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Nitrogen fixation rate of *Acacia mangium* Wild at mid rotation in Brazil is higher in mixed plantations with *Eucalyptus grandis* Hill ex Maiden than in monocultures

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Abstract

· *Key message* Inter-specific interactions with eucalypts in mixed plantations increased N_2 fixation rate of acacia trees compared to monocultures. N_2 fixation was higher during the wet summer than during the dry winter both in acacia monocultures and in mixed plantations.

· *Context* Introducing N-fixing trees in fast-growing tropical plantations may contribute to reducing the long-term requirements of N fertilizers. Management practices established in forest monocultures should be revisited in mixed-species plantations.

· *Aims* This field experiment aimed to compare N_2 fixation rates of *Acacia mangium* Wild in monospecific stands and in mixed-species stands with *Eucalyptus grandis* W. Hill ex Maiden. A secondary objective was to gain insight into the seasonal variations of N_2 fixation.

· *Methods* ^{15}N was applied to acacia and eucalypt monocultures and mixed-species with a 1:1 ratio at mid rotation. Leaves were collected in autumn, winter, spring, and summer to determine the foliar N concentrations and ^{15}N atom fraction. The N content in

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the above-ground biomass was estimated as well as the percentage of N derived from atmospheric N_2 (%Ndfa) using eucalypts in monoculture as reference plants.

• **Results** %Ndfa values averaged over the year were 14% in monoculture and 44% in mixed-species stands. While the stocking density of acacia trees was twice as high in monoculture as in mixture, the amounts of N fixed in above-ground biomass of acacia trees were close (35–39 kg N ha^{-1}) at 39 months after planting. %Ndfa values were higher during the wet summer than the dry winter both in acacia monocultures and in mixed plantations.

• **Conclusion** The stocking density of acacia trees can be reduced in mixed plantations with eucalypts in comparison to acacia monocultures with a low influence on the input of N to soil through biological fixation.

Keywords Symbiotic N_2 fixation · Seasons · Mixed-species plantations · Competition · Forest rotation

1 Introduction

In the tropics, nitrogen-fixing trees (NFT) may fix large amounts of atmospheric N_2 (Binkley and Giardina 1997; Nygren et al. 2012). Associating NFTs with non-nitrogen fixing trees can increase biomass production of plantations (Piotto 2008; Bouillet et al. 2013; Santos et al. 2016), soil carbon sequestration (Resh et al. 2002), microbial diversity (Rachid et al. 2013), and soil nutrient availability (Voigtlaender et al. 2012; Koutika et al. 2014). NFTs are likely to improve the N status of companion species rapidly, through below-ground pathways (Nygren and Leblanc 2015; Paula et al. 2015), and in the long term, through decomposition of N-rich litter and pruning residues (Beer 1988; Forrester et al. 2006).

N_2 fixation is regulated by numerous factors such as water availability in the soil, temperature, nutrient availability, and interactions between plants and soil microorganisms (Rastetter et al. 2001; Vitousek et al. 2002; Soussana and Tallec 2010; Diagne et al. 2013; Augusto et al. 2013). High soil temperatures and water content in autumn and spring led to an increase in nodule biomass and N fixation for *Acacia dealbata* Link seedlings growing in 2-year-old *Eucalyptus regnans* F. Muell. stands (Adams and Attiwill 1984).

The percentage of N derived from atmospheric N_2 (%Ndfa) is highly dependent on plant age and species (Parrotta et al. 1996; Isaac et al. 2011). %Ndfa values ranging from 10 to 100% have been found for tropical NFTs (Binkley and Giardina 1997; Forrester et al. 2006; Nygren et al. 2012). The gradual decrease in %Ndfa that occurs after planting has been mainly associated with the ability of NFTs to regulate N_2 fixation depending on their N requirements and changes in soil N availability (Vitousek et al. 2002; Barron et al. 2011; Sheffer et al. 2015). Regulation of N_2 fixation has been reported for natural tropical and temperate forests (Pfautsch et al. 2009; Barron et al. 2011) and may account for the temporal changes in %Ndfa in mixed-species planted forests (Parrotta et al. 1996; Balieiro et al. 2008; Bouillet et al. 2008).

Intra- and inter-specific competition for N, light, and water may influence N_2 fixation (Vitousek et al. 2002; Forrester et al. 2007; Wurzburger and Miniat 2014; Sheffer et al. 2015). Non-leguminous species with highly competitive soil N uptake may increase the %Ndfa values of the associated NFTs, as has been observed for *Pseudosamanea guachapele* (Kunth) Harms in mixed-species plantations with *Eucalyptus grandis* Hill ex Maiden (Balieiro et al. 2008) and for several acacia species growing with grasses in African savannas (Cramer et al. 2007). The competition for light and water may limit plant growth and N_2 fixation rates, as reported for *Pisum sativum* L. associated with *Hordeum vulgare* L. (Jensen 1996) and *A. mangium* mixed with *E. grandis* (Bouillet et al. 2008). However, inter-specific competition for light and water by *Pinus palustris* Mill increased the root nodule biomass and N_2 fixation of *Morella cerifera* (L.) Small (Hagan and Jose 2011).

N_2 fixation rates depend largely on environmental factors. Understanding the factors controlling N_2 fixation in tropical planted forests could help to improve soil preparation and weed control techniques as well as fertilization regimes in order to optimize the N inputs to the soil over the rotation cycle. Our study set out to assess the seasonal variability of %Ndfa for *A. mangium* trees grown in monoculture and in association with *E. grandis* trees. These two species are widely planted in tropical regions (FAO 2010) and mixed-species plantations with eucalypts and acacias may be an alternative to eucalypt monocultures in N-deficient soils (Bouillet et al. 2013; Forrester et al. 2006). We tested the hypotheses that (1) %Ndfa of *A. mangium* trees is higher in mixed-species plantations with *E. grandis* trees than in monospecific stands and (2) %Ndfa is higher during the rainy summer than during the dry winter both in mixed plantations and in *A. mangium* monoculture.
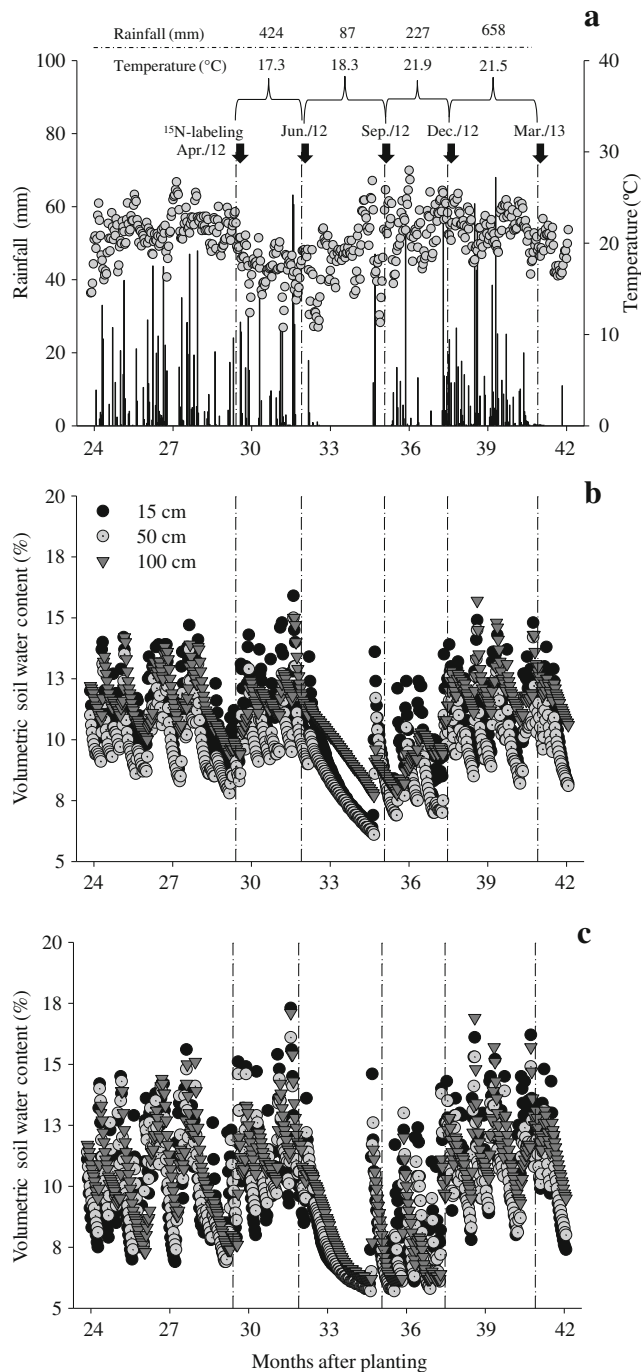
Fig. 1 Rainfall (vertical bars) and mean air temperature (circle) in an open area 100 m from the field trial (**a**) and volumetric soil water content at a depth of 15 cm (black circle), 50 cm (gray circle), and 100 cm (triangle) in the monospecific stand (**b**) and the mixed-species stand (**c**) of *Acacia mangium* over the study period. Volumetric soil water content was monitored in one block of the experiment using three Campbell CS616 probes per depth. Measurements were taken every half hour and averaged over the day. Soil was ^{15}N-labeled in one block on April 15th and 16th, 2012. Leaves were sampled in June 2012, September 2012, December 2012, and March 2013 (black arrows)

2 Material and methods

2.1 Site description

The study was carried out at the Itatinga experimental station of São Paulo University (23°02′ S, 48°38′ W). The landscape is typical of the western plateau of São Paulo state, with smoothly undulating topography. The site was on the top of a hill (slope < 3%) at an elevation of 860 m ASL. The soils were Ferralsols (FAO classification) and were typical of large areas planted with eucalypts in Brazil (Gonçalves et al. 2013). The soil texture was uniform below a depth of 1 m with clay content of 13% in the A_1 layer and ranging from 20 to 25% between 1 and 3 m in depth. The main soil characteristics down to a depth of 3 m in the experiment can be found in Voigtlaender et al. (2012). The same soil type under eucalypt plantations was characterized in detail (including micro-nutrients and soil solution chemistry) down to a depth of 3 m in a separate experiment (Maquère 2008). The soil pH_{H20} ranged from 5.4 to 5.9 depending on the soil layer. Carbon (C) concentrations were 17.6, 6.4, 5.0, and 3.5 g kg^{-1} in soil layers 0–0.05, 0.05–0.15, 0.15–0.5, and 0.5–1 m, respectively, total N concentrations were 0.9, 0.3, 0.4, and 0.2 g kg^{-1}, available phosphorus concentrations (P-resin) were 4.0, 2.5, 1.9, and 1.3 mg kg^{-1} and cation exchange capacities (CEC) were 1.76, 0.95, 0.75, and 0.58 cmol$_c$ kg^{-1} (Voigtlaender et al. 2012). Low nutrient stocks in the soil (exchangeable K$^+$, Na$^+$, Ca^{2+}, Mg^{2+} contents < 0.2 mmol$_c$ kg^{-1} below a depth of 0.15 m), related to the long period of eucalypt cultivation, made the area a promising environment for studying mixed-species plantations associating NFTs and eucalypts (Laclau et al. 2008; Voigtlaender et al. 2012).

Temperature is relatively low during the dry season (from June to September). Our study was carried out from November 2011 to April 2013. During this period, the cumulative rainfall collected in an open area at 100 m from the field trial was 2237 mm and the average air temperature was 20.2 °C (Fig. 1).

2.2 Experimental design

Our experiment used three treatments and three blocks of the large-scale experiment described in Laclau et al. (2008). The treatments were 100A (*A. mangium* monoculture), 100E (*E. grandis* monoculture), and 50E:50A (a 1:1 ratio of *E. grandis* and *A. mangium*, the two species being planted alternately in the row and offset in adjacent rows). The stand density was 1111 trees ha^{-1} (3 m × 3 m spacing). The fertilizer doses applied at planting were 40 g P plant^{-1} (buried at 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},

Table 1 Ranges of basal area and height of the trees sampled for the estimation of biomass and N$_2$ fixation in the monospecific stands of *Acacia mangium* and *Eucalyptus grandis* (100A and 100E, respectively) and the mixed-species stands (50E:50A–*Acacia* and 50E:50A–*Eucalyptus*), at 39 months of age

Treatment/species	Trees sampled for biomass quantification		Trees sampled for N$_2$ fixation analysis	
	Basal area (cm^2 tree^{-1})	Height (m)	Basal area (cm^2 tree^{-1})	Tree height (m)
100A	30.6–310.9	6.7–10.7	42.1–215.3	8.7–10.2
100E	82.0–249.6	14.4–19.1	53.8–187.2	14.8–19.1
50E:50A–*Acacia*	29.9–232.1	7.3–9.9	66.1–226.0	9.5–10.9
50E:50A–*Eucalyptus*	107.8–280.8	14.0–19.5	37.5–254.1	12.4–18.6

and 1 g Mn plant^{-1}. No N fertilization was applied. Fertilization trials at the study site and in nearby commercial forests on the same soil type showed that, with the exception of N, the amounts of nutrients applied were non-limiting for eucalypt tree growth (Gonçalves et al. 2008; Laclau et al. 2009).
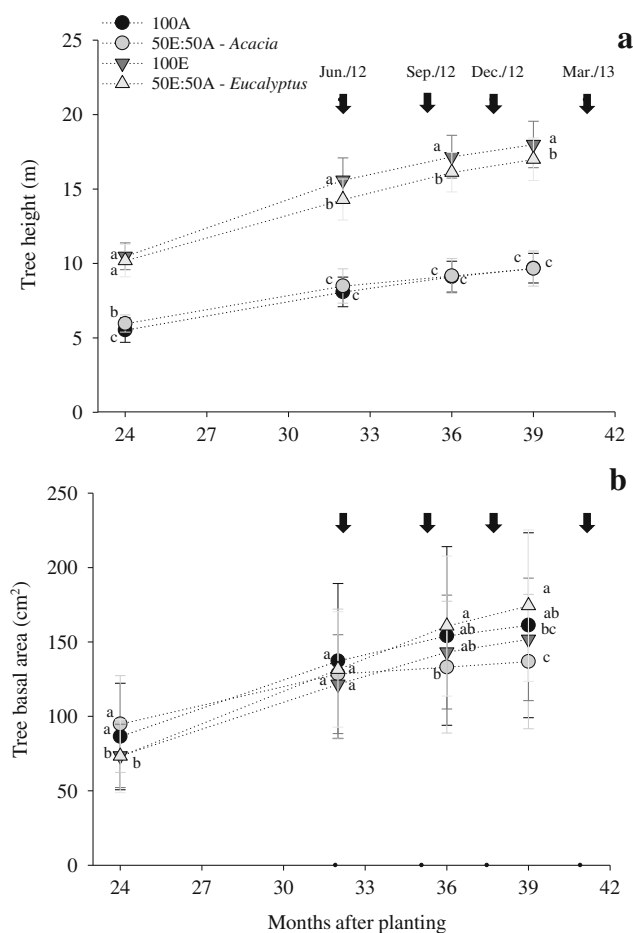
Fig. 2 Mean tree height (**a**) and basal area (**b**) of *Acacia mangium* and *Eucalyptus grandis* in the monospecific stands (100A and 100E, respectively) and the mixed stand (50E:50A–*Acacia*/50E:50A–*Eucalyptus*). Vertical bars indicate standard deviations between blocks ($n = 3$). No statistical differences were found between blocks ($P \geq 0.3$). For a given date, different letters indicate differences between treatments ($p < 0.05$)

Only the boles were harvested in May 2009, and the residues were spread uniformly within each plot. Seedlings of *A. mangium* and *E. grandis* were planted in the same planting rows and the same plots on November 2009, following the same experimental design and protocol. *A. mangium* seeds were inoculated with *Rhizobium* strains (BR 3609T and BR6009 provided by EMBRAPA Agrobiologia, Seropédica-Rio de Janeiro state) selected for their high N$_2$ fixation efficiency.

The volumetric soil water content was monitored in one block at depths of 0.15, 0.50 and 1.00 m, using 3 Campbell CS616 probes per depth in 100A and 50E:50A. Measurements were taken every half hour and averaged over the day. The volumetric soil water content in the 0–100 cm soil layer was similar in 50E:50A and 100A, on average 11.3% in autumn, 7.9% in winter, 7.8% in spring, and 11.4% in summer (Fig. 1b, c).

2.3 Tree growth, stand biomass, and N accumulation

Tree height and diameter at 1.3 m (DBH) were measured in three blocks 24, 32, 36, and 39 months after planting. For multi-stem trees, the DBH of each individual stem was measured and the individual basal area was calculated. The growth of acacia trees covering the range of diameters was also monitored monthly in one block with band dendrometers (accuracy ± 0.2 mm) in 100A ($n = 15$) and 50E:50A ($n = 10$) from September 2012 (34 months after planting) to March 2013 (41 months after planting).

The above-ground biomass was estimated in January 2013 (at 39 months) sampling 10 acacias and/or eucalypts from each of the 100A, 100E, and 50E:50A treatments (total of 40 trees). The trees were taken from all three blocks for each species and each treatment with two to four trees sampled in each block. The sampled trees were equally distributed within the range of basal areas calculated from the stand inventory (Table 1). The trees were separated into leaves, branches, stemwood, and stembark. Diameters, lengths, and weights were

measured in the field. Tree foliage was collected from three sections of the crown (lower, intermediate, and upper). Sub-samples were taken from all the components, dried at 65 °C to constant weight, and ground in a Willey mill (0.8 mm mesh). The mill was carefully cleaned between each milling using a vacuum cleaner, compressed air jet and ethyl alcohol. The N concentrations of the leaves, branches, stembark, and stemwood were determined using the Kjeldahl method with wet digestion using concentrated sulfuric acid and distillation using sodium hydroxide 18 M (TE36/1and TE36/3 analyzers–Tecnal Co., Piracicaba, Brazil). Allometric models established from destructive tree sampling (Tables 2, 3) were applied to the stand inventory to assess above-ground biomass and N content of *A. mangium* and *E. grandis* trees in the monospecific stands and the mixed-species stands in three blocks.

2.4 N$_2$ fixation

Ammonium sulfate with 98.9 $x(^{15}N)$ (Coplen 2011) was applied at a rate of 0.3 kg N ha^{-1} on April 15th and 16th, 2012, 29 months after planting. The fertilizer was diluted in water and applied uniformly to the soil litter using a watering can. We considered that the low amount of applied N did not affect the N$_2$ fixation of acacias (Parrotta et al. 1996; Bouillet et al. 2008). Only one block was labeled owing to operational limitations (^{15}N fertilizer and $^{15}N/^{14}N$ analysis costs and the need to use scaffolding to sample leaves up to a height of 18 m). Tree heights and basal areas were not significantly different between the blocks (Fig. 2).

In each treatment and for each species, we selected four trees evenly distributed over the range of basal areas in April 2012 (Table 1). Leaves were sampled in June 2012 (end of autumn), September 2012 (end of winter), December 2012 (end of spring) and March 2013 (end of summer). For each tree and each sampling date, we divided the crown into three tiers of equal height and all the leaves of two pairs of opposite branches in the crown (north-south and east-west) were collected from each tier of the canopy and pooled to make a composite sample representative of the crown (35 g of leaves collected on average). The sampled leaves were then dried at 65 °C and ground. 10 mg of dry material (Barrie and Prosser, 1996) were put in tin capsules for isotopic analysis. N concentrations and $x(^{15}N)$ values of the leaves were determined using a Hydra 20–20 mass spectrometer coupled to an automatic N analyzer (ANCA-GSL, SERCON Co., Crewe, UK). The precision of the isotopic measurements was 0.0001 $x(^{15}N)$.

The percentage of N derived from N$_2$ fixation (%Ndfa) for each acacia was calculated using the equation (Fried and Middelboe 1977):

$$\%Ndfa = \left(1 - \frac{x^E_{Fx}}{x^E_{Refx}}\right) \times 100 \tag{1}$$

where x^E_{Fx} was the excess atom fraction in the acacia leaves at each sampling period and x^E_{Refx} was the mean value ($n = 4$) of $x^E(^{15}N)$ in the eucalypt leaves sampled at the same dates in 100E treatment. At each sampling date, the excess atom fraction was calculated for each individual tree using the equation:

$$x^E = x(^{15}N)_{leaves} - x(^{15}N)_{air} \tag{2}$$

A previous study during the first rotation of our experiment showed that the %Ndfa values were roughly similar using the ^{15}N labelling method sampling only the foliage or the whole tree (Bouillet et al. 2008). We considered that this equation was valid for estimating %Ndfa as the labeled stand was young and the $^{15}N:^{14}N$ ratio in the fertilizer was high (Hardarson and Danso 1993; Chalk and Ladha 1999; Bouillet et al. 2008).

2.5 Calculations and statistical analysis

Allometric equations were established for estimating the dry matter and N content of each tree component (Tables 2 and 3 in "Appendices"). The biomass and N content linear models were tested and adjusted using the packages *plyr* (Wickham and Francois 2015) and *leaps* (Lumley 2009). Differences between treatments and blocks in tree height, tree basal area, above-ground biomass, and N content were tested at each sampling date using two-way ANOVA. Differences between the DBH growth rates of acacias in 100A and in 50E:50A were tested at each sampling date using one-way ANOVA. The differences for $x(^{15}N)$ in the acacia and eucalypt leaves were tested over the year using ANOVA with treatment as fixed factor, sampling date as a repeated measurement factor, and interaction treatment × sampling date. Differences between the %Ndfa values of acacia trees in 100A and in 50E:50A over the year were tested using ANOVA with treatment as fixed factor, sampling dates as repeated measures, and interaction treatment × sampling date. The homogeneity of variance was tested by Levene's test, and the normal distribution of residuals was tested using the Shapiro-Wilks test. The values were log-transformed when the variances were unequal. When

ANOVA indicated that the effects were significant, the means were compared using the Tukey test. The significance level was 0.05. The statistical analyses were performed with R (R core Team 2015).
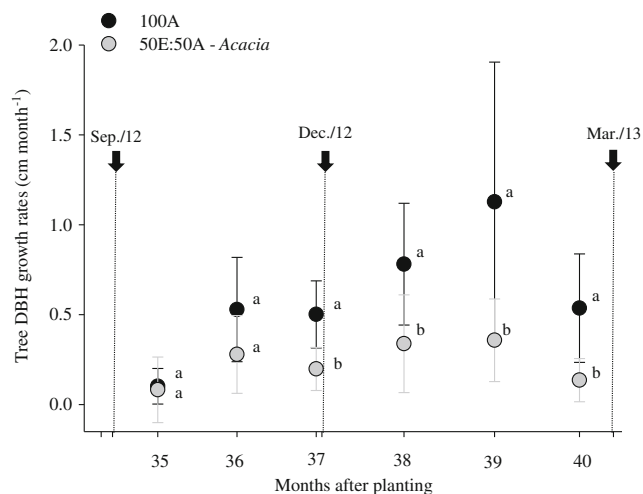
3 Results

3.1 Tree growth

Eucalypts were significantly taller than acacias regardless of the sampling date (Fig. 2a). At 24 months after planting, the height of the eucalypts was not significantly different between 100E and 50E:50A. However, from 32 months onwards, the eucalypts were significantly taller in 100E than in 50E:50A. At 24 months after planting, the acacias were significantly taller in 50E:50A than in 100A with heights of 6.0 and 5.5 m, respectively. For older trees, there was no significant difference.

At 24 months, the basal area was significantly greater for acacias than eucalypts regardless of the treatment (Fig. 2b). From 36 months onwards, the individual basal area of acacias and eucalypts was not significantly different between 100A and 100E, but acacias in 50E:50A had the lowest basal areas. At 39 months, the basal area of eucalypts was significantly higher in 50E:50A than in 100E (Fig. 2b).

The DBH growth rate of acacias was low between September and December 2012 with mean values of 0.4 cm month^{-1} in 100A and 0.2 cm month^{-1} in 50E:50A (Fig. 3) and was significantly higher in 100A than in

50E:50A from December 2012 to March 2013. The highest DBH growth rate of acacias was between January and March 2013 with mean values of 0.8 cm month^{-1} in 100A and 0.3 cm month^{-1} in 50E:50A.

3.2 Above-ground biomass and N content

At 39 months, the above-ground biomass of eucalypts was not significantly different between 100E and 50E:50A, with mean values of 63.2 and 68.3 kg tree^{-1}, respectively (Fig. 4a). The above-ground biomass of acacias was 43% higher in 100A than in 50E:50A, with mean values of 40.6 and 28.3 kg tree^{-1}, respectively (Fig. 4a).
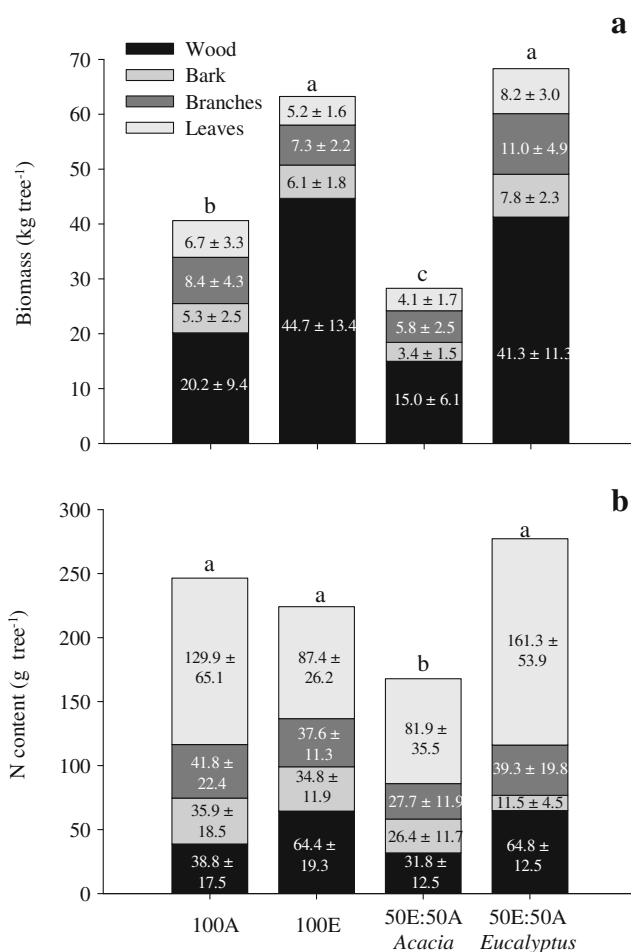
Fig. 4 Mean biomass (**a**) and N content (**b**) ($\pm$ standard deviation) of above-ground compartments of *Acacia mangium* and *Eucalyptus grandis* trees in the monospecific stands (100A and 100E, respectively) and the mixed-species stand (50E:50A–*Acacia* and 50E:50A–*Eucalyptus*) at 39 months of age. No statistical differences were found between blocks ($p \geq 0.16$). Different letters indicate differences between treatments ($p < 0.05$) in the total tree biomass and N content of the above-ground compartments. Stem biomass in 100A, 100E, 50E:50A–*Acacia* and 50E:50A–*Eucalyptus* were 19.9, 49.6, 8.0, and 22.9 Mg ha^{-1}, respectively
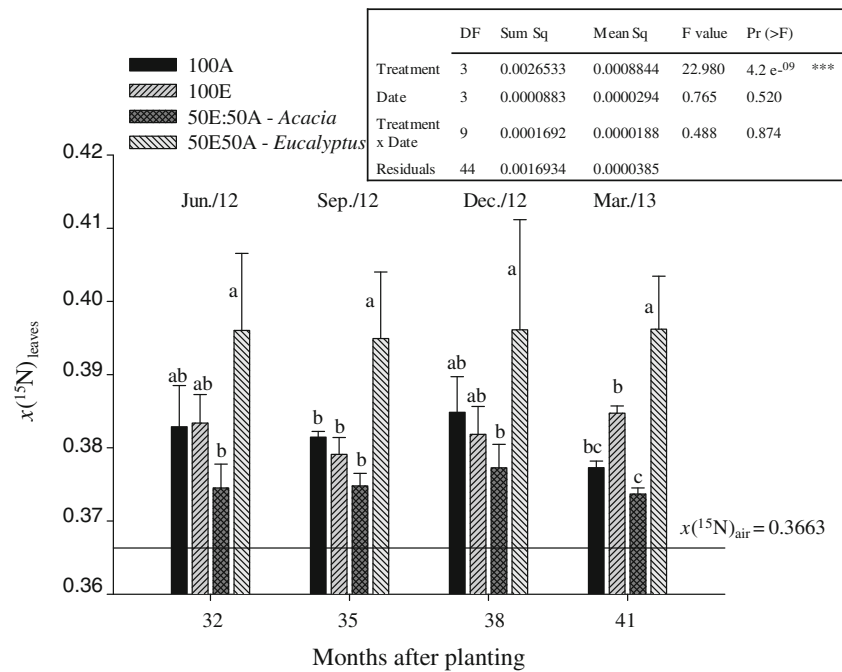

Fig. 3 Mean DBH growth rate of *Acacia mangium* trees in monospecific stand (100A) and mixed stand (50E:50A) measured monthly with band dendrometers. Vertical bars indicate standard deviations between trees ($n = 15$ in 100A; $n = 10$ in 50E:50A). The sampling dates of acacia leaves are indicated by arrows and vertical dotted line. For a given date, different letters indicate differences between treatments ($p < 0.05$)

Fig. 5 Seasonal variation of $x(^{15}N)$ in the leaves of *Acacia mangium* and *Eucalyptus grandis* trees in monospecific stands (100A and 100E, respectively) and mixed-species stand (50E:50A *Acacia* and 50E:50A–*Eucalyptus*). Vertical bars indicate standard deviations between trees ($n = 4$). Different lower case letters indicate significant differences ($p < 0.05$) between treatments at each sampling date

	DF	Sum Sq	Mean Sq	F value	Pr (>F)	
Treatment	3	0.0026533	0.0008844	22.980	4.2 e^{-09}	***
Date	3	0.0000883	0.0000294	0.765	0.520	
Treatment x Date	9	0.0001692	0.0000188	0.488	0.874	
Residuals	44	0.0016934	0.0000385			

At 39 months, the N content in the above-ground biomass of individual trees was not significantly different between eucalypt trees in 50E:50A (277.3 g N tree^{-1} on average) and acacia trees in 100A (246.4 g N tree^{-1}) or between eucalypt trees in 100E (224.1 g N tree^{-1}) and acacia trees in 100A. However, the above-ground N content in individual acacias was significantly lower (167.8 g N tree^{-1}) in 50E:50A than 100A (Fig. 4b).

3.3 Seasonal variations of $x(^{15}N)$ and %Ndfa

The $x(^{15}N)$ values of the acacia and eucalyptus leaves did not change significantly throughout the year (Fig. 5). The $x(^{15}N)$ of the acacia leaves was significantly lower in 50E:50A than in 100A with mean values across the sampling dates of 0.3751 and 0.3816, respectively. The $x(^{15}N)$ of the eucalypt leaves was significantly higher in 50E:50A than in 100E with mean values across the sampling dates of 0.3958 and 0.3823, respectively. Acacia and eucalypt leaves in monospecific stands had similar $x(^{15}N)$ values in all seasons except in summer (March 2013) when $x(^{15}N)$ values were on average 0.3847 in eucalypt leaves and 0.3773 in acacia leaves.

The N_2 fixation rate of acacias varied significantly throughout the year with the highest %Ndfa values found in March 2013 (end of summer) (Fig. 6). The N_2 fixation rate of acacias was significantly higher in 50E:50A than in 100A, and the seasonal behavior was similar in the two treatments (non-significant interaction treatment × sampling date). %Ndfa in 100A ranged from 0 to 14%

in June, September, and December. Over the same period, the %Ndfa in 50E:50A ranged from 30 to 52% (Fig. 6). %Ndfa values averaged over the study period were ~ 3 times higher in 50E:50A than in 100A (Fig. 6).
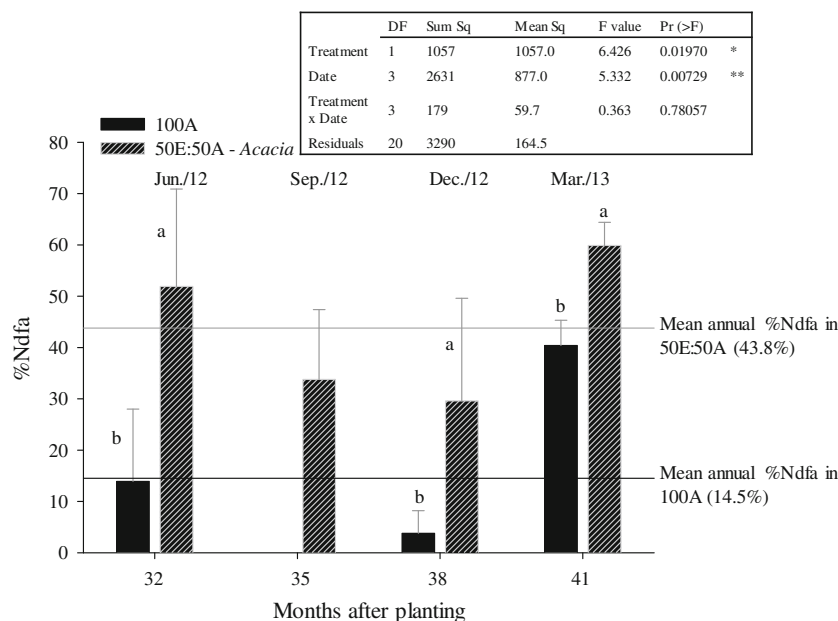
The total N derived from N_2 fixation in the above-ground biomass (i.e., leaves, branches, bark, and wood) of acacia trees sampled 39 months after planting was estimated at 35.1 kg N ha^{-1} in 100A and 39.1 kg N ha^{-1} in 50E:50A, whereas the stocking density of acacia trees was twice as high in 100A as in 50E:50A.

Statement on data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

4 Discussion

4.1 Above and below-ground growth of acacia and eucalypt trees

Tree height and above-ground biomass were higher for eucalypt trees than for acacia trees both in monospecific and mixed-species stands, as observed in the first rotation of this experiment which was set up in 2003 after 60 years of eucalypt plantation management without fertilizer amendment (Laclau et al. 2008). Climatic conditions limit *A. mangium* growth in the study region leading to rapid overtopping by *E. grandis* in mixed-species stands

	DF	Sum Sq	Mean Sq	F value	Pr (>F)	
Treatment	1	1057	1057.0	6.426	0.01970	*
Date	3	2631	877.0	5.332	0.00729	**
Treatment x Date	3	179	59.7	0.363	0.78057	
Residuals	20	3290	164.5			

Fig. 6 Seasonal variation of the percentage of N derived from fixation (%Ndfa) in *Acacia mangium* trees growing in monospecific stands (100A) and mixed-species stand with *Eucalyptus grandis* trees (50E:50A–*Acacia*). Vertical bars indicate standard deviations between trees ($n = 4$). Different letters indicate significant differences in %Ndfa ($p < 0.05$) between treatments at each sampling date. The %Ndfa values of acacias changed significantly throughout the year ($p < 0.05$). %Ndfa was taken to be 0 when the $x(^{15}N)$ value was higher in individual acacias than the mean $x(^{15}N)$ value in the eucalypts

(Nouvellon et al. 2012). The individual basal area of eucalypt trees was higher in 50E:50A than in 100E, as a result of less competition for light by acacia trees than by eucalypt trees in the mixed stands (le Maire et al. 2013), as well as the increase in N availability for the eucalypts (Paula et al. 2015).

The similar foliar $x(^{15}N)$ values in 100A and 100E in June, September, and December 2012 might be explained by a high capacity of both species for taking up soil N. During the first rotation of this experiment, the fine root biomass (diameter < 1 mm) in the 0–0.5-m-soil layer was lower in 100E than in 100A (Silva et al. 2009; Laclau et al. 2013), with values of 100 g m^{-2} in 100E and 144 g m^{-2} in 100A at 5 years after planting. High fine root densities for acacias in the upper soil layer of the mixed stands (50 g m^{-2} compared with the 100 g m^{-2} for eucalypt trees in 100E with double the stocking density) strongly suggest that both species had access to the ^{15}N applied in 50E:50A. While nitrogen fixation by *A. mangium* trees probably led to $x(^{15}N)$ values in the leaves close to that of the air (Bouillet et al. 2008) and could consequently decrease the $x(^{15}N)$ signature of *E. grandis* trees in mixed stands, foliar $x(^{15}N)$ values of eucalypt trees were higher in 50E:50A than in 100E at each sampling date. This pattern suggests that, for an individual *E. grandis* tree, a higher proportion of the ^{15}N applied in fertilization was taken up in 50E:50A than in 100E. A reduction of competition for soil N for each

E. grandis tree in 50E:50A relative to 100E might result from the N$_2$ fixation of *A. mangium* trees, which decreased the demand of soil N for *A. mangium* trees and therefore increased the availability of ^{15}N applied for *E. grandis* trees. Moreover, *E. grandis* trees could be more competitive than *A. mangium* trees in capturing the ^{15}N applied as fertilizer, which could lead to higher accumulation rates of $x(^{15}N)$ in each *E. grandis* tree in 50E:50A than in 100E. Specific root length and specific root area were greatly enhanced for both species in mixed stands relative to the monocultures in our experiment, which probably contributed to increasing the capacity of the trees to take up soil N (Germon et al. 2017). Sap flow measurements in the same plots and stand age showed that transpiration of eucalypts was on average 20% higher in 50E:50A than in 100E (unpublished data), which suggests a higher capacity of eucalypts to take up soil resources in mixed stands than in monocultures. A glasshouse experiment showed that *Acacia mangium* and *Eucalyptus* seedlings take up the same form of mineral N (Epron et al. 2016) and the ^{15}N natural abundance was similar in acacia and eucalyptus leaves at age 30 months in the first rotation of our experiment (Bouillet et al. 2008), which suggests that different N nutrition patterns for eucalypt and acacia were unlikely to change foliar $x(^{15}N)$ values after labelling. Therefore, higher foliar $x(^{15}N)$ values for *E. grandis* trees in 50E:50A than in 100E probably reflected both

enhanced capture of soil resources and differences in availability of ^{15}N in the soil. The enhanced uptake of soil N for *E. grandis* trees in 50E:50A relative to 100E did not increase the above-ground biomass, which results in particular from changes in C partition between above- and below-ground tree components (Nouvellon et al. 2012; Epron et al. 2013).

4.2 Factors accounting for higher N_2 fixation of acacia trees in mixture than in monoculture

Two potentially interacting factors might account for higher N_2 fixation of acacia trees in the mixture than in the monoculture: large differences in mineral N availability in the soil between treatments and/or higher competition with eucalypt trees in the mixture to take up soil N than between acacia trees in monoculture. Changes in soil N mineralization might contribute to explaining the temporal variability in %Ndfa observed in 100A throughout the year. %Ndfa values averaged over the year of 14% for 3-year-old acacia trees in the second rotation was lower than for the same age trees in the first rotation. A comparison of isotopic methods in another treatment at the same experimental site suggested that %Ndfa would have been >60% in the first rotation in 100A if the ^{15}N dilution method had been used instead of the natural abundance method (Bouillet et al. 2008). The lower %Ndfa in 100A for the second rotation than in the first rotation is consistent with a sharp increase in soil N availability. Soil N mineralization was twice as high in 100A as in 100E at the end of the first rotation (Voigtlaender et al. 2012) and input-output budgets showed that the harvest of the first rotation led to an increase in soil N stock of 460 kg N ha^{-1} in 100A relative to 100E (unpublished data). Soil N mineralization rates are commonly higher in *Acacia* monoculture and in mixed plantations than in *Eucalyptus* monoculture, with strong seasonal variations (Wang et al. 2013; Mo et al. 2016). A decline in %Ndfa for *Leucaena leucocephala* (Lam.) mixed with *Eucalyptus robusta* L.E. Smith from 100 to 40% between 1 and 3.5 years after planting could be a result of the incorporation into the soil of large amounts of N fixed over this period by *L. leucocephala* plants (Parrotta et al. 1996). A down-regulation of N_2 fixation depending on soil N availability has been reported for tropical legume trees growing in natural conditions (Barron et al. 2011). An increase in soil N content was associated with a decrease in N_2 fixation rates of acacias growing with *Eucalyptus regnans* in natural mixed-species stands (Pfautsch et al. 2009). A similar pattern was reported in a field experiment in the Ivory Coast where the %Ndfa of *A. mangium* trees was 2.4 times lower in the most fertile block than in the least

fertile block (Galiana et al. 2002). The relatively low maximum values of %Ndfa in this experiment during the summer (59.9% in 50E:50A and 40.4% in 100A) might reflect the increase in soil N from the establishment of the experiment in 2003 (Voigtlaender et al. 2012).

The high N requirements of the eucalypts in 50E:50A led to strong competition for soil N with the acacias, which probably increased the N_2 fixation rates. The mean N content in the above-ground biomass of the eucalypts was 20% higher in 50E:50A than in 100E and 11% higher than that of the acacias in 100A. The higher N accumulation in eucalypts in 50E:50A than in 100E could be explained by the higher soil N mineralization rates in 50E:50A than in 100E for the two first years after replanting with mean values of 91 and 68 kg N ha^{-1} year^{-1}, respectively (Voigtlaender 2012). Soil N mineralization was 136 kg N ha^{-1} year^{-1} in 100A over the same period, which was 49% higher than in 50E:50A (Voigtlaender 2012). The competition for soil N acquisition was, therefore, probably higher in 50E:50A than in 100A for acacias, contributing to the reduction in the above-ground biomass and N content of the acacias in 50E:50A. The higher %Ndfa of acacias in 50E:50A than in 100A was, therefore, consistent with an upregulation of N_2 fixation as soil N availability decreases as commonly reported for NFTs (Vitousek et al. 2002; Barron et al. 2011; Sheffer et al. 2015). This could explain why the %Ndfa of *P. guachapele* plants, growing as understory in *E. grandis* stands, was much higher than in *P. guachapele* monocultures (47 vs 26%) (Balieiro et al. 2008). However, a recent study showed that competition for a limited N pool is not always the sole mechanism increasing nodulation and N_2 biological fixation, and that facilitative root-root interactions might also be involved. Maize root exudates increased nodulation and the expression of genes stimulating N_2 fixation in faba beans (Li et al. 2016). Further studies are needed to assess whether root exudates in mixed-species forests are likely to increase nodulation and N_2 fixation of leguminous tree species.

Studies have shown the importance of sharing soil N pools with similar ^{15}N/^{14}N ratios for the two species when estimating %Ndfa in forest ecosystems (Parrotta et al. 1996; Chalk and Ladha 1999). Using eucalypts in 100E as reference plant fulfilled this condition as the total N concentration and the $x(^{15}N)$ values in the upper 15 cm of soil were not statistically different at the end of the first rotation between 100A, 100E, and 50E:50A (Voigtlaender et al. 2012). Using eucalypts growing in 100E instead of 50E:50A may have also prevented an underestimation of %Ndfa owing to the direct transfer of unlabeled N of atmospheric origin from *A. mangium*

tree to *E. grandis* tree as recently observed in the same experiment (Paula et al. 2015). However, using eucalypts growing in 50E:50A could have prevented an overestimation of %Ndfa if there was multidirectional N transfer between acacias and eucalypts, as has been shown for mixed herbaceous species (Carlsson and Huss-Dunell 2014). We checked that using eucalypts in 50E:50A instead of 100E as reference plant did not change the main findings of our study (seasonal changes of %Ndfa and large differences between monoculture and mixture). %Ndfa values averaged over the study period were ~ 1.5 and ~3 times higher in 50E:50A than in 100A, using reference plants in 50E:50A and 100E, respectively. Our results show that monitoring seasonal changes of foliar $x(^{15}N)$ provide more realistic estimates of annual N_2 fixation rates than only one sampling made several months after ^{15}N soil labelling.

4.3 Seasonal variation in N_2 fixation by acacia trees in monospecific and mixed stands

In agreement with our second hypothesis, there were noticeable variations in %Ndfa through the year depending on the climatic conditions and the changes in N demand of the acacia trees (Fig. 6). Leaf life span in the early growth stages is around 240 days for *E. grandis* (Epron et al. 2012) and 270 days for *A. mangium* (Nouvellon et al. 2012) at our study site. As all the leaves of six branches per tree were collected at each sampling date, the average age of the sampled leaves was approx. 120–140 days for both species. Foliar $x(^{15}N)$ values, therefore, depended on the amounts of ^{15}N taken up from the soil by eucalypts and acacias over the 4–5-month period before sampling. However, N from tree reserves may also be used during leaf expansion (Proe et al. 2000; Weatherall et al. 2006) and most of the ^{15}N taken up in the soil was probably accumulated in the early phase of leaf growth (Jordan et al. 2001). Sampling all tree compartments, and not only the leaves, would have made it possible to disentangle the effects of the internal N recycling and of the seasons on the temporal change in $x(^{15}N)$ of acacias leaves. However, the short duration of our study (only 1 year after ^{15}N application) and the low difference between N_2 fixation rates estimated sampling only leaves or the whole trees during the first rotation of the same experiment (Bouillet et al. 2008) suggest that ^{15}N recycling within trees was unlikely to modify the seasonal patterns of %Ndfa. The %Ndfa values of acacias in both monospecific and mixed stands were lower in the leaves sampled in September and December, which were produced during the dry season. Tree growth was low from July to December as found for the first rotation of the same experiment (Laclau et al. 2008; Bouillet et al. 2013). Tree demand for N was probably low during the periods of low growth, which might lead to low %Ndfa. Low soil water content from July to December during the experiment might also help to explain the low %Ndfa in leaves sampled in September and December in both 50E:50A and 100A. The specific activity of nodules has been reported as lower during drought for *P. sativum* (Prudent et al. 2016) and *Casuarina equisetifolia* J. R. and G. Forst (Srivastava and Abasht 1994). The %Ndfa values were highest in summer both in 100A and 50E:50A which might be a result of the high N demand to achieve high growth rates. The N_2 fixation was also maxima during the hot, rainy season for *Inga jinicuil* G. Don (Roskoski and van Kessel 1985), *Acacia spp.* (Adams and Attiwill 1984) and *C. equisetifolia* (Srivastava and Abasht 1994).

5 Conclusion

Our study based on ^{15}N labelling at mid rotation in Brazilian forest plantations suggests that soil N availability as well as the trees' N demand are likely to influence N_2 fixation of *A. mangium*. Lower %Ndfa values in the second rotation than in the first rotation for *A. mangium* monocultures were consistent with an increase in soil N mineralization. The competition for soil mineral N likely led to higher rates of N_2 fixation by acacias growing in association with eucalypts than in monoculture. %Ndfa was low during the dry season when the *A. mangium* tree growth was limited by the climatic conditions. The management of *A. mangium* trees to improve the soil N status should take account of the variations in N_2 fixation over successive rotations that are different between monocultures and mixed-species stands.

Compliance with ethical standards

Declaration on conflicts of interest The authors declare no conflicts of interest.

Appendices

Table 2 Allometric equations of the above-ground tree compartments for *Acacia mangium* in the monoculture (100A) and the mixed stand (50E:50A–*Acacia*) and for *Eucalyptus grandis* in the monoculture (100E) and the mixed stand (50E:50A–*Eucalyptus*), at 39 months after planting

Treatment	Compartment	Model	R^2	RMSE	n
100A	Leaves	$DM = -1.037 + 48.482 \times CBH^2$	0.929	0.560	22
	Branches	$DM = -1.592 + 64.687 \times CBH^2$	0.806	1.312	23
	Bark	$DM = -0.587 + 35.450 \times CBH^2$	0.930	0.407	22
	Wood	$DM = -1.6 + 125.819 \times CBH^2$	0.970	0.857	21
100E	Leaves	$DM = 27.703 \times CBH^2$	0.969	0.310	10
	Branches	$DM = 38.922 \times CBH^2$	0.864	0.988	9
	Bark	$DM = 32.309 \times CBH^2$	0.961	0.382	9
	Wood	$DM = 238.226 \times CBH^2$	0.938	3.785	10
50E:50A–*Acacia*	Leaves	$DM = -0.427 + 31.634 \times CBH^2$	0.920	0.268	23
	Branches	$DM = -0.855 + 48.657 \times CBH^2$	0.910	0.462	21
	Bark	$DM = -0.405 + 27.266 \times CBH^2$	0.943	0.205	18
	Wood	$DM = -1.090 + 107.963 \times CBH^2$	0.952	0.688	20
50E:50A–*Eucalyptus*	Leaves	$DM = 22.274 \times (DBH^2 \times Ht)$	0.910	0.904	10
	Branches	$DM = -7.187 + 82.516 \times CBH^2$	0.923	1.566	9
	Bark	$DM = 1.716 + 16.613 \times (DBH^2 \times Ht)$	0.965	0.386	8
	Wood	$DM = 11.026 + 82.749 \times (DBH^2 \times Ht)$	0.898	3.784	9

DM dry matter in kg tree^{-1}, *CBH* circumference at breast height in m, *DBH* diameter at breast height in m, *Ht* tree height in m. *RMSE* root-mean-square error, *n* number of samples used to fit the model. All the regressions were significant ($p < 0.001$)

Table 3 Allometric equations of the N content in above-ground tree compartments for *Acacia mangium* in the monoculture (100A) and the mixed stand (50E:50A–*Acacia*) and for *Eucalyptus grandis* in the monoculture (100E) and the mixed stand (50E:50A–*Eucalyptus*), at 39 months after planting

Treatment	Compartment	Model	R^2	RMSE	n
100A	Leaves	$NC = -21.569 + 958.799 \times CBH^2$	0.930	11.025	22
	Branches	$NC = -9.932 + 347.379 \times CBH^2$	0.813	6.620	23
	Bark	$NC = -6.984 + 278.289 \times CBH^2$	0.911	3.633	22
	Wood	$NC = -1.912 + 226.421 \times CBH^2$	0.962	1.902	21
100E	Leaves	$NC = 466.196 \times CBH^2$	0.919	8.556	10
	Branches	$NC = 200.301 \times CBH^2$	0.896	4.368	9
	Bark	$NC = 99.924 \times (DBH^2 \times Ht)$	0.864	4.947	9
	Wood	$NC = 343.156 \times CBH^2$	0.955	4.611	10
50E:50A–*Acacia*	Leaves	$NC = -10.827 + 669.620 \times CBH^2$	0.954	4.279	23
	Branches	$NC = -3.566 + 224.583 \times CBH^2$	0.896	2.324	21
	Bark	$NC = -4.152 + 226.401 \times CBH^2$	0.932	1.873	18
	Wood	$NC = -2.235 + 227.968 \times CBH^2$	0.928	1.838	20
50E:50A–*Eucalyptus*	Leaves	$NC = 767.453 \times CBH^2$	0.960	11.034	10
	Branches	$NC = -24.394 + 298.559 \times CBH^2$	0.953	4.691	9
	Bark	$NC = 32.459 \times (DBH^2 \times Ht)$	0.788	2.275	8
	Wood	$NC = 32.319 + 88.979 \times (DBH^2 \times Ht)$	0.879	3.529	9

NC N content in g tree^{-1}, *CBH* circumference at breast height in m, *DBH* diameter at breast height in m, *Ht* tree height in m. *RMSE* root-mean-square error, *n*: number of sample used to fit the model. All the regressions were significant ($p < 0.001$, except for bark for 50E:50A–*Eucalyptus*: $p = 0.011$)

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