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Mixed-species plantations of *Acacia mangium* and *Eucalyptus grandis* in Brazil 2: Nitrogen accumulation in the stands and biological N_2 fixation

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Abstract

The sustainability of plantation forests is closely dependent on soil nitrogen availability in short-rotation forests established on low-fertility soils. Planting an understorey of nitrogen-fixing trees might be an attractive option for maintaining the N fertility of soils. The development of mono-specific stands of *Acacia mangium* (100A:0E) and *Eucalyptus grandis* (0A:100E) was compared with mixed-species plantations, where *A. mangium* was planted in a mixture at a density of 50% of that of *E. grandis* (50A:100E). N_2 fixation by *A. mangium* was quantified in 100A:0E and 50A:100E at age 18 and 30 months by the ^{15}N natural abundance method and in 50A:100E at age 30 months by the ^{15}N dilution method. The consistency of results obtained by isotopic methods was checked against observations of nodulation, Specific Acetylene Reduction Activity (SARA), as well as the dynamics of N accumulation within both species. The different tree components (leaves, branches, stems, stumps, coarse roots, medium-sized roots and fine roots) were sampled on 5–10 trees per species for each age. Litter fall was assessed up to 30 months after planting and used to estimate fine root mortality. Higher N concentrations in *A. mangium* tree components than in *E. grandis* might be a result of N_2 fixation. However, no evidence of N transfer from *A. mangium* to *E. grandis* was found. SARA values were not significantly different in 100A:0E and 50A:100E but the biomass of nodules was 20–30 times higher in 100A:0E than in 50A:100E. At age 18 months, higher $\delta^{15}N$ values found in *A. mangium* tree components than in *E. grandis* components prevented reliable estimations of the percentage of N derived from atmospheric fixation (%Ndfa). At age 30 months, %Ndfa estimated by natural abundance and by ^{15}N dilution amounted to 10–20 and 60%, respectively. The amount of N derived from N_2 fixation in the standing biomass was estimated at 62 kg N ha^{-1} in 100A:0E and 3 kg N ha^{-1} in 50A:100E by the ^{15}N natural abundance method, and 16 kg N ha^{-1} in 50A:100E by the ^{15}N dilution method. The total amount of atmospheric N_2 fixed since planting (including fine root mortality and litter fall) was estimated at 66 kg N ha^{-1} in 100A:0E and 7 kg N ha^{-1} in 50A:100E by the ^{15}N natural abundance method, and 31 kg N ha^{-1} in 50A:100E by the ^{15}N dilution method. The most reliable estimation of N_2 fixation was likely to be achieved using the ^{15}N dilution method and sampling the whole plant.

Keywords: *Eucalyptus*; *Acacia*; Mixed-species plantations; N_2 biological fixation; ^{15}N natural abundance; ^{15}N dilution; ARA; Brazil

1. Introduction

Large areas of forest plantations have been established on low-fertility soils. The sustainability of those plantations causes concern when current emphasis on productivity and profitability leads to intensive silvicultural practices (improved germplasm, short rotations, change of species, etc.) and increases nutrient demands at the site. Input-output budgets suggest that the extent of tree response to nitrogen (N) inputs might be largely dependent on the characteristics of organic matter in the soil (content and properties) accumulated before afforestation, and the amount of N removed from the ecosystem in intensively managed plantations (Corbeels and McMurtrie, 2002; Laclau et al., 2005). Additional N inputs are needed to

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ensure sustainable production for many forest plantations managed in short rotations. However, N fertilizers account for a significant share of the silvicultural costs in Brazil and little information is available about their environmental impacts through nitrate leaching, NH_3 volatilization and nitrogen oxide emission in tropical forest plantations (Fisher and Binkley, 2000). A more environmentally sound option for supplying the N requirements of fast growing plantations and improving soil N status might be achieved by introducing N-fixing trees (NFT) in mixed-species plantations (Rothe and Binkley, 2001).

Various experiments have shown that mixed-species plantations of eucalypts with NFT have the potential to increase stand productivity when compared to eucalypt monocultures (Khanna, 1997; Binkley et al., 2003; Forrester et al., 2004; Wichiennopparat et al., 1998; Forrester et al., 2006). However, other studies have shown no impact, or a depressive effect, of mixtures on eucalypt growth (Parrotta et al., 1996; Forrester et al., 2006). A major difficulty in interpreting the effects of NFT in mixed-species plantations is estimating N inputs in the ecosystem through atmospheric N_2 fixation. Field-based estimates of N_2 fixation by perennial NFT are scarce (Binkley and Giardina, 1997; Forrester et al., 2006). That flux can be assessed by isotopic methods, Acetylene Reduction Activity (ARA) measurements, xylem ureide analysis, or comparison of N accumulation in N-fixing and non-fixing trees (Boddey et al., 2000). However, most of these methods provide only qualitative information. Reference methods for quantifying N fixation rates are based on ^{15}N isotopic measurements (Shearer and Kohl, 1986; Högberg, 1997; Dommergues et al., 1999; Boddey et al., 2000). Despite the importance of that flux for understanding the overall functioning of mixed-species plantations, the scarcity of field measurements might be explained by the difficulty in: (i) assessing a valid spatial-temporal sampling of tree components, (ii) selecting appropriate reference species, (iii) accounting for factors affecting $\delta^{15}N$ values within trees (sources of N, chemical form and depth of N taken up in the soil, mycorrhizal symbioses, etc.), and (iv) applying ^{15}N labelled fertilizer on a sufficient area owing to cost limitations (Boddey et al., 2000).

A combination of additive and replacement series was tested in Brazil, comparing mono-specific stands of *Eucalyptus grandis* (Hill ex Maid) and *Acacia mangium* (Wild) with mixed plantations in proportions of 1:1, and other stands with different densities of *A. mangium* for the same density of *E. grandis*. This article sets out to quantify N_2 biological fixation throughout the early growth of mixed-species plantations, comparing ^{15}N natural abundance and dilution methods. The consistency of results obtained from isotopic methods was checked against observations of nodulation, ARA, and N accumulation dynamics within both species.

2. Materials and methods

2.1. Site characteristics

The study was carried out at the Itatinga experimental station belonging to São Paulo University (23°02′S, 48°38′W). A complete description of the site is given by Laclau et al. (2008). The mean annual rainfall is 1360 mm with a cold season from June to September, and the average annual temperature is 19 °C. The relief is typical of the São Paulo Western Plateau, with a gentle undulating topography. The experiment was located on the top of a hill (slope < 3%). The soils were Ferralsols (FAO classification) developed on cretaceous sandstone, Marília formation, Bauru group. Textural uniformity was high (clay content ranging from 13% in the A_1 horizon to 25% at a depth of 6 m). The soil pH was acidic and the amounts of bioavailable nutrients were quite low.

The experiment was set up in a *Eucalyptus saligna* (Sm.) plot managed as a coppice, without fertilizer application from 1940 to 1997. The stumps were devitalized and *E. grandis* seedlings were planted in 1998 with low fertilizer inputs (300 kg ha^{-1} NPK 10:20:10). High levels of nutrient exports with the boles from 1940 to 1998 made this a suitable area for expecting a eucalypt response to N inputs.

2.2. Experiment

The *E. grandis* stand was harvested in December 2002. Only the boles were removed from the plot and slashes were spread uniformly in the field. A complete randomized block design was set up in May 2003, with 7 treatments and 4 blocks, in order to assess the influence of an *A. mangium* understorey on the growth of *E. grandis* seedlings (mono-progeny from the Suzano Company). Each plot had a total area of 30 m × 30 m and an inner plot of 18 m × 18 m with two buffer rows. The treatments were:

T1: 100A:0E: *A. mangium* planted at a spacing of 3 m × 3 m, without N fertilization;
T2: 0A:100E: *E. grandis* planted at a spacing of 3 m × 3 m, without N fertilization;
T3: 0A:100E + N: *E. grandis* planted at a spacing of 3 m × 3 m, with application of 120 kg N ha^{-1};
T4: 25A:100E: *E. grandis* planted at a spacing of 3 m × 3 m + *A. mangium* planted in a mixture at a density of 25% of the *E. grandis* density;
T5: 50A:100E: *E. grandis* planted at a spacing of 3 m × 3 m + *A. mangium* planted in a mixture at a density of 50% of the *E. grandis* density;
T6: 100A:100E: *E. grandis* planted at a spacing of 3 m × 3 m + *A. mangium* planted in a mixture at a density of 100% of the *E. grandis* density;
T7: 50A:50E: Mixture in a proportion of 1:1 between *E. grandis* and *A. mangium* (555 trees per hectare of each species), without N fertilization.

The *E. grandis* seedlings were planted in the interrow after subsoiling (depth 40 cm). *A. mangium* seedlings were inoculated with rhizobium strains selected by EMBRAPA (Rio de Janeiro) for their N_2 fixation capacities, and exhibited high levels of nodulation in the nursery. They were planted at mid-distance between *E. grandis* trees in 25A:100E, 50A:100E and 100A:100E, in the same planting rows, to avoid modifying

accessibility in the stand. Fertilizer inputs were representative of commercial silviculture in that area and previous experiments showed that they were not limiting for tree growth. Two tonnes per hectare of dolomitic limestone were applied on planting and 40 g P plant^{-1} were dug in 20 cm from the plants, as well as 9 g K plant^{-1}, 3 g B plant^{-1}, 6 g Fe plant^{-1}, 3 g Zn plant^{-1}, and 1 g Mn plant^{-1}. In 0A:100E + N, 30 kg N ha^{-1} were applied (ammonium nitrate form) on planting. Three complementary fertilizations were applied, with 25 kg K ha^{-1} at 6, 12 and 18 months after planting in all treatments, as well as 30 kg N ha^{-1} in 0A:100E + N only, on the same application dates (total application of 120 kg N ha^{-1}).

Treatments selected for N accumulation estimations were also established in about 10 inner buffer rows either side of each block, in order to carry out sequential destructive samplings without disturbing stand growth inside the trial. However, the number of acacias in 50A:100E was not sufficient in the buffer rows and several trees were sampled from age 18 months in the 50A:100E plot of block 4.

2.3. Biomass and N accumulation

2.3.1. Aboveground tree components

Circumference at breast height and height were measured excluding 2 buffer rows in each plot (36–72 trees per plot measured) at ages 4, 9, 12, 17, 24, 29 and 37 months. Crown diameter was measured at 9 and 12 months of age for each tree in 2 perpendicular directions. Most of the *A. mangium* trees were multi-stem trees and the circumference of all the stems with a diameter at breast height >2 cm was measured on each inventory.

A complete description of sampling methods was described in Laclau et al. (2008). In brief, aboveground biomass was estimated sampling 6 trees distributed over the range of heights in 100A:0E and 0A:100E at age 6 months. At ages 12, 18 and 30 months, respectively, 6, 8 and 10 trees of each species were sampled over the range of basal areas in three treatments (100A:0E, 0A:100E and 50A:100E). The trees were separated into components: leaves, living branches, dead branches, stemwood and stembark. Diameters, lengths and weights were measured in the field. Tree foliage was divided in 3 thirds according to tree height at 18 and 30 months after planting. Sub-samples were taken from all the components, dried at 65 °C to constant weight, and ground for determination of total N concentrations. Biomass and N content regressions were established for each component from age 6 to 30 months, as polynomials of the circumference at breast height and age (Laclau et al., 2008). Allometric equations established at 6 months, sampling *E. grandis* and *A. mangium* trees in 100A:0E and 0A:100E, were applied for the same species in 50A:100E, since it was found that competition did not influence tree growth at that age.

2.3.2. Belowground tree components

Stumps and coarse roots (diameter >1 cm) were excavated at 18 months for all the trees sampled aboveground. At 30 months after planting, stumps and coarse roots were excavated for 5 *E. grandis* trees covering the range of basal areas (among the 10 trees sampled aboveground), and all the *A. mangium* trees sampled aboveground in 100A:0E, 0A:100E and 50A:100E (total of 10 eucalypts and 20 acacias). Medium-sized roots (diameter between 3 and 10 mm) were sampled in 4 pits for each treatment 100A:0E, 0A:100E and 50A:100E, at ages 18 and 30 months. After removing carefully adhering soil by hand, stumps and roots were weighed and sampled. The samples of each component were dried at 65 °C to constant weight and ground for chemical analysis. Fine root biomass was quantified at ages 6, 12, 18 and 30 months in 100A:0E, 0A:100E and 50A:100E. Fine roots were sampled at 5–12 distances from selected trees with a root auger in twelve plots (3 treatments × 4 blocks) down to a depth of 1 m at ages 6 and 12 months and down to a depth of 2 m at 18 and 30 months. Composite samples of each root size were calcinated at 450 °C for 4 h and belowground biomasses were corrected to reach an ash content of 3% corresponding to the mean value found for the aerial components not contaminated by soil particles.

2.4. Return of N to soil

The amount of N taken up by the trees since planting was the sum of N accumulated in the standing biomass with the flux of N returning to the soil through litter fall and fine root mortality.

2.4.1. Litter fall

Litter fall was collected every 4 weeks in 9 plots (100A:0E, 0A:100E and 50A:100E in 3 blocks) up to age 36 months. Leaves, flowers and fruits were collected from 5 litter-traps (52 cm × 52 cm) systematically located in the stands to sample representatively different distances from the trees (15 traps per treatment). Bark and branches were collected in a 9 m^2 area delimited between 4 trees in each plot. The samples of each component were dried at 65 °C to constant weight and ground for chemical analysis.

2.4.2. Root mortality

Fine root mortality was not measured in this experiment and studies quantifying that flux are scarce in tropical forest plantations. Measurements in Congolese eucalypt stands highlighted values of fine root turnover of the same extent as leaf turnover at age 2 years and the same trend was observed in an experiment with contrasted N inputs at the Itatinga experimental station (unpublished data). The ratio between fine root mortality and fine root living biomass throughout each development stage was then considered to be identical to that measured between leaf litter fall and leaf standing biomass (Cotrufo, 2006). Fine root production was estimated summing the increment in fine root biomass throughout each period with fine root mortality assessed from leaf turnover. The amount of N taken up to produce fine roots was calculated by multiplying N concentration within living fine roots by the mass of fine roots produced (mean living fine root biomass + mortality estimation over the period).

2.5. Estimation of N_2 fixation

The fixation of atmospheric N_2 by *A. mangium* was estimated by three different methods.

2.5.1. Acetylene Reduction Activity

Acetylene Reduction Activity measurements were carried out according to Hardy et al. (1968) on fresh nodules using a gas chromatograph (Agilent 6850 Series GC System) to determine nitrogenase activity. That method cannot give quantitative reliable figures for N_2 fixation but may provide an insight into the relative intensity of atmospheric nitrogen fixation by NFT depending on the treatments applied (Fisher and Binkley, 2000; Watt et al., 2003). Nodules were collected at 18 and 30 months after planting in the 0–20 cm soil layer at 8 distances from four *A. mangium* trees sampled for biomass estimation. For each position, roots with attached nodules were placed in 150 ml glass jars containing air/acetylene (9:1, v/v), and 10 ml gas samples were removed after 20- and 40-min incubation periods in the field for C_2H_4 analysis. The biomass of *A. mangium* nodules was quantified by auger sampling down to a depth of 2 m in 100A:0E and 50A:100E at 18 months and 30 months after planting. Five distances from an average *A. mangium* tree were sampled in 8 plots (2 treatments × 4 blocks) and the nodules separated by hand-picking in the 0–10, 10–30, 30–50, 50–100, 100–150, and 150–200 cm layers. The samples were dried at 65 °C and weighed after removing carefully adhering soil by hand. Specific Acetylene Reduction Activity (SARA) was calculated as the ratio between ARA and the dry biomass of nodules.

2.5.2. ^{15}N natural abundance

The suitability of the area for assessing N_2 fixation by the natural abundance method was investigated before setting up the experiment. A grass species and eucalypt sprouts sampled systematically throughout the area exhibited mean $\delta^{15}N$ values of 4.9 and 3.8‰, respectively, with standard deviations of 1.0 and 0.4‰ ($n = 6$ for each species).

Inter-block variability of $\delta^{15}N$ was assessed in 100A:0E, 0A:100E and 50A:100E from leaf samples of both *E. grandis* and *A. mangium* trees. Recent fully expanded leaves were sampled in the higher third of the canopy of 6 trees per plot at ages 18 and 30 months. ^{15}N determinations were performed for 4 composite samples (4 blocks) per species and per treatment.

N concentrations and $\delta^{15}N$ values within aerial tree components (leaf, branch, stemwood, stembark) were determined in 100A:0E, 0A:100E and 50A:100E, for 4 trees per species at age 18 months and 10 trees per species at 30 months after planting. Additional leaf samples were collected at 18 months on 4 trees per species in each treatment. Belowground parts (stump, coarse roots, medium-sized roots, and fine roots) were sampled on 3 trees per species covering the range of basal areas in each treatment at age 18 months and 4 trees at age 30 months.

The percentage of N derived from atmospheric N_2 (%Ndfa) was calculated according to the following equation (Shearer and Kohl, 1986):

$$\%\text{Ndfa} = \frac{100(\delta^{15}N_{REF} - \delta^{15}N_F)}{(\delta^{15}N_{REF} - B)} \quad (1)$$

where $\delta^{15}N_{species} = [(^{15}N/^{14}N)_{species} - (^{15}N/^{14}N)_{air}]/(^{15}N/^{14}N)_{air}$, $\delta^{15}N_{REF}$ was the relative natural isotopic abundance of *E. grandis*, chosen as the reference non-fixing tree, $\delta^{15}N_F$ the relative isotopic abundance of *A. mangium*, and B the relative isotopic abundance of *A. mangium* growing on N-free medium. The mean B value of −0.3‰ measured by Galiana et al. (2002) for *A. mangium* was used for Ndfa% calculations in this study.

$\delta^{15}N$ values were not significantly correlated with tree size, irrespective of the component and the species (data not shown). In each tree component, mean values of $\delta^{15}N$ for the treatment were then used to estimate the %Ndfa at each age. No significant differences in $\delta^{15}N$ were observed between *E. grandis* growing in pure stands (EP) and in a mixture (EM), irrespective of the tree component. The relative natural isotopic abundance was then calculated as the mean of the $\delta^{15}N$ values measured in *E. grandis* trees sampled both in 0A:100E and 50A:100E.

A weighted average of the relative isotopic abundance of the sampled tree was calculated from the following equation (modified from Guinto et al., 2000):

$$\delta^{15}N_{tree} = \frac{\begin{array}{l}(\delta^{15}N_{leaves}N_{leaves} + \delta^{15}N_{branches}N_{branches} + \delta^{15}N_{stem}N_{stem} + \delta^{15}N_{stump}N_{stump} + \delta^{15}N_{coarse\,roots}N_{coarse\,roots} \\ + \delta^{15}N_{medium\,roots}N_{medium\,roots} + \delta^{15}N_{fine\,roots}N_{fine\,roots})\end{array}}{(N_{leaves} + N_{branches} + N_{stem} + N_{stump} + N_{coarse\,roots} + N_{medium\,roots} + N_{fine\,roots})} \quad (2)$$

where $N_{component}$ was the N content of the tree component estimated on a stand level by applying N allometric equations to the stand inventory, except for medium-sized and fine roots (hectare-basis mean of the N contents of roots estimated by auger sampling and excavation in 100A:0E, 0A:100E and 50A:100E). Stembark and stemwood were analysed separately at age 30 months but not at age 18 months. A weighted average $\delta^{15}N$ for the stem was therefore calculated at 30 months after planting from the following equation:

$$\delta^{15}N_{stem} = \frac{(\delta^{15}N_{stemwood}N_{stemwood} + \delta^{15}N_{bark}N_{bark})}{(N_{stemwood} + N_{bark})} \quad (3)$$

As *A. mangium* litter fall primarily consisted of leaves over the study period, the $\delta^{15}N$ values of leaves and litter fall were considered as equals. Moreover, it was also assumed that the

$\delta^{15}N$ values of living and dead fine roots were equal. Component-specific %Ndfa values were then calculated from Eq. (1) for litter fall and dead fine roots.

2.5.3. ^{15}N dilution

The study was conducted in a 0.20 ha area planted with the 50A:100E design and separated from block 4 by five buffer rows. Ammonium sulphate (0.5 atom% ^{15}N) was applied 18 months after planting, at a rate of 20 kg N ha^{-1}. The fertilizer was diluted in water and applied uniformly to the soil using a watering can.

Four *A. mangium* and four *E. grandis* covering the range of basal areas were sampled at 30 months after planting in the inner plot. Representative samples of the different biomass components (leaf, branch, stemwood, stembark, stump and coarse root) were collected for each tree. For a given tree component %Ndfa was calculated from the following equation (Fried and Middelboe, 1977):

$$\%\text{Ndfa} = 100\left[1 - \frac{(\text{AE}_{\text{F30}})}{\text{AE}_{\text{REF30}})}\right] \quad (4)$$

where $AE = {}^{15}N \times 100/({}^{15}N + {}^{14}N) - 0.003663$, AE_{F30} was the percentage atom excess of *A. mangium* at age 30 months and AE_{REF30} was the percentage atom excess of *E. grandis* at age 30 months. The natural abundance methodology was used to estimate %Ndfa of medium and fine roots. A weighted average AE for the standing trees was estimated from Eq. (2). The rate of N_2 fixation was considered unchanged before and after soil labelling, and not significantly influenced by the application of 20 kg N ha^{-1} (Parrotta et al., 1994a).

2.6. Chemical and isotopic analysis

Total N was analysed individually for all tree components sampled at each age, by acid base volumetry after Kjeldahl mineralization. ^{15}N natural abundance was measured using a mass spectrophotometer (Delta Plus, Thermo Electron, Bremen, Germany) coupled to an elemental analyser (Carlo Erba NA 1110 CHNS, CE Instruments, Rodano, Italy). The precision of the measurement was 0.5‰. Isotopic analyses were performed for the ^{15}N dilution experiment with a 20–20 mass spectrometer coupled to an automatic N analyser, model ANCA-SL, from PDZ Europe, Crewe, United Kingdom. The analytical precision was ±0.001 atom% ^{15}N.

2.7. Statistical analysis

Pearson correlation coefficients were calculated using the SAS PROC COR procedure. Homogeneity of variances was tested at each age by Levene's test and original values were log transformed when variances were unequal. Differences between treatments were tested using one-way or two-way ANOVA (SAS, 1998). The probability threshold used to determine significance was $P < 0.05$. When the ANOVA indicated significant effects, the means were compared with Newman Keuls' multiple comparison tests, up to age 18 months. From that age onwards, the 50A:100E plot in block 4 was excluded from the analysis and the PDIFF statement of PROC GLM was used.

3. Results

3.1. N concentrations in trees

A. mangium in 50A:100E (AM) exhibited higher N concentrations than *E. grandis* growing both in a pure stand (EP) and mixed plantations (EM). The differences were statistically significant except for stems and branches at 12 months, and medium-sized roots at 30 months after planting (Table 1). A similar trend was observed for *A. mangium* in 100A:0E (AP) which exhibited significantly higher N concentrations than *E. grandis*, except for leaves, stems and branches at 18 months. In those components, AP displayed lower N concentrations than *E. grandis* but the differences between the two species were not significant. N concentrations in all tree components were not significantly different between EP and EM, except for stemwood at 30 months. N concentration among components decreased in the following order: leaf > bark > fine root > branch > stump/coarse roots/medium-sized roots > stemwood. A larger decrease in N concentrations from age 18 months to age 30 months was observed for AM than for AP in all the tree components, except leaves. A downward trend in N concentration with stand age was also observed in *E. grandis* tree components.

Table 1
N concentration (g kg^{-1}) in the tree components of *A. mangium* for 100A:0E (AP) and 50A:100E (AM), and of *E. grandis* for 0A:100E (EP) and 50A:100E (EM)

Component	12 months				18 months				30 months			
	AP	EP	AM	EM	AP	EP	AM	EM	AP	EP	AM	EM
Leaves	28.8a	20.8b	29.4a	20.4b	18.5b	19.1b	24.0a	18.8b	21.5b	17.7c	24.7a	18.3c
Stemwood (1)									2.5b	1.5d	2.7a	1.7c
Bark (2)									9.0b	3.4c	13.1a	3.5c
Stem = (1) + (2)	11.7a	2.1b	7.2ab	2.5b	4.4b	5.8b	10.6a	3.3b	3.8b	1.9c	4.8a	2.0c
Branches	9.1a	4.1b	8.4ab	4.4b	7.2b	8.6b	13.7a	5.3b	5.3b	3.3c	6.7a	3.3c
Stump + coarse roots					6.3a	4.0c	5.2b	3.2c	4.9a	2.8b	4.4a	2.5b
Medium-sized roots					11.1a	4.4b	9.5a	4.4b	9.1a	3.2b	5.6b	2.8b
Fine roots					16.7a	8.0b	16.7a	8.1b	13.7a	6.1c	9.0b	5.3c

Different letters (a–c) indicate significant differences ($P < 0.05$) between species for a given age and tree component.

Table 2
Nitrogen content (kg N ha^{-1}) accumulated at 30 months after planting in the stands and total uptake from planting, including the returns to soil with litter fall and fine root mortality in 100A:0E, 0A:100E, and 50A:100E

Tree components	100A:0E	0A:100E	50A:100E	
	Acacia	Eucalypt	Acacia	Eucalypt
Leaves	123.6a	89.9b	11.8c	101.3b
Stem	77.0a	52.2b	6.6c	61.6b
Branches	43.9a	22.7b	3.8c	20.0b
Stump + coarse roots	15.1b	24.1a	1.4c	24.7a
Medium-sized roots	5.2a	2.5b	0.1c	1.7b
Fine roots	38.8a	19.4b	2.3c	15.2b
Total N in standing biomass	303.6a	210.8b	27.2c	223.5b
Litter fall	90.0b	105.0a	15.8c	95.0b
Fine root mortality[a]	51.8	59.7	9.5	39.7
Total N uptake since planting[a]	445.4	375.5	52.5	358.2

Different letters (a–c) indicate significant differences ($P < 0.05$) between species for a given age and tree component.
[a] Root mortality estimation did not allow any statistical analysis.

3.2. N accumulation

N content in the aboveground parts was significantly higher in the mixed-species stand (50A:100E) than in mono-specific stands (100A:0E and 0A:100E) up to 12 months after planting (data not shown). Thereafter, N accumulation was higher in the pure *A. mangium* stand (100A:0E) than in 50A:100E, despite a lower stocking density. Thirty months after planting, N accumulation in the leaves amounted to 90, 113 and 124 kg ha^{-1} in 0A:100E, 50A:100E and 100A:0E, respectively, representing 40–45% of the amount of N in the standing biomass (Table 2). The nitrogen content of stumps and roots at age 30 months ranged from 45 to 59 kg N ha^{-1}, representing about 20% of the total accumulation of N in the stands. The total amount of nitrogen taken up by trees over 30 months after planting in 100A:0E, 0A:100E and 50A:100E was about 445, 375 and 410 kg N ha^{-1}, respectively. Litter fall and fine root mortality were two major fluxes of the N cycle, with a total input to soil throughout the 30-month period estimated at about 140 kg N ha^{-1} in 100A:0E, 165 kg N ha^{-1} in 0A:100E and 160 kg N ha^{-1} in 50A:100E. The flux of nitrogen provided by fine root mortality over that period was estimated at about 17 and 35% of the amount of N accumulated in the standing biomass of AP and AM respectively, at age 30 months.

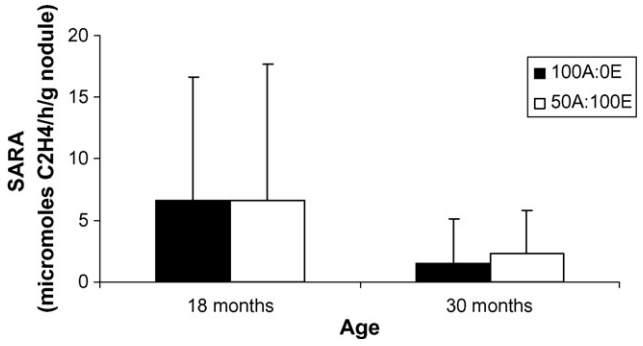

Fig. 1. Mean Specific Acetylene Reduction Activity (SARA) values at 18 and 30 months after planting for *A. mangium* growing in 100A:0E and in 50A:100E. Bars represent standard deviations ($n = 32$).

3.3. Estimation of N_2 fixation

3.3.1. ARA

High within-treatment variability in SARA was observed but no significant correlation was found between SARA values and tree size or distance from *A. mangium* stems. ARA in AP and AM were not significantly different and values were 3–4 times lower at 30 months after planting than at age 18 months (Fig. 1). For a given dry biomass of nodules, the N_2 fixation potential was then roughly equivalent in the two treatments. Nodules were observed down to a depth of 2 m, but 85 and 40% of the nodule biomass was found in the 10–50 cm soil layer at 18 and 30 months of age, respectively. Nodule biomass was not significantly different at 18 and 30 months after planting in each treatment. However, nodule biomass was 20–30 times higher in 100A:0E than in 50A:100E, whereas the stocking density of *A. mangium* was only twice as high in 100A:0E (Fig. 2a and b). The *A. mangium* trees growing in a monoculture then had a much greater potential to fix atmospheric N_2 than in a mixture with *E. grandis*.

3.3.2. ^{15}N natural abundance

Comparisons of $\delta^{15}N$ values in composite samples of leaves collected at 18 and 30 months after planting in 100A:0E, 0A:100E and 50A:100E showed that the inter-block variability

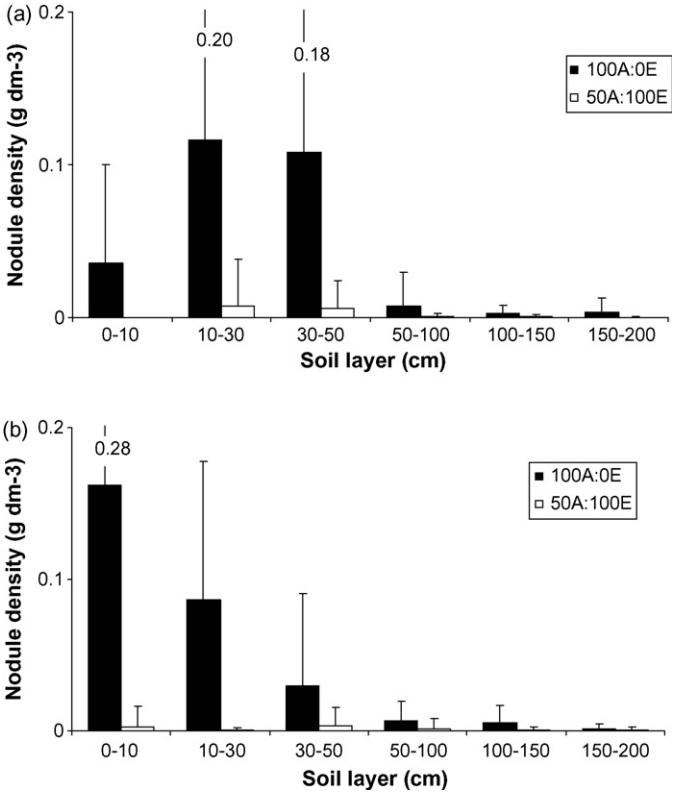

Fig. 2. Changes in nodule biomass with soil depth for *A. mangium* growing in 100A:0E and in 50A:100E. Observations at 18 months (a) and at 30 months after planting (b). Bars represent standard deviations ($n = 20$).

Table 3
Relative ^{15}N natural abundance ($\delta^{15}N$ expressed in ‰) in biomass components of *A. mangium* in 100A:0E (AP) and 50A:100E (AM), and *E. grandis* in 0A:100E and 50A:100E (E)

Tree components	18 months			30 months		
	AP	AM	E	AP	AM	E
Relative natural abundance ($\delta^{15}N$)						
Leaves	4.09a	3.39a	3.37a	1.89a	1.88a	1.95a
Stem	2.58a	2.06a	2.69a	1.02a	1.33a	1.40a
Branches	2.73a	2.51a	2.75a	0.06a	0.86ab	1.20b
Stump + coarse roots	2.10a	2.21a	2.02a	1.25a	1.90a	2.20a
Medium-sized roots	3.35a	2.51a	2.94a	0.72a	0.48a	1.22a
Fine roots	0.46a	1.82ab	2.26b	0.79a	0.36a	0.81a
Within-tree range	3.63	1.57	1.35	1.83	1.42	1.39
Ratios of $\delta^{15}N$						
R leaves	1.21	1.01		0.97	0.96	
R stem	0.96	0.77		0.73	0.95	
R branches	0.99	0.91		0.05	0.61	
R stump + coarse roots	1.04	1.09		0.57	0.86	
R medium-sized roots	1.14	0.85		0.59	0.39	
R fine roots	0.20	0.81		0.98	0.44	

Within-tree ranges of $\delta^{15}N$ among components and ratio (*R*) between $\delta^{15}N$ in *A. mangium* and *E. grandis* are indicated. Different letters (a and b) for a given age and tree component indicate significant differences ($P < 0.05$) between species.

was not significant, irrespective of the species and sampling age (data not shown). Mean $\delta^{15}N$ values were low, most of them ranging from 0.5 to 3‰, with a global downward trend from 18 to 30 months after planting (Table 3). Even though differences were not significant, $\delta^{15}N$ values in leaves and stump + coarse roots were lower in *E. grandis* trees than in AP and AM at age 18 months, and in medium-sized roots of *E. grandis* trees than in AP. By contrast, $\delta^{15}N$ values were lower in *A. mangium* than in *E. grandis* at 30 months after planting, irrespective of tree components. For a given species and treatment, a high variability in $\delta^{15}N$ was observed among tree components. Large differences in the ratio (*R*) between the $\delta^{15}N$ values measured in *A. mangium* and *E. grandis* were also found between the tree components and the sampling ages (Table 3).

At age 18 months, the higher $\delta^{15}N$ values measured for *A. mangium* than for *E. grandis* in various tree components prevented reliable estimations of %Ndfa. By contrast, even though most of the differences between the two species were not significant in the 30-month-old stands, the %Ndfa was estimated at that age since $\delta^{15}N$ values in all the components of *A. mangium* trees were lower than those in *E. grandis* trees.

The amounts of atmospheric N_2 fixed accounted for 10–20% of the amount of N accumulated in the standing biomass and cumulated over 30 months of growth (Table 4). The corresponding amounts of N derived from atmospheric fixation were much higher for AP (62.3 and 65.8 kg N ha^{-1} respectively) than for AM (2.8 and 7.1 kg N ha^{-1} respectively).

Table 4
Estimation of the percentage of nitrogen derived from atmospheric N_2 (%Ndfa) in *A. mangium* in 100A:0E (AP) and 50A:100E (AM), at 30 months after planting

	^{15}N natural abundance		^{15}N dilution
	AM	AP	AM
%Ndfa			
Standing biomass	10.2%	20.5%	58.8%
Since planting	13.5%	14.8%	58.3%
N derived from atmospheric N_2			
N fixed in standing biomass	62.3	2.8	16.0
Total N fixed since planting	65.8	7.1	30.6

Values (kg N ha^{-1}) of N derived from atmospheric N_2 in the standing biomass and in the total amount of N accumulated since planting are indicated. The ^{15}N natural abundance and ^{15}N dilution methods were used.

3.3.3. ^{15}N dilution

The mean values of AE ranged from 0.0015 to 0.0091‰, with marked variability among tree components (Table 5). *E. grandis* exhibited significantly higher AE values than *A. mangium*, irrespective of tree components. The *R* ratio ranged from 0.22 to 0.74, but only from 0.33 to 0.48 when stemwood and stembark were not considered separately.

The ^{15}N dilution method showed higher percentages of N_2 fixation than the ^{15}N natural abundance method (Table 4). The amount of nitrogen derived from atmospheric N_2 fixation was estimated at 16.0 kg N ha^{-1} in the 30-month-old stand and 30.6 kg N ha^{-1} including N returns to soil since planting, with litter fall and fine root mortality. The corresponding %Ndfa amounted to about 60%.

3.3.4. Use of leaves or whole-tree components to assess N_2 fixation

High variability in $\delta^{15}N$ was found among tree components, as well as in the ratio of $\delta^{15}N$ between *A. mangium* and *E. grandis* trees. Moreover, as both species exhibited great variations in N content among tree components, the use of leaf $\delta^{15}N$ instead of whole-tree $\delta^{15}N$ led to differences of 70–90% in the estimation of N_2 fixation by the ^{15}N natural abundance method (Table 6). Those differences were less marked (15–20%) when the ^{15}N dilution method was used.

Table 5
^{15}N atom excess (AE in ‰) in tree components of *A. mangium* and *E. grandis* in 50A:100E measured at 30 months after planting

Component	^{15}N atom excess (AE)	
	A. mangium	*E. grandis*
Leaves	0.0019a	0.0058b
Stemwood	0.0068a	0.0091b
Bark	0.0015a	0.0068b
Stem = stemwood + bark	0.0040a	0.0084b
Branches	0.0023a	0.0068b
Stump + coarse root	0.0028a	0.0080b
Maximum difference	0.0066	0.0049
	Ratios of ^{15}N atom excess	
R leaves	0.33	
R stemwood	0.74	
R stembark	0.22	
R stem	0.48	
R branch	0.34	
R stump + coarse roots	0.35	

Ratios (*R*) of AE between *A. mangium* and *E. grandis* trees in 50A:100E are indicated. Different letters (a and b) indicate significant differences ($P < 0.05$) between *A. mangium* and *E. grandis*.

Table 6
Amounts of N derived from atmospheric N_2 (kg N ha^{-1}) for *A. mangium* in 100A:0E (AP) assessed by the ^{15}N natural abundance, and in 50A:100E (AM) assessed by the ^{15}N natural abundance and ^{15}N dilution methods, at age 30 months

	^{15}N natural abundance				^{15}N dilution	
	AP		AM		AM	
	L	WT	L	WT	L	WT
N derived from atmospheric N_2 in standing biomass	7.9	62.3	0.8	2.8	18.4	16.0
Total N fixed since planting	11.6	65.8	1.5	7.1	36.2	30.6

Comparison of estimates based on %Ndfa of leaves (L) and on whole-tree weighted average %Ndfa (WT).

4. Discussion

4.1. Nitrogen concentration and N transfer from *A. mangium* to *E. grandis* trees

The higher N concentration found in most of the N-fixing species tree components (*A. mangium*) than in non-N-fixing species (*E. grandis*) might be a result of N_2 fixation. The same trend has been observed in other mixed-species plantations, e.g. *A. mangium* vs. *Eucalyptus urophylla* (Blake) (Galiana et al., 2002); *Inga edulis* (Mart.) vs. *Terminalia amazonia* (Gmel.) (Nichols and Carpenter, 2006); *Casuarina equisetifolia* (L.) vs. *Eucalyptus robusta* (Sm.) (Parrotta et al., 1994a). However, the higher N concentrations in AM than in AP might be partly explained by the lower growth of AM (Laclau et al., 2008), which might lead to a higher proportion of living tissues. The decrease in N concentration observed from 18 to 30 months after planting, both for *A. mangium* and *E. grandis*, might be a dilution effect in the biomass of the trees, reflecting internal retranslocations of that element from older to younger tissues (Laclau et al., 2001). Whereas Khanna (1997) observed that N concentrations in *Eucalyptus globulus* (Labill.) fine roots at 33 months after planting were higher when that species was planted in a mixture with *Acacia mearnsii* (De Wild.) than in mono-specific stands, N concentrations in *E. grandis* trees were not influenced by the mixture with *A. mangium* until age 30 months in our study. The lack of significant N transfer from N-fixing trees to the non-fixing trees in 50A:100E was also suggested by the similar values of $\delta^{15}N$ natural abundance for *E. grandis* trees in 0A:100E and 50A:100E. A large increment in soil N availability through N_2 fixation in mixed-species plantations requires: (i) N_2 fixation by the bacteria in symbiosis with the legume species, (ii) N accumulation in the living tissues of the NFT, (iii) returns to the soil of organic matter enriched in N, and (iv) mineralization of legume-derived organic nitrogen. Even in fast growing tropical plantations, such a process takes time.

The small amount of N accumulated in AM from 18 to 30 months after planting (≈3 kg N ha^{-1}) was mainly explained by low tree growth, with an increment in NPP of 2.6 t ha^{-1} yr^{-1} of dry matter (Laclau et al., 2008), and a decrease in N concentrations within biomass components. By contrast, AP exhibited an increment of 87 kg N ha^{-1} over the same period owing to an increment in NPP that was 12 times higher (30.8 t ha^{-1} yr^{-1} of dry matter) and a smaller decrease in N concentrations than AM.

At 30 months after planting, N contents were 44 and 19% higher in the standing biomass of 100A:0E and 50A:100E, respectively, than in the *E. grandis* monoculture. The differences decreased to 19 and 9% for cumulated N fluxes since planting (including litter fall and fine root mortality). Leaves were the main sink of N in both *A. mangium* and *E. grandis* trees, containing 40–45% of the amount of N accumulated in the 30-month-old stands. For both species, the N content in stumps and roots amounted to about 20% of total N in the standing biomass and dead fine root accounted for 12–16% of the cumulated fluxes of N since planting. The order of magnitude of those fluxes highlights the fact that an accurate estimation of N dynamics in belowground biomass is essential for gaining an insight into the N cycle in mixed-species plantations including N-fixing trees. Such a feature was shown for other legume species in glasshouse experiments (Khan et al., 2002) and tropical forest plantations (Harmand et al., 2004).

4.2. Qualitative information on N_2 fixation

Nodule sampling and SARA estimations were performed in November 2004 and November 2005 right at the beginning of the rainy season. The adverse conditions over the winter (June–September) prevented optimum development of nodules and might explain the low SARA values measured. Mean SARA values ranged from 3 to 6 μmol C_2H_4 h^{-1} g^{-1} of nodules but high standard deviations at 18 months after planting displayed high spatial variability. Such values were found by Van Kessel and Roskoski (1981) on *Inga jinicuil* (Schlechter). However,

SARA from 11 to 27 μmol C_2H_4 h^{-1} g^{-1} of nodules were measured by Van Kessel et al. (1983) for 6 species of leguminous trees while Sutherland and Sprent (1993) observed a production of 48 μmol C_2H_4 h^{-1} g^{-1} of nodules on *Leucaena leucocephala* (Lam.). Ribet and Drevon (1996) measured values from 25 to 100 μmol C_2H_4 h^{-1} g^{-1} of nodules for *A. mangium* seedlings growing in a solution culture. SARA was not measured at the end of the rainy season in this study but more favourable environmental conditions might lead to much higher N-fixing activities than those indicated by SARA measurements made in November here.

The much lower biomass of nodules observed in 50A:100E might be a result of high inter-specific competition with *E. grandis* for natural resources limiting *A. mangium* growth (Laclau et al., 2008), and leading to a drier soil (unpublished data). Likewise, the decrease in SARA measured from 18 to 30 months after planting in both 100A:0E and 50A:100E might be a result of an increment in intra- and inter-specific competition for water and nutrients throughout the development of the stands (Dommergues et al., 1999).

Nodules are usually collected in the upper soil layer (0–10 or 0–20 cm) where they are assumed to find the best conditions for their development (Van Kessel and Roskoski, 1981; Hingston et al., 1982). However, nodules were lacking in the 0–10 cm layer at 18 months after planting in 50A:100E and only 30% of the total nodule biomass was found in that layer at age 30 months. Moreover, from 40 to 85% of the total biomass of nodules was found in the 10–50 cm soil layer in 100A:0E and 50A:100E. Those results showed that reliable ARA estimations should take into account the possible occurrence of nodules beyond a depth of 20 cm.

4.3. Quantitative estimation of N_2 fixation

4.3.1. ^{15}N natural abundance method

E. grandis was chosen as a non-fixing reference plant because it is a perennial species likely to explore the same soil layers as the *A. mangium* and therefore to utilise the same pool of soil N (Galiana et al., 2002). However, the mycorrhizal status of a given species has an influence on its access to different soil nitrogen sources and the fractionation associated with N uptake (Shearer and Kohl, 1986; Spriggs et al., 2003). Högberg (1990) showed that tree species associated with ectomycorrhiza (ECM) generally had a higher foliar $\delta^{15}N$ than species associated with endomycorrhiza (VAM). We did not check the mycorrhizal habit of *A. mangium* and *E. grandis* in this trial, but the association of both tree species with ECM and VAM is well documented (Duponnois and Bâ, 1999). The bias in the interpretation of $\delta^{15}N$ values due to the mycorrhiza status of both species should therefore be low.

Low values of $\delta^{15}N_{REF}$, as observed in our study, prevented efficient use of the ^{15}N natural abundance method (Dommergues et al., 1999). The suggestion of Högberg (1997) that the mean ^{15}N abundance of reference species should be at least 5‰ higher than the *B* value to estimate N fixation by that method was not met here. The low ^{15}N natural abundance values measured in *E. grandis* and the limited number of trees sampled at each age (5–10) might explain inconsistent findings such as: (i) higher $\delta^{15}N$ values at age 18 months in *A. mangium* than in *E. grandis* for several tree components or (ii) marked changes in $\delta^{15}N$ observed between 18 and 30 months for some biomass components of both species. Further investigations would be necessary to assess the factors controlling the ^{15}N natural abundance values in each species.

4.3.2. ^{15}N dilution method

Eq. (4) is only valid if the %Ndfa is similar after soil labelling and over the period from planting to the date of ^{15}N-enriched fertilizer application, otherwise the following formula should be used:

$$\%\text{Ndfa} = 100\left(1 - \frac{[(N_{\text{Ffinal}}AE_{\text{Ffinal}} - N_{\text{Finitial}}AE_{\text{Finitial}})/(N_{\text{Ffinal}} - N_{\text{Finitial}})]}{[(N_{\text{REFfinal}}AE_{\text{REFfinal}} - N_{\text{REinitial}}AE_{\text{REFinitial}})/(N_{\text{REFfinal}} - N_{\text{REFinitial}})]}\right) \quad (5)$$

where N_{Ffinal}, N_{Finitial}, N_{REFfinal}, and $N_{\text{REFinitial}}$ are the N content of the NFT on harvesting, the N content of the NFT on soil labelling, the N content of the reference species on harvesting, and the N content of the reference species on soil labelling, respectively. AE_{Ffinal}, AE_{Finitial}, AE_{REFfinal}, and $AE_{\text{REFinitial}}$ are the percentage atom excess of the NFT on harvesting, the percentage atom excess of the NFT on soil labelling, the percentage atom excess of the reference species on harvesting, and the percentage atom excess of the reference species on soil labelling, respectively.

One way of avoiding bias when using Eq. (4) instead of Eq. (5) is to apply fertilizer with a high $^{15}N/^{14}N$ ratio and to label the soil at young stand stages. In that case, Eqs. (5) and (4) are equivalent as AE_{Ffinal} and AE_{REFfinal} are much higher than AE_{Finitial} and $AE_{\text{REFinitial}}$, respectively, and N_{Ffinal} and N_{REFfinal} are much higher than N_{Finitial} and $N_{\text{REFinitial}}$, respectively.

High rates of ^{15}N enrichment (5–10% ^{15}N atom excess) are usually used in ^{15}N dilution experiments (Liyanage et al., 1994; Parrotta et al., 1994a,b; Guinto et al., 2000). High AE_{Ffinal} and AE_{REFfinal} are then observed, and can be measured with a low sensitivity mass spectrometer. However, field experiments with fast growing tree species require the application of labelled fertilizer on dozens of square metres and ^{15}N enrichment is then limited by the cost of the application. In this study, the low ^{15}N enrichment (0.5% ^{15}N atom excess) of the fertilizer led to low AE values. The stability of the *R* ratio among tree components in this study suggests that a reliable assessment of N_2 fixation was obtained in the field from low values of fertilizer ^{15}N enrichment. However, application of 1% ^{15}N atom excess fertilizer should be preferred in future studies.

Table 7
Estimation of N_2 fixation by nitrogen fixing tree species, using ^{15}N methods

Legume species	Location	Plantation age[a,b] (yr)	%Ndfa[c]	N fixed (kg ha^{-1} yr^{-1})	Method used	Authors
Acacia angustifolia	**Zimbabwe**	**2**	**56**	**61**	**N. abundance**	Chikowo et al. (2004)
Acacia caven	Chile	1	14	0.5	Labelling	Ovalle et al. (1996)
	Chile	2	86	9	Labelling	Ovalle et al. (1996)
	Chile	6	50	9.5	Labelling	Aronson et al. (2002)
Acacia dealbata	**Australia**	**5**	**59**	**50**	**N. abundance**	May and Attiwill (2003)
Acacia holosericea	Senegal	10	39	nd	N. abundance	Ndiaye and Ganry (1997)
Acacia mangium	Ivory Coast	2	50	nd	N. abundance	Galiana et al. (2002)
Acacia melanoxylon	Australia	2.3	43	<1	N. abundance	Hamilton et al. (1993)
Acacia mucronata	Australia	2.3	48	<1	N. abundance	Hamilton et al. (1993)
Acacia senegal	Sudan	4	24–61	7–12	N. abundance	Raddad et al. (2005)
Alnus glutinosa	France	>15	94	nd	N. abundance	Beaupied et al. (1990)
Alnus incana spp. *rugosa*	USA	Nd	85–100	43	N. abundance	Hurd et al. (2001)
Alnus incana	France	5–6	75	nd	N. abundance	Domenach et al. (1989)
Calliandra calothyrsus	Australia	1	50	76	N. abundance	Purwantari et al. (1996)
Calliandra calothyrsus	Australia	2	38–65	67–93	Labelling	Stahl et al. (2002)
Calliandra calothyrsus	Kenya	0.6	36–54	24	N. abundance	Gathumbi et al. (2002)
Casuarina equisetifolia	**Puerto Rico**	**2 M**	**42–67**	**39–62**	**Labelling**	Parrotta et al. (1994a)
		2	**42–67**	**82–94**		
Casuarina equisetifolia	Senegal	3	38	15	N. abundance	Mariotti et al. (1992)
Cytisus scoparius	**New Zealand**	**1–2**	**81**	**111**	**N. abundance**	Watt et al. (2003)
Erythrina lanceolata	Costa Rica	1	0–53	0–72	N. abundance	Salas et al. (2001)
Faidherbia albida	Senegal	1	15–23	nd	Labelling	Gueye and Ndoye (2000)
Flemingia macrophylla	Burundi	1	–	10	N. abundance	Snoeck (1995)
Gliricidia sepium	Senegal	10	0–17	nd	N. abundance	Ndiaye and Ganry (1997)
Gliricidia sepium	French W. Indies	–	54–92	204	N. abundance	Nygren et al. (2000)
Hardwickia binata	French W. Indies	–	0–22	nd	N. abundance	Nygren et al. (2000)
Inga pilosula	French Guiana	–	49–80	–	N. abundance	Koponen et al. (2003)
Inga oerstediana	**French Guiana**	**1–3**	**20**	**–**	**N. abundance**	Grossman et al. (2006)
Leucaena leucocephala	**Porto Rico**	**2 M**	**70**	**51–71**		Parrotta et al. (1994b)
		2	**70**	**103**	**Labelling**	
Leucaena leucocephala	Nigeria	3	62–75	98–119	Labelling	Sanginga et al. (1996)
						Sanginga et al. (1990)
Prosopis alba	Chile	1	25	0.4	Labelling	Ovalle et al. (1996)
	Chile	2	52	1.8	Labelling	Ovalle et al. (1996)
	Chile	6	10	0.55	Labelling	Aronson et al. (2002)
Prosopis chilensis	Chile	1	31	0.5	Labelling	Ovalle et al. (1996)
	Chile	2	70	2	Labelling	Ovalle et al. (1996)
	Chile	6	30	0.8	Labelling	Aronson et al. (2002)
Prosopis cineraria	Senegal	10	21	nd	N. abundance	Ndiaye and Ganry (1997)
Prosopis glandulosa	**USA**	**1**	**41–63**	**40**	**N. abundance**	Shearer and Kohl (1991)
Pterocarpus lucens	Senegal	–	26–49	11–29	N. abundance	Sylla et al. (2002)
Pterocarpus officinalis	French Guiana	–	35–58	–	N. abundance	Koponen et al. (2003)
Robinia pseudoacacia	Austria	2	90	110	Labelling	Danso et al. (1995)
Zygia cataractae	French Guiana	–	31–50	–	N. abundance	Koponen et al. (2003)

Information related to mixed stands is in bold. N. abundance: Natural abundance.
[a] M: Mixed plantation with *Eucalyptus* sp.
[b] nd: Not determined.
[c] %Ndfa: Nitrogen derived from atmosphere.

4.3.3. Within-tree sampling

This study showed the need to sample the whole plant for an accurate estimate of N_2 fixation when using the ^{15}N natural abundance method. Such a requirement was highlighted by Boddey et al. (2000) and Khan et al. (2002) and illustrated by Sanginga et al. (1990) who found that roots of *L. leucocephala* contained about 60% of the N_2 fixed in the whole plant. However, it is often assumed that the relative ratio of $\delta^{15}N$ measured in the leaves of the NFT and of the reference species is the same as if whole trees were sampled (Galiana et al., 2002). Such a hypothesis may therefore lead to marked bias in the estimation of N_2 fixation, as observed in our study. By contrast, in the ^{15}N dilution experiment, there were lower discrepancies in the estimates of N_2 fixation when %Ndfa was assessed from the AE of leaves only. That finding was consistent with the low within-tree AE variability found by Parrotta et al. (1994a) and Liyanage et al. (1994). In such cases, the use of foliar AE instead of whole-tree AE would not be too detrimental for obtaining an accurate estimate of N derived from atmospheric N_2.

4.3.4. Comparison of natural abundance and dilution methods

Under our conditions, the dilution method was likely to give the best estimation of N_2 fixation. The values of %Ndfa were about 60% for both standing biomass and total N uptake since planting. Those results were consistent with those of Galiana et al. (2002) who estimated a %Ndfa of about 50% for 2-year-old stands of *A. mangium* in Ivory Coast, using ^{15}N natural abundance.

4.3.5. Uncertainty of N_2 fixation estimates

Several limitations appeared in this study for using the ^{15}N natural abundance and ^{15}N dilution methods to estimate N_2 fixation by *A. mangium*:

- The *B* value of −0.3% was calculated from above ground parts of 10 twelve-month-old *A. mangium* seedlings inoculated with the *Bradyrhizobium* sp. strain *Aust13c* and grown in greenhouse (Galiana et al., 2002). Additional studies should be carried out to take into account the $\delta^{15}N$ of the roots. Likewise the *B* value should be estimated for older trees, since a decrease in $\delta^{15}N$ was observed throughout the development of trees in our study. The *B* value of *A. mangium* exposed to the same rhizobium strains as those used in this experiment should also be assessed, as it was found that different strains might affect the ^{15}N abundance of legume shoot tissues (Boddey et al., 2000).
- We did not quantify the possible variations in N_2 fixation and ^{15}N discrimination over the year (Watt et al., 2003), which might have biased the %Ndfa estimations.
- We observed that both *E. grandis* and *A. mangium* growing in monocultures developed a dense root system in the topsoil (data not shown). By contrast, *E. grandis* exhibited a higher biomass of fine roots than *A. mangium* in the surface soil layer of the mixed stands and that difference in root exploration might have biased the estimation of N_2 fixation. Variations in nitrate and ammonium availability might occur with soil depth, leading to differences in $\delta^{15}N$ of the mineral nitrogen taken up by the two species, as NH_4^+ is less depleted in ^{15}N than NO_3^- (Boddey et al., 2000). Moreover, a lower proportion of N derived from the ^{15}N-enriched fertilizer might have been taken up by *A. mangium* than *E. grandis* since the fertilizer was applied at the soil surface where the *A. mangium* fine root density was low. However, the ^{15}N-enriched fertilizer was applied during the rainy season in a free draining coarse-textured soil and large amounts of rain should have led to a re-distribution of ^{15}N in the soil profile.
- The *A. mangium* fine root mortality was a major process in incorporating N in the soil in both 100A:0E and 50A:100E. But two factors led to great uncertainties in the estimation of N_2 fixed in that component: (1) fine root mortality was estimated roughly from observations made on leaf turnover, and (2) only 4 bulk samples for each treatment were analysed at each age to assess the relative isotopic abundance of fine roots. Two conditions must therefore be fulfilled to obtain accurate estimations of the amount of atmospheric N_2 fixed by *A. mangium*: (1) reliable quantification of fine root mortality and (2) intensive sampling of fine roots for both *E. grandis* and *A. mangium*. Moreover, nitrogen retranslocation occurring during root senescence and possible differences in $\delta^{15}N$ values between living and dead roots were not taken into account and might lead to a slight bias of the N_2 fixation estimations. However, the available information suggests that N retranslocation in fine roots is limited (Gordon and Jackson, 2000).

4.4. Field quantification of N_2 fixation in forest plantations

There are few references for field estimates of atmospheric N_2 fixation by NFT (Table 7). Marked variability in %Ndfa and fixed N is observed among species or sometimes for a given species. That finding may be explained by the limitation in using ^{15}N isotopic methods previously pointed out. Moreover, only leaves have been sampled in most of these studies and none of them have tested both natural abundance and dilution methods. Further studies need to be carried out to obtain reliable figures for N_2 fixation, which are essential for interpreting inter-specific interactions in mixed plantations with NFT.

5. Conclusion

The ^{15}N dilution method was likely to give more reliable estimates of N_2 fixation by *A. mangium* trees than the ^{15}N natural abundance method. The amount of atmospheric N_2 fixed by *A. mangium* over 30 months of growth was estimated at about 30 kg N ha^{-1} by the ^{15}N dilution method in 50A:100E, and 65 kg N ha^{-1} by the ^{15}N natural abundance method in 100A:0E. However, a comparison of atmospheric N_2 fixation estimations provided by the ^{15}N natural abundance and ^{15}N dilution methods in 50A:100E showed that estimation by the ^{15}N natural abundance method might be underestimated in the study area. Nitrogen input in the mixed-species stand was not insubstantial in this N depleted soil, since *A. mangium* survived under the *E. grandis* canopy and it is likely that despite low growth (Laclau et al., 2008), *A. mangium* would continue to fix N_2 until the end of the rotation. Moreover, the ecological conditions in the Itatinga region (4-month dry season, low temperatures during the winter) were not optimum for *A. mangium* growth and N_2 fixation. The same design was duplicated at 5 sites in Brazil and at 2 sites in Congo to assess the potential of that legume tree to increase the productivity of eucalypt plantations through a facilitation interaction, improving N availability. Other experiments will be set up in Brazil to test other legume species, as well as various combinations of both *E. grandis* and *A. mangium* trees to help in gaining a general picture of the potential of mixed-species plantations.

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