N \cdot m^{-2} \cdot a^{-1}$ in a total application of fertilizer at the rate of 2 g $N \cdot m^{-2} \cdot a^{-1}$. Such an amount is unlikely to decrease N_2 fixation appreciably, yet has given relatively good results for observing isotopic dilution. Use of higher enrichments of ^{15}N would increase precision and simplify analysis, but would significantly increase costs.

3.1. Location of the reference species

When isotopic dilution methodology is used to estimate N_2 fixation in an annual crop, the reference species usually occupies a separate plot from that of the fixing species. Whether the two species are mixed or occupy separate plots, either the same results or only slightly different results are obtained for the $^{15}N/^{14}N$ ratio in the reference species. This is generally true, even with mixed swards of perennial forage crops, although under certain conditions and with certain mixtures greater differences may be expected. However, with forage crops the above ground biomass is harvested at each cutting and totally removed. This is not generally true for tree crops and, more importantly, the biomass left standing after coppicing or harvesting of a tree crop may be a considerable source of available N. Also, litter is significant for tree crops. The litter affects the available soil N supply and because the litter of N_2 fixing species will have a different $^{15}N/^{14}N$ ratio and a different total amount of N, the soil N supply and the $^{15}N/^{14}N$ ratio under a pure stand of non-fixing trees would soon differ from that under fixing trees [3]. Such a situation compounded over time will result in large errors in estimating pNdfa. Therefore, when applying the isotopic dilution method to perennial species in longer term studies, the reference species must be planted in close proximity to the fixing species so that it will be influenced over time by the same environmental and biological factors which influence the fixing species.

3.2. Timing of fertilizer applications

In general, repeated applications of small quantities of enriched fertilizer have been used with success. Such a practice allows the $^{15}N/^{14}N$ ratio of the available soil N to fluctuate only modestly over time and reduces the potential problems associated with the differential pattern of uptake of N by the fixing and reference species. Figure 2 illustrates the fluctuation in % ^{15}N atom excess in leaves over 3 years of ^{15}N enriched fertilizer applications of the same enrichment. In early applications of ^{15}N fertilizer, there were rapid fluctuations in the foliar ^{15}N concentration. With time, the ^{15}N enrichment in the soil became more stable and the trees grew and built up a large internal reserve of N. Fluctuations associated with fertilizer applications were considerably reduced.

3.3. Labelling area

Because tree roots are more likely to explore a very large soil volume than are herbaceous annual crops or even perennial forage legumes, the size of labelled plots is important. Very small plots (<4 m^2) require root isolation and therefore are unlikely to provide sufficient additional information beyond what could be gained from short term glasshouse studies. Larger plots become prohibitive because of the cost of the isotopically enriched fertilizer. A suitable alternative is the use of miniplots (≥ 9 m^2) within a larger treatment plot of trees. Isolating the soil of the miniplot by inserting a plastic barrier in a trench around the perimeter of the miniplot is a prudent technique to prevent roots from within the miniplot growing out and those outside from growing in. However, if the trees are uniformly spaced in the entire plot, labelled N is applied over the entire rooting area and, if reference and

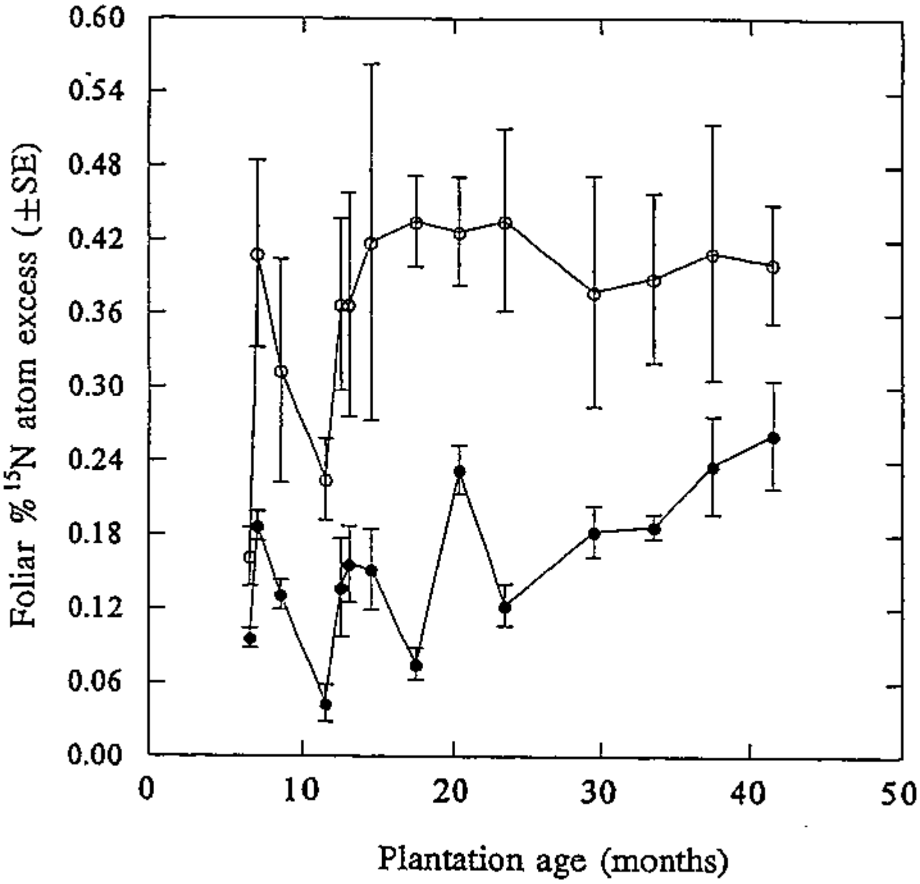

FIG. 2. Foliar ^{15}N content in Eucalyptus *(o) and* Leucaena *(•) over a period of time in which ^{15}N enriched fertilizer was applied. Fertilizer application occurred at 6, 12, 18, 24, 30, 34 and 38 months.*

fixing trees are properly paired with respect to position within the labelled miniplot (i.e. planted in a checkerboard pattern), trenching and use of a plastic barrier may not be necessary. Figure 3 illustrates the problem associated with too small a labelling area (16 m^2) and no soil barrier to root growth out of the labelled area. After 24 months, the untrenched *Eucalyptus* was obtaining soil N from outside the labelled plot area. This N is at low natural enrichment and is decreasing the % ^{15}N atom excess in the plant. A similar pattern of ^{15}N accumulation was not observed in the fixing species (Fig. 4) and therefore estimation of N_2 fixation using data from the untrenched plots would be erroneous.

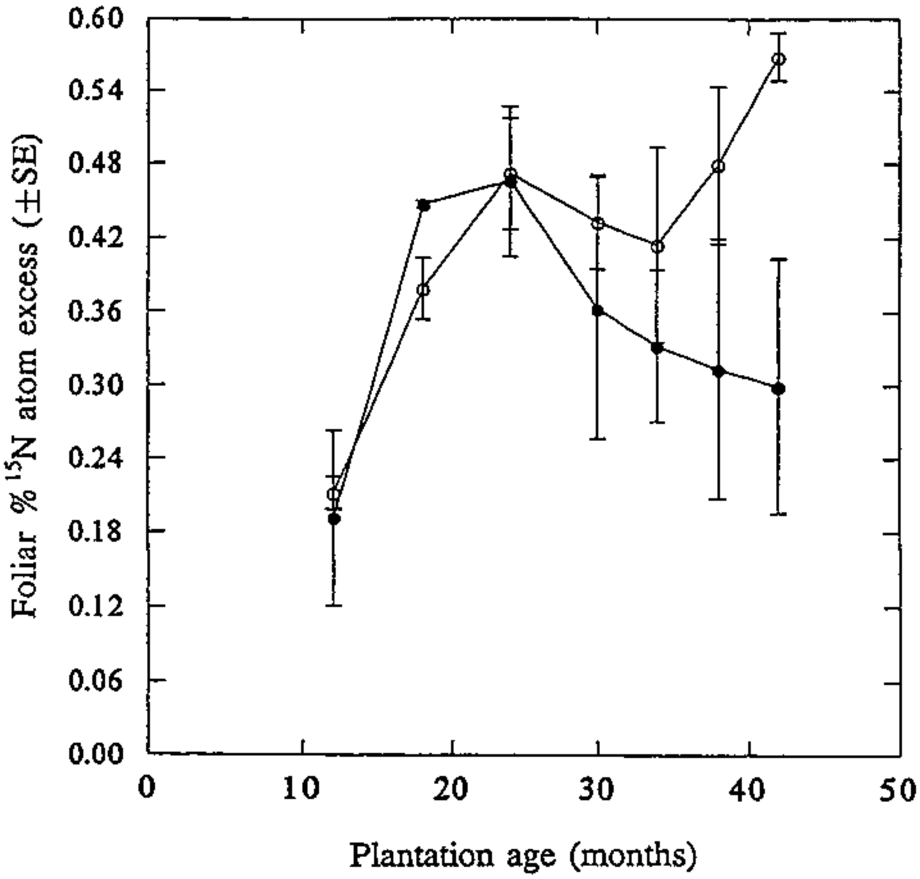

FIG. 3. Foliar ^{15}N content in Eucalyptus *interplanted with* Casuarina. *The pattern of ^{15}N assimilation was similar in trenched (○) and untrenched (●) plots up to approximately 24 months. Thereafter, the $^{15}N/^{14}N$ ratio of the untrenched plants began to decrease, probably as a result of root growth out of the labelled plot area.*

3.4. Isotopic enrichment and N recycling

When the isotope dilution method is used to determine N_2 fixation, the $^{15}N/^{14}N$ ratio of *accumulated* N (ΔN) during the time interval studied should be determined for both species. For more mature trees, the amount and proportion of N derived from internal tree reserves may differ for the fixing and reference species. For the fixing and reference species, the $^{15}N/^{14}N$ ratio of the stored N must be considered in the calculation of N_2 fixation by this method. To determine the $^{15}N/^{14}N$ ratio of the ΔN, estimation of total N as well as determination of the $^{15}N/^{14}N$ ratio

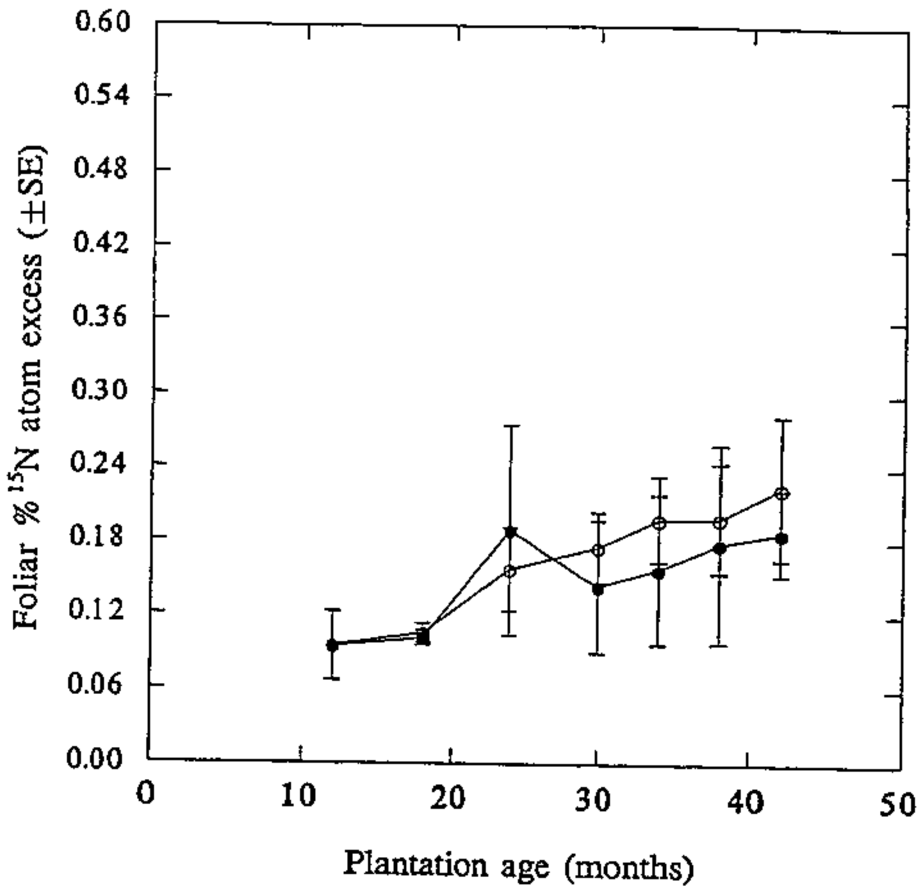

FIG. 4. Foliar ^{15}N content in Casuarina *interplanted with* Eucalyptus. *The pattern of ^{15}N accumulation was similar in trenched (○) and untrenched (●) plots throughout the duration of the study.*

would have to be made at the beginning of the experiment and at each sampling time for both the fixing and reference species. Subsequent sampling and analysis will allow calculation of ΔN using Eq. (1). The $^{15}N/^{14}N$ atom excess of the accumulated N for each species during each study period can be calculated using Eqs (2) and (3) (ae is atom excess)

$$ae^{WT} = \frac{[(N^{rt} \times ae^{rt}) + (N^{st} \times ae^{st}) + (N^{lv} \times ae^{lv})]}{N^{rt} + N^{st} + N^{lv}} \quad (2)$$

where rt, st and lv refer to roots, stems and leaves, respectively; and ae^{WT} is the whole tree weighted average of the % ^{15}N atom excess of the tree at any sampling time; and

$$ae^{\Delta N} = \frac{[(N^{WT} \times ae^{WT})_{t_1} + (N^{lr} \times ae^{lr})_{t_0-t_1} - (N^{WT} \times ae^{WT})_{t_0}]}{\Delta N} \tag{3}$$

where $ae^{\Delta N}$ is the % ^{15}N atom excess of the accumulated N between time t_0 and time t_1.

Once the ΔN atom excess is determined for both the fixing and reference species, these values can be used to calculate the pNdfa for the time interval (i.e. for the N accumulated during the observation period) by using a modified version of the formula of Fried and Middelboe [5]

$$\text{pNdfa} = \left[1 - \frac{ae^{\Delta N}_{Fix}}{ae^{\Delta N}_{Ref}} \right] \times 100 \tag{4}$$

TABLE I. ESTIMATIONS OF pNdfa BASED ON DIFFERENT METHODS OF CALCULATION[a]

Time interval (months)	pNdfa^{FM}	pNdfa^{MOD}
18–24	62.06	72.52
24–30	55.73	−4.25
30–34	49.74	35.51
34–38	54.09	70.68
38–42	59.02	72.35
18–30	55.73	48.50
24–38	54.09	46.63
30–42	59.02	60.38
12–42	59.02	61.23

[a] pNdfa^{FM} = calculation of pNdfa using the formula of Fried and Middelboe [5]; pNdfa^{MOD} = calculation of pNdfa using modified formulas accounting for ΔN.

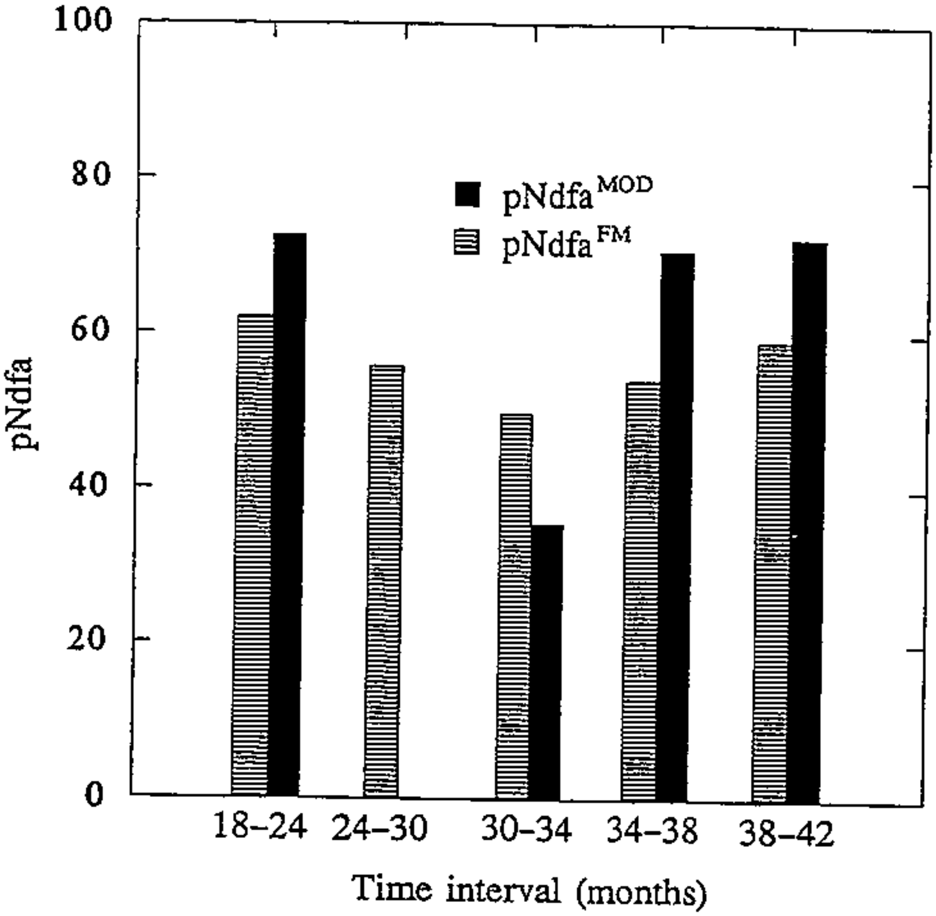

FIG. 5. pNdfa of Casuarina *interplanted with* Eucalyptus *in a trenched plot, calculated by the equation of Fried and Middelboe ($pNdfa^{FM}$) and Eq. (4) ($pNdfa^{MOD}$) for time intervals between two ^{15}N fertilizer applications.*

Table I compares the differences observed in calculating pNdfa using formula (4) with a comparison to the traditional Fried and Middelboe [5] formula. Figure 5 illustrates these differences visually for small time intervals (4–6 months) corresponding to the ^{15}N enriched fertilizer applications. In Fig. 6, the same data have been recalculated over longer time intervals.

The pNdfa calculated by the Fried and Middelboe [5] formula gives an integrated (cumulative) value over the entire lifetime of the tree. The results in Table I suggest that fixation in this *Casuarina* plantation continued at the same rate up to at least an age of 42 months. Estimations calculated by the equations above showed a similar pattern for longer time intervals (Fig. 6), but not always for shorter time intervals (Fig. 5).

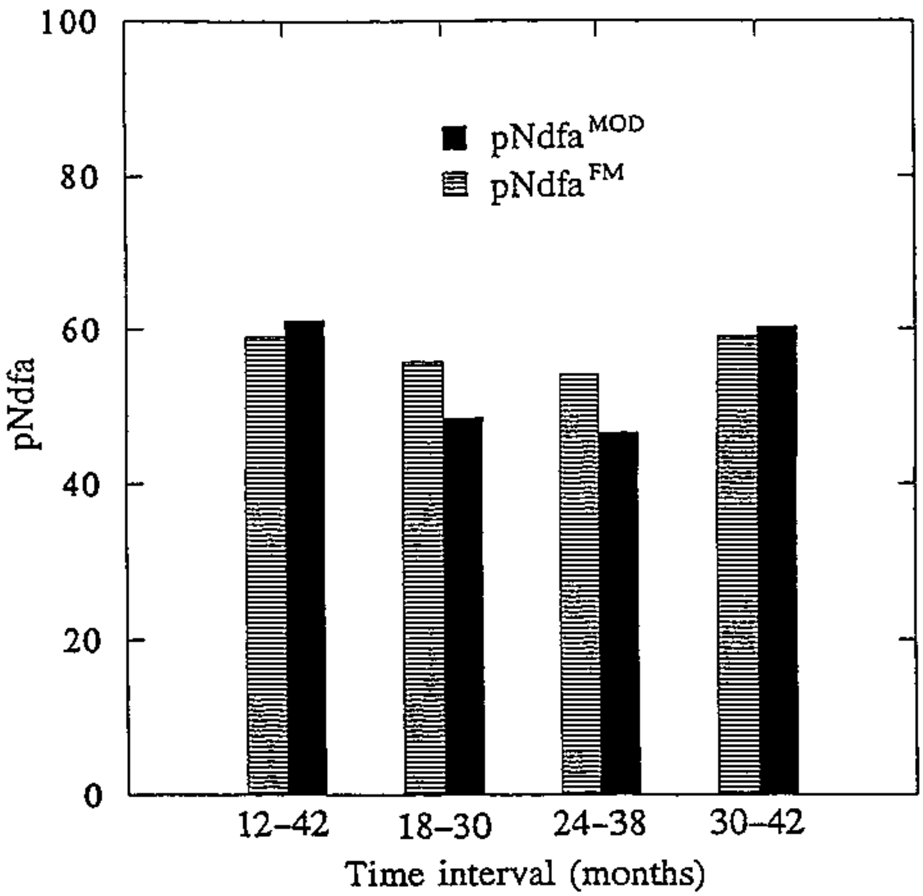

FIG. 6. The pNdfa of Casuarina *interplanted with* Eucalyptus *in a trenched plot, recalculated for time intervals covering numerous ^{15}N fertilizer applications.*

Determining pNdfa by isotope dilution of ΔN suffers from the potential error of estimating the differences in N content between two time periods. Since this estimate is based on a formula derived from measurements of the height, basal diameter, etc. of living trees, all of which are known to include significant variance, the estimate itself is subject to possible serious error. Furthermore, an estimate within a short time period is a small difference between two large numbers, both of which are subject to variance. Using longer time intervals (Fig. 6) appreciably improves the estimate. Because destructive harvesting was performed at 12 and 42 months in our study, the pNdfa^{MOD} for the time interval 12-42 months (Table I and Fig. 6) is based on actual measurements and does not suffer from the errors of estimation associated with data from the other time points.

The data presented herein is only an illustration of the estimation problems using data from an actual field study subject to all of the errors mentioned. Yet it must be realized that if one wants to know if a 5 year old tree is still fixing N, one may have to harvest whole trees or find ways of accurately estimating both the ΔN during a particular time interval and the ^{15}N atom excess of that ΔN.

Considering the short time intervals utilized in our study and the large potential errors associated with each data point, it is probably surprising that only one data point (Table I) was completely erroneous. Even this erroneous estimate can be corrected by calculating pNdfa over longer time intervals (Fig. 6) in which the errors of biomass estimation are considerably reduced. The results of applying Eq. (4) to the data of this one experiment suggest that under proper circumstances this approach can be utilized to determine the proportion of N derived from fixation of trees of more advanced age when reasonably accurate measurements of ΔN can be determined in a suitably designed $^{15}N/^{14}N$ isotope experiment.

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