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Evidence of short-term belowground transfer of nitrogen from *Acacia mangium* to *Eucalyptus grandis* trees in a tropical planted forest

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ABSTRACT

The short-term belowground transfer of nitrogen from nitrogen-fixing trees to companion trees has never been studied in the field. A ^{15}N pulse-labeling study was conducted in a mixed plantation of *Acacia mangium* and *Eucalyptus grandis* at the peak of leaf area, 26 months after planting. ^{15}N–NO_3^- was injected into the stem of one big *Acacia* tree in three plots. ^{15}N was traced over 2 months in the labeled *Acacia* tree as well as in neighboring *Eucalyptus* trees. For both species, young leaves were sampled, as well as fine roots and the rhizosphere at a distance of 0.75 m and 2.25 m from the labeled tree. The ^{15}N atom% was also determined in the wood, bark, branches and total foliage of the 3 labeled *Acacia* trees and 9 *Eucalyptus* trees, 60 days after labeling. Most of the leaves, fine roots and rhizosphere samples of both species were ^{15}N enriched from 5 days after labeling. The $\delta^{15}N$ values were higher at a distance of 0.75 m than at 2.25 m in *Acacia* roots, but were similar at both distances in *Eucalyptus* roots and the rhizospheres. The wood and bark of *Eucalyptus* trees sampled at a distance of 6.2 m from the labeled *Acacia* trees were ^{15}N enriched. This shows belowground N transfer from *Acacia* to *Eucalyptus* trees in the field during the first few days after labeling. This facilitation process may provide a significant amount of the nitrogen requirements of trees close to N-fixing trees in mixed forests.

1. Introduction

Recent studies show that associating nitrogen-fixing trees (NFT) and non-nitrogen-fixing species (non-NFS) can be beneficial for biomass production (Piotto, 2008; Forrester, 2014), soil carbon sequestration (Forrester et al., 2013; Blaser et al., 2014), soil fauna and microbial diversity (Bini et al., 2013; Manhães et al., 2013; Rachid et al., 2013) and soil nutrient availability (Voigtlaender et al., 2012; Blaser et al., 2014; Koutika et al., 2014). In tropical forest ecosystems, up to 90% of the nitrogen (N) in NFTs is derived from symbiotic fixation (Parrotta et al., 1996; Binkley and Giardina, 1997; Nygren et al., 2012). This N input is likely to improve the N status of associated non-NFS in forests established on degraded land (Nichols and Carpenter, 2006), agroforestry systems (Daudin and Sierra, 2008), mixed forest plantations (Binkley et al., 2003; Richards et al., 2010), and tropical forests (Batterman et al., 2013).

Decomposition of aboveground litter, pruning residues and roots is commonly considered to be the main N pathway from NFTs to non-NFS (Mafongoya et al., 1998; Munroe and Isaac, 2014). However, some studies have shown that direct belowground transfer of N without transformation of the N source (Munroe and Isaac, 2014) can also provide substantial amounts of N for non-NFS. Low-molecular N weight compounds, such as ammonium, nitrate and amino-acids (Wacquant et al., 1989; Paynel et al., 2001; Paynel and Cliquet, 2003), exuded by legume roots may be taken up by the

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companion plants (Jalonen et al., 2009; Fustec et al., 2010; Munroe and Isaac, 2014) provided that the plant uptake occurs before these compounds are taken up or mineralized by soil microorganisms, a process that is very fast (Cliquet et al., 1997; Lipson and Näsholm, 2001; Jones et al., 2004). Direct transfer of N can also occur via common mycorrhizal networks (CMN) (He et al., 2004, 2009; Kähkölä et al., 2012; Fellbaum et al., 2014).

Short-term (i.e within a few days or weeks) belowground transfer of N has been observed from NFTs to grasses and agricultural crops. The percentage of N of the grass *Dichantium aristatum* derived from transfer (%NDFT) from N-fixing *Gliricidia sepium* seedlings ranged from 2 to 15% through root exudates, and from 0.5 to 2% via CMNs over 8 weeks in a greenhouse experiment (Jalonen et al., 2009). Sierra and Daudin (2010) estimated that, in the field, 45–80% of the N content in *D. aristatum* was derived from belowground transfer from *G. sepium* trees planted 1–5 m apart, 84 days after cutting the grass. After 21 days of contact between the root systems of *Triticum durum* seedlings and 2-month-old *Acacia senegal* seedlings growing in pots, the %NDFT of the wheat reached 14% (Isaac et al., 2012). In a greenhouse experiment, Catchpoole and Blair (1990) estimated that 4% and 8% of the amount of labeled ^{15}N in *Leucaena leucocephala* seedlings was transferred to *Panicum maximum* grass in 6 and 12 weeks, respectively. In a pot study carried out over 4 weeks, Rao and Giller (1993) estimated that 3–4% of the amount of N in 3-month-old *Leucaena diversifolia* seedlings was transferred to *Cenchrus ciliaris* grass.

Although short-term belowground N transfer from NFTs to grass is now well documented, so far as we are aware (e.g. review from Chalk et al., 2014), such a fast transfer has never been confirmed between NFTs and non-NFTs in the field. The significance of this facilitation process in mixed species forests is still under discussion owing to the lack of experimental data (Chalk et al., 2014). In a 1-month pot experiment, the percentage of N in 6-month-old *Eucalyptus maculata* seedlings that was derived from *Casuarina cunninghamia* plants via CMNs ranged from 1 to 9% depending on the nodulation status (He et al., 2004). This percentage varied from 4 to 30% in 12-month-old seedlings (He et al., 2005). Field studies suggest that N transfer could occur belowground from *Inga edulis* to *Theobroma cacao* (Nygren and Leblanc, 2009, 2015) and from *Acacia* sp. to *Eucalyptus* sp. (Hoogmoed et al., 2014) but the amount and the speed of the belowground N transfer were not estimated.

This study set out to assess whether there was short-term N transfer between NFTs and non-NFTs in a mixed plantation of *Eucalyptus grandis* Hill ex Maid. and *Acacia mangium* Wild. Both *E. grandis* and *A. mangium* are widely planted in tropical regions (FAO, 2010), and mixed plantations of *Eucalyptus* and *Acacia* might be an alternative to *Eucalyptus* monoculture (Forrester et al., 2013). A fast response of *Eucalyptus* trees to N fertilization has been observed in southeast Brazil (Gonçalves et al., 2013). Association with *A. mangium* may create facilitation processes involving atmospheric N_2 fixation and N transfer to *Eucalyptus* trees (Bouillet et al., 2013). This experiment was designed to assess the possible short-term belowground N-transfer from *Acacia* to *Eucalyptus* neighbors up to a distance of 6.2 m from ^{15}N labeled *Acacia* trees. ^{15}N–NO_3^- was injected in the stem of one big *Acacia* tree in three plots. The $\delta^{15}N$ values of recently-expanded leaves, fine roots and the rhizosphere (i.e soil adhering to the root, Hinsinger et al., 2003) of the two species were measured from March 5th to April 30th, 2012, and trees were destructively sampled 2 months after labeling. This study tested the hypotheses that (1) short-term belowground N transfer occurs between *Acacia* and *Eucalyptus*, which would be consistent, with the high densities of fine roots of both species and their intermingling in mixed plantations (Laclau et al., 2013a), and (2) N-transfer is not restricted to *Eucalyptus* trees located very close to *Acacia* trees as *Eucalyptus* trees are able to develop extensive root systems rapidly (O'Grady et al., 2005; Laclau et al., 2013b), to access resource-rich soil patches several meters from the trees (Bouillet et al., 2002; Sudmeyer and Simons, 2008; Silva et al., 2011).

2. Material and methods

2.1. Site description

The study was carried out at the Itatinga experimental station of São Paulo University, Brazil (23°02′S, 48°38′W), at an elevation of 860 m asl. The soils were Ferralsols (FAO classification). The texture was very uniform below a depth of 1 m with clay content around 13% in the A1 layer and ranging from 20% to 25% between 1 m and 6 m in depth. The 0–10 cm soil layer had a cation exchange capacity (CEC) < 2 cmolc kg^{-1} soil, with a mean total N concentration of 0.6 g kg^{-1} soil (Voigtlaender et al., 2012). The soils were typical of large areas planted with *Eucalyptus* in Brazil (Gonçalves et al., 2013). During the study period, the average air temperature was 20.4 °C and the cumulative rainfall, collected in an open area 100 m from the field trial, was 209.9 mm (Fig. 1). The soil water content was monitored in the first block of the field trial. The soil water content at 15 cm was measured every half hour using 3 Campbell CS616 probes, and the values were then averaged over the day.

2.2. Site layout

A complete randomized block design with 7 treatments and 4 blocks was set up in May 2003 to compare monospecific and mixed species stands of *A. mangium* and *E. grandis*. A detailed description of the original experimental layout can be found in Laclau et al. (2008). This study was carried out in 3 plots (1 plot per block of the original experiment) where the two species were planted alternately at 1.5 m spacing in the row, with 3 m between rows, giving a total stocking density of 2222 trees ha^{-1}. Only the boles were harvested in May 2009 and the residues were spread uniformly within each plot. *Eucalyptus* stem wood biomass at harvest time was typical of productive commercial plantations (Bouillet et al., 2013). *A. mangium* and *E. grandis* seedlings were re-planted

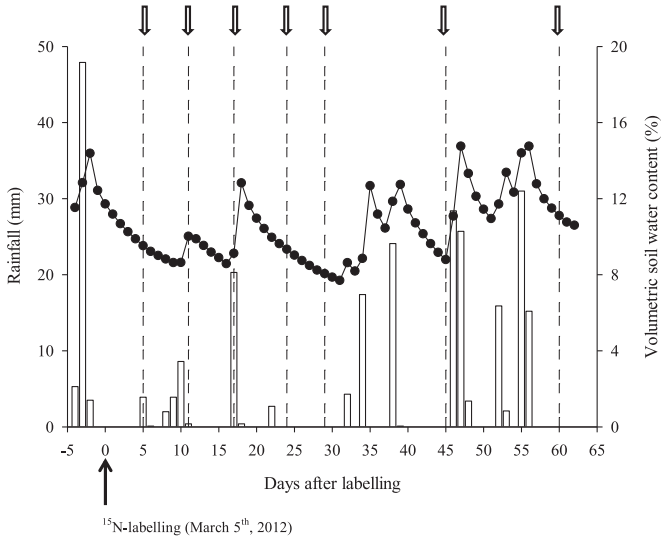

Fig. 1. Rainfall (vertical bars) and volumetric soil water content (black circles) at a depth of 15 cm over the 60 day study period. Sampling dates are indicated by arrows and dotted lines. Five days after *Acacia* labeling, there was rainfall after samples had been collected.

in the same plots on November 2009. Factorial fertilization trials at the study site and in nearby commercial forests on the same soil type showed that the amounts of nutrients applied were non-limiting for *Eucalyptus* tree growth (Laclau et al., 2009), except for N. The fertilizers applied on planting were 40 g P plant^{-1} (buried at 20 cm from the plants), as well as 9 g plant^{-1} K, 3 g plant^{-1} B, 6 g plant^{-1} Fe, 3 g plant^{-1} Zn, and 1 g plant^{-1} Mn. Additional potassium was applied at a rate of 115 kg ha^{-1} K 6 months after planting.

2.3. ^{15}N labeling of Acacia trees

In each plot (3 plots in separate blocks), a ^{15}N labeled solution was injected into the stem of one *Acacia* tree 26 months after planting. This corresponded to the peak leaf area in these fast-growing plantations (le Maire et al., 2013). The labeled trees had a single stem and were selected from the 10% biggest acacias in each stand (mean height of 7.4 m). No tree mortality was observed within a radius of 6.2 m around the labeled *Acacia* trees (Fig. 2). The injection technique was based on a passive uptake design (Proe et al., 2000) with ^{15}N injected into the xylem vessels. For each *Acacia* tree to be labeled, a hole (6 mm in diameter and 25 mm in depth) was drilled into the stem, 1 m above the ground. The drill was lubricated using distilled water to prevent damage to the xylem vessels. Immediately after removing the drill, a polyethylene tube (6 mm in diameter), attached to a bottle containing 100 ml of distilled water, was pushed 20 mm into the drilled hole (Fig. 3). This bottle was then connected to a second bottle containing 0.9 g of N (98 atom% ^{15}N–NO_3^-) as potassium nitrate, dissolved in 400 ml of distilled water. Using two bottles in this way prevented any litter or soil contamination by ^{15}N when inserting the tube into the hole. ^{15}N contamination was also avoided by packing the bark around the tube with non-toxic mineral putty (Terostat®) to prevent the solution leaking, and by covering the soil around the labeled trees with a plastic bag. In addition, a cellulose sheet was placed just below the hole in the stem to check that there were no leaks. The 500 ml solution was absorbed by the stem in 12–36 h depending on the tree. When the solution had been completely absorbed, the hole was filled with Terostat putty. A few *Acacia* leaves fell during the study period and were removed from the experimental area within 24 h of falling.

Fig. 3. ^{15}N-labeling by injection into the stem of 26-month-old *Acacia mangium* (block 1). The bottle at the bottom contained 100 ml distilled water before connection to the second bottle above which contained 0.9 g of N (98 atom% ^{15}N–NO_3^-) dissolved in 400 ml of distilled water.

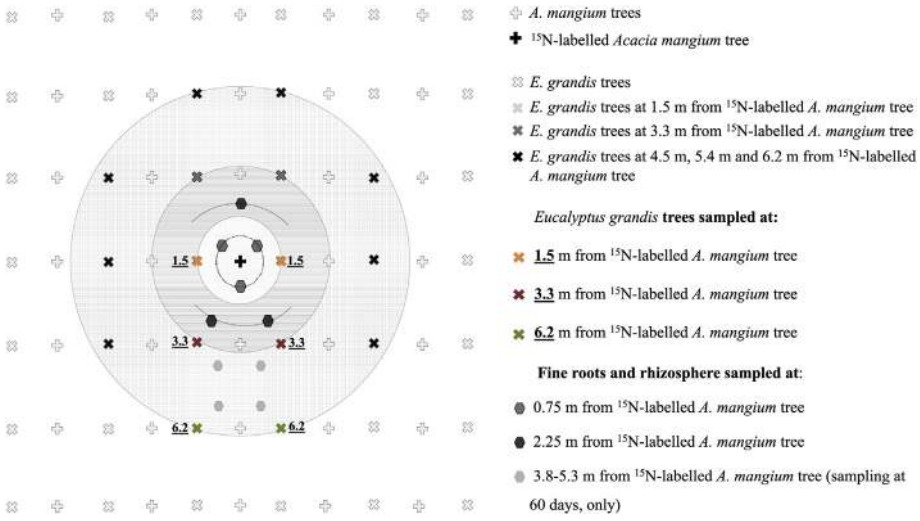

Fig. 2. Sampling plan. Recently expanded leaves in the upper part of the crown, fine roots and rhizosphere of *Acacia mangium* and *Eucalyptus grandis* trees were sampled at various dates after ^{15}N-labeling of *Acacia* trees, as shown in Fig. 1. Two, 4 and 10 *Eucalyptus* trees were located at distances of 1.5 m, 3.3 m and between 4.5 m and 6.2 m, respectively from the ^{15}N-labeled *Acacia* trees. The areas between 0 and 1.5 m from the labeled *Acacia* trees, 1.5–3 m and 3–6.2 m were 7.1 m^2, 21.2 m^2 and 92.5 m^2, respectively.

2.4. Sampling

In each block, scaffolding was used to sample recently expanded leaves (hereafter referred to as young leaves) in the upper third of the crown of the ^{15}N-labeled acacia and of 3 sets of *Eucalyptus* trees (6 per block) located at distances of 1.5 m, 3.3 m and 6.2 m from the acacia (Fig. 2). The mean height of the *Eucalyptus* trees across the 3 blocks was 12.6 m, 10.3 m and 12.6 m at distances of 1.5 m, 3.3 m and 6.2 m respectively from the labeled *Acacia* trees. Five, 11, 17, 24, 29, 45 and 60 days after ^{15}N labeling, 3 leaves were collected from each labeled acacia in each block and 5 leaves from each sampled *Eucalyptus* tree at different angles around the tree. These leaf samples were then bulked for each tree. On the same dates, three soil samples were collected from each block using a PVC tube (5 cm in diameter and 10 cm in length) at distances of 0.75 m and 2.25 m from the labeled *Acacia* trees (Fig. 3). These soil samples were then bulked for each distance and for each block. Sixty days after ^{15}N labeling, four additional soil samples were taken at a distance of 3.8 and 5.3 m from each labeled *Acacia* tree (Fig. 2) and bulked. The soil samples were taken immediately to the laboratory to separate the fine roots (diameter < 2 mm) of each species as in previous studies (Silva et al., 2009; Laclau et al., 2013a). Small fragments of roots were discarded and only the fine roots that could be clearly identified as belonging to one or other of the two species (fine *Eucalyptus* roots had a higher degree of branching and were darker than fine *Acacia* roots (Laclau et al., 2013a)) were used for chemical and isotopic analyses. The rhizosphere was then collected by gently shaking off any soil still adhering to the roots. Before labeling, samples had been taken in the same way to measure the $\delta^{15}N$ background values of the young leaves, the fine roots and the rhizosphere of the two species, except for young *Eucalyptus* leaves which were bulked for each distance from the *Acacia* trees that were to be labeled (3 samples of *Eucalyptus* tree leaves per block).

At the end of the experiment, the labeled *Acacia* tree and one *Eucalyptus* tree at each distance from the labeled *Acacia* were harvested in each block (3 *Acacia* trees and 9 *Eucalyptus* trees in total). The N concentrations and $\delta^{15}N$ values of leaves, living branches, stem wood and stem bark were determined for all the sampled trees. A detailed description of the methodology can be found in Bouillet et al. (2008). $\delta^{15}N$ background values for each tree compartment (Table 1) had been measured for one *Acacia* tree and one *Eucalyptus* tree per block just before labeling.

2.5. Sample preparation and isotopic analyses

The fine roots were washed in tap water. All plant materials were dried at 65 °C to constant weight. The bark, wood and branches were ground in a Wiley mill (0.8 mm mesh) and the leaves, fine roots, and rhizosphere were ground in a porcelain mortar. The $\delta^{15}N$ values and N concentration were determined using a Hydra 20-20 mass spectrometer coupled to an automatic N analyzer (ANCA-GSL, SERCON Co., Crewe, UK) using 10 mg of dry plant material samples and 15 mg of soil samples (Barrie and Prosser, 1996). The precision of the isotopic measurements was 0.001 ^{15}N atom%. The $\delta^{15}N$ value of a given sample was expressed as:

$$\delta^{15}N_{sample}(‰) = \left(\frac{(^{15}N/^{14}N)sample - (^{15}N/^{14}N)air}{(^{15}N/^{14}N)air}\right) \times 1000 \quad (1)$$

2.6. N transfer calculations

2.6.1. Mass balance

The proportion of the ^{15}N injected into the *Acacia* stem that was transferred to the foliage, branches, stem wood and stem bark of the *Eucalyptus* trees sampled at the end of the study period was estimated as:

$$P_{compartment}(\%) = \Big(N\ content^{*}\Big(^{15}N\ atom\%_{60\ days} - {}^{15}N\ atom\%_{0\ day}\Big)\Big/{}^{15}N\Big)^{*}100 \quad (2)$$

Where:

- N content is the N content 60 days after *Acacia* labeling
- $^{15}N\ atom\%_{60\ days}$ is the ^{15}N atom% 60 days after *Acacia* labeling
- $^{15}N\ atom\%_{0\ day}$ is the ^{15}N atom% before *Acacia* labeling
- ^{15}N is the mass of ^{15}N injected = 0.882 g

The underlying assumption of Eq. (2) is that the N content of the *Eucalyptus* compartments did not change from the beginning to the end of the experiment, or that such change was negligible in relation to the ^{15}N enrichment. A marked increase in N content during the study period could lead to a significant underestimation of $P_{compartment}$ (%).

The proportion of the injected ^{15}N transferred to the aboveground compartments of a given *Eucalyptus* tree was estimated as:

$$P_{above}(\%) = P_{foliage}(\%) + P_{branches}(\%) + P_{wood}(\%) + P_{bark}(\%) \quad (3)$$

The proportion of ^{15}N transferred to the foliage, branches, wood and bark of *Eucalyptus* trees within a 6.2 m radius around the labeled *Acacia* was calculated on the assumption that 2, 4 and 10 *Eucalyptus* trees were located at 1.5 m, 3.3 m and 4.5–6.2 m, respectively (Fig. 3):

$$P_{above\_(0-6.2\ m)}(\%) = 2P_{above\_1.5\ m}(\%) + 4P_{above\_3.3\ m}(\%) + 10P_{above\_6.2\ m}(\%) \quad (4)$$

Table 1
Mean $\delta^{15}N$ (‰) values ± standard error (n = 3) in aboveground compartments of *Acacia mangium* and neighboring *Eucalyptus grandis* before (background values) and 60 days after *Acacia* ^{15}N-labeling. For a given tree compartment and a given distance,* indicates significantly higher $\delta^{15}N$ values than background values (P < 0.05). For a given Eucalyptus tree compartment, different letters indicate significant differences between distances (P < 0.05).

Compartments	*A. mangium*		*E. grandis*			
			Background	Distance from *A. mangium*		
	Background	60 days		1.5 m (60 days)	3.3 m (60 days)	6.2 m (60 days)
Total foliage	3.90 ± 0.78	2946.03* ± 1066.13	0.00 ± 0.00	0.35a ± 0.37	−0.29b ± 0.24	−0.34b ± 0.22
Branches	4.33 ± 0.84	2364.20* ± 395.73	0.01 ± 0.01	0.64a ± 0.64	−0.05a ± 0.78	−0.24a ± 0.45
Wood	8.10 ± 1.08	2169.22* ± 495.32	0.01 ± 0.00	4.13*a ± 1.11	2.82*a ± 0.87	2.74*a ± 0.75
Bark	5.15 ± 0.67	971.27* ± 66.95	0.01 ± 0.00	4.12*a ± 1.26	3.04*a ± 0.86	3.17*a ± 0.90

Where:

$P_{above\_1.5m}$ (%), $P_{above\_3.3m}$ (%), $P_{above\_6.2m}$ (%) are the values of P_{above} (%) for the *Eucalyptus* trees at 1.5 m, 3.3 m, and 6.2 m from the labeled *Acacia* tree.

The proportion of injected ^{15}N transferred to fine *Eucalyptus* roots within a radius of 6.2 m around the labeled *Acacia* trees was estimated as:

$$P_{roots\_(0-6.2\,m)}(\%) = 7.1P_{roots\_0.75\,m}(\%) + 21.2P_{roots\_2.25\,m}(\%) + 92.5P_{roots\_3.8-5.3\,m}(\%) \quad (5)$$

Where P_{roots_i} (%) is defined as:

$$P_{roots\_i}(\%) = \left(FR_N\ content * \left(^{15}N\ atom\%_{60\ days} - {}^{15}N\ atom\%_{0\ day}\right)\Big/0.882\right) * 100 \quad (6)$$

Where:

- FR_N is the N content per m^2 of fine roots at a distance i from the labeled *Acacia* tree
- $^{15}N\ atom\%_{60\ days}$ is the ^{15}N atom% of the fine roots collected 60 days after labeling
- $^{15}N\ atom\%_{0\ day}$ is the ^{15}N atom% of the fine roots collected before labeling
- FR_N was estimated by multiplying the mean N concentration in fine roots, collected 60 days after labeling, by a fine root biomass density of 178.5 g m^{-2} as estimated for the 0–2 m soil layer at the same site in plots with the same proportion of *Eucalyptus* and *Acacia* trees (Laclau et al., 2013a). The area considered was 7.1 m^2 between 0 and 1.5 m, 21.2 m^2 between 1.5 m and 3 m, and 92.5 m^2 between 3 m and 6.2 m for fine *Eucalyptus* roots sampled at 0.75 m, 2.25 m, and 3.8–5.3 m, respectively (Fig. 3).

The proportion of injected ^{15}N transferred to *Eucalyptus* trees within a radius of 6.2 m around labeled *Acacia* trees was estimated as:

$$P_{eucalypt\_(0-6.2\,m)}(\%) = P_{above\_(0-6.2\,m)}(\%) + P_{roots\_(0-6.2\,m)}(\%) \quad (7)$$

2.6.2. N derived from transfer

The proportion of *Eucalyptus* N derived from transfer (%NDFT) from *Acacia* was estimated at the collection date using equation (8) (Jalonen et al., 2009; Isaac et al., 2012):

$$\%NDFT(t) = \left(\frac{\delta^{15}N_{Euc}(0) - \delta^{15}N_{Euc}(t)}{\delta^{15}N_{Euc}(0) - \delta^{15}N_{Ac}(t)}\right) \times 100 \quad (8)$$

Where:

- $\delta^{15}N_{Euc}$ is the $\delta^{15}N$ value of fine *E. grandis* roots collected at 0.75 m or 2.25 m from the ^{15}N labeled *Acacia*.
- $\delta^{15}N_{Ac}$ is the $\delta^{15}N$ value of fine *A. mangium* roots collected at 0.75 m or 2.25 m from the ^{15}N labeled *Acacia*.
- $\delta^{15}N_{Euc}(0)$ and $\delta^{15}N_{Euc}(t)$ are the $\delta^{15}N$ values of fine *Eucalyptus* roots before *A. mangium* labeling, and at the end of each collection date, respectively
- $\delta^{15}N_{Ac}(t)$ is the $\delta^{15}N$ value of fine *Acacia* roots at the end of each study period.

The mean value of %NDFT was calculated for each sampling date (2 distances × 3 blocks). The mean value of %NDFT for the 60 day study period was calculated as the mean of %NDFT for each sampling date (n = 7).

2.7. Statistical analyses

For each collection date, each species and each distance (0.75 m and 2.25 m, and 3.8–5.3 m at 60 days), the ^{15}N enrichment of the tree material and rhizosphere was tested against ^{15}N background values using a one-tailed paired t test (analysis 1). For each species and each collection date, a two-way analysis of variance was used to test for differences in the $\delta^{15}N$ values of tree components and rhizosphere due to the distances from the labeled *Acacia* trees (0.75 m, 2.25 m, and 3.8–5.3 m at 60 days) and blocks (analysis 2). A linear mixed model was used (analysis 3) to test the effects of the distance from the labeled *Acacia* tree and the blocks (as fixed effects) as well as the sampling dates (as random effects) on the $\delta^{15}N$ values of fine *Acacia* roots, *Acacia* rhizosphere, fine *Eucalyptus* roots, *Eucalyptus* rhizosphere and young *Eucalyptus* leaves. Additionally, for each sampling date, the increase in N concentration in *Acacia* leaves and fine roots and leaves after ^{15}N stem injection was tested against the initial N concentration values using a one-tailed paired t test (analysis 4). The GLM procedure of SAS 9.3 was used (SAS Institute, Cary, NC, USA) for the fixed effects models. ASReml (Gilmour et al., 2005) was used for the linear mixed models. The homogeneity of variances was tested using Levene's test, and the normal distribution of residuals was tested using Shapiro–Wilks test. The values were log-transformed when variances were unequal. Bonferroni's test was used for comparisons for fixed effects models. Wald's test was used for mixed effects models (Kenward and Roger, 1997). The significance level was 0.05.

3. Results

3.1. $\delta^{15}N$ in labeled Acacia trees

The $\delta^{15}N$ of the young leaves increased significantly after labeling *Acacia* trees (analysis 1) to reach values > 650‰ from 5 days after labeling (Fig. 4). The $\delta^{15}N$ values within young leaves varied between the labeled *Acacia* trees and between the sampling dates, from 11 days after labeling. Fine *Acacia* roots had higher mean $\delta^{15}N$ values than the background values of $\delta^{15}N$ at distances of 0.75 m and 2.25 m from the labeled *Acacia* trees (Fig. 5a) with significant differences (analysis 1) on five collection dates at 0.75 m and two collection dates at 2.25 m. The $\delta^{15}N$ values in fine *Acacia* roots were higher at a distance of 0.75 m from the ^{15}N labeled *Acacia* trees than at a distance of 2.25 m (analysis 3), with significant differences

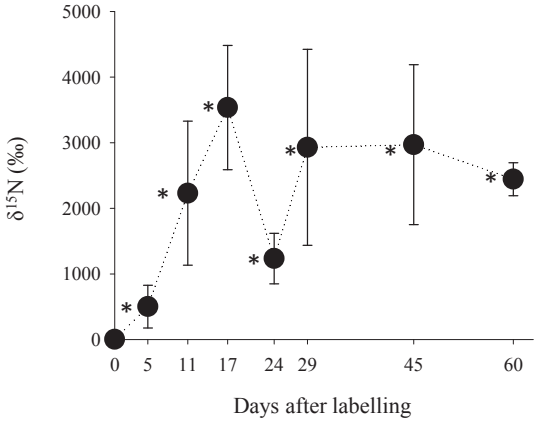

Fig. 4. $\delta^{15}N$ values of *Acacia mangium* young leaves after ^{15}N injection in *A. mangium* stem. Background values are indicated for the labeling date (day 0). Vertical bars indicate standard errors between blocks (n = 3). For a given collection date, * indicates significant differences from $\delta^{15}N$ background values ($P < 0.05$). The mean background $\delta^{15}N$ value was 2.68 ± 0.40‰ (n = 3).

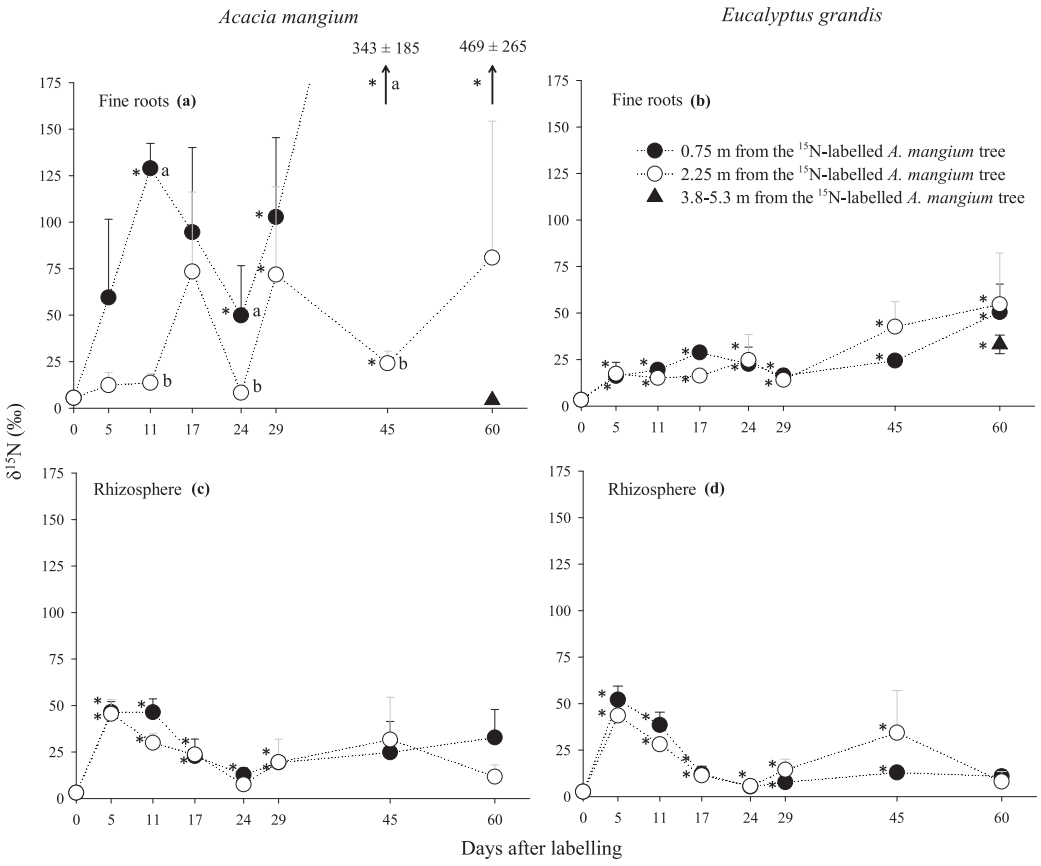

Fig. 5. $\delta^{15}N$ values over the 60 day period of fine roots (a, b) and rhizosphere (c, d) of *Acacia mangium* and *Eucalyptus grandis* collected at a distance of 0.75 m and 2.25 m from the ^{15}N-labeled *A. mangium*. The $\delta^{15}N$ values of fine roots collected at 3.8–5.3 m are given at 60 days. Background values are given at the labeling date (day 0). Vertical bars indicate standard errors between blocks (n = 3). At a given collection date and a given distance, * indicates significant differences from $\delta^{15}N$ background values ($P < 0.05$). The mean background $\delta^{15}N$ values ± standard error (n = 3) were 6.38 ± 0.44‰, 2.38 ± 0.28‰, 3.16 ± 1.02‰, and 3.17 ± 0.38‰, for fine *Acacia* roots, fine *Eucalyptus* roots, *Acacia* rhizosphere and *Eucalyptus* rhizosphere, respectively. At 24 days, significant differences in $\delta^{15}N$ values of *Acacia* rhizosphere were found only at 2.25 m (Fig. 5d). For a given collection date, different letters indicate significant differences between distances ($P < 0.05$). The $\delta^{15}N$ values ± standard error (n = 3) of fine *A. mangium* roots at 0.75 m from the ^{15}N-labeled *A. mangium* tree were 343 ± 185‰ and 469 ± 265‰, at 45 and 60 days respectively.

between the two distances (analysis 2) 11, 24, and 45 days after labeling (Fig. 5a). 60 days after labeling, fine *Acacia* roots had lower mean $\delta^{15}N$ values than $\delta^{15}N$ background values at a distance of 3.8–5.3 m from the labeled trees (Fig. 5a), and all the aboveground compartments of the labeled *Acacia* trees had much higher $\delta^{15}N$ values (Table 1).

3.2. $\delta^{15}N$ in the rhizosphere

The rhizosphere of both species had higher $\delta^{15}N$ values after labeling than the background values, at all distances from the labeled *Acacia* trees, with significant differences (analysis 1) on most of the sampling dates (Fig. 5c, d). The ^{15}N enrichment of the rhizosphere was not significantly affected by the sampling distances, for all species and on all collection dates (analyses 2 and 3). The time series of $\delta^{15}N$ values in the rhizosphere showed a similar pattern for both species with a rapid initial increase after labeling, followed by a marked decrease from 5 to 24 days. Over the study period, the *Eucalyptus* rhizosphere had only slightly lower ^{15}N enrichment than the *Acacia* rhizosphere with mean $\delta^{15}N$ values of 20.4‰ and 26.8‰, respectively.

3.3. $\delta^{15}N$ in Eucalyptus trees

Fine *Eucalyptus* roots were significantly ^{15}N-enriched (analysis 1) at distances of 0.75 m and 2.25 m from the labeled *Acacia* trees from 5 days after labeling, as well as at a distance of 3.8–5.3 m from the labeled *Acacia* trees at 60 days after labeling (Fig. 5b). Over the study period, the change in $\delta^{15}N$ values of the fine *Eucalyptus* roots was not significantly different at distances of 0.75 m and 2.25 m from the labeled *Acacia* trees (analyses 2 and 3). 60 days after labeling fine *Eucalyptus* roots had mean $\delta^{15}N$ values of 50‰ at a distance of 0.75 m from the labeled *Acacia* trees, 55‰ at 2.25 m, and 33‰ at 3.8–5.3 m, and the effect of the distance from the labeled tree was not significant (Fig. 5b).

On most of the sampling dates, the $\delta^{15}N$ values in young *Eucalyptus* leaves were higher than the background values (Fig. 6). Differences were significant (analysis 1) 5 days after labeling at a distance of 1.5 m from the labeled *Acacia* trees and 45 and 60 days after labeling, at distances of 3.3 m and 6.2 m. There were no significant differences in the $\delta^{15}N$ values of young *Eucalyptus* leaves at different distances from the labeled *Acacia* trees (analyses 2 and 3). However, the mean $\delta^{15}N$ values of 2.95‰ at 1.5 m, 2.55‰ at 3.2 m, and 2.39‰ at 6.2 m showed a slight decrease with the distance from the labeled *Acacia* trees. The $\delta^{15}N$ values of young *Eucalyptus* leaves also decreased slightly with distance from 1.5 m to 6.2 m from the labeled *Acacia* trees on four of the seven collection dates.

60 days after labeling the *Acacia* trees, the wood and bark of neighboring *Eucalyptus* trees had a significantly higher $\delta^{15}N$ (analysis 1) but without significant variation with distance (analysis 2) from the labeled *Acacia* trees (Table 1). The branches and the total foliage of the same *Eucalyptus* trees did not have

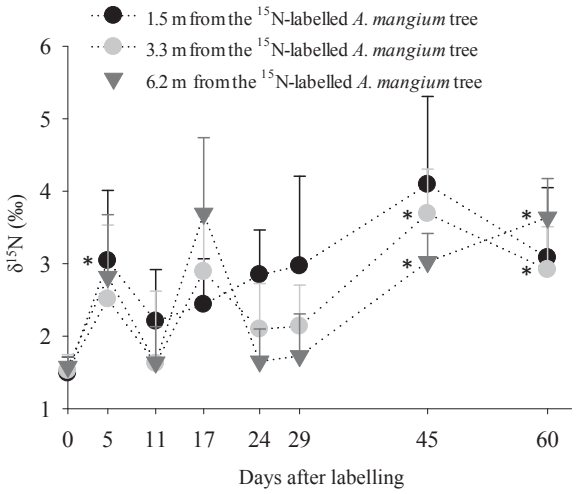

Fig. 6. $\delta^{15}N$ values over the 60 day period of young leaves of *Eucalyptus grandis* pairs located at a distance of 1.5 m, 3.3 m and 6.2 m from the ^{15}N-labeled *Acacia mangium*. The background values are indicated for the labeling date (day 0). Vertical bars indicate standard errors between blocks (n = 3). At a given collection date and a given distance, * indicates significant differences with $\delta^{15}N$ background values ($P < 0.05$). The mean background $\delta^{15}N$ values ± standard error (n = 3) were 1.49 ± 0.36‰, 1.53 ± 0.35‰, and 1.58 ± 0.21‰, at a distance of 1.5 m, 3.3 m and 6.2 m, respectively.

significantly higher $\delta^{15}N$. However, the $\delta^{15}N$ values for the total foliage were significantly higher at a distance of 1.5 m than at distances of 3.3 m and 6.2 m from the labeled *Acacia* trees (analysis 2).

3.4. ^{15}N transferred to Eucalyptus trees

The percentage of the ^{15}N injected into *Acacia* trees that was transferred within 60 days to the *Eucalyptus* trees within a radius of 6.2 m around the labeled *Acacia* trees was estimated at 3.33 ± 1.24% (2.25%, 0.85%, 0.16%, 0.04% and 0.03% for fine roots, wood, bark, foliage and branches, respectively).

The percentage of N of *Eucalyptus* trees derived from transfer (%NDFT) from *Acacia* trees estimated using Eq. (8) ranged from 15.8% to 78.2% depending on the sampling dates, with an average value of 43.2% over the experimental period of 60 days (Fig. 7).

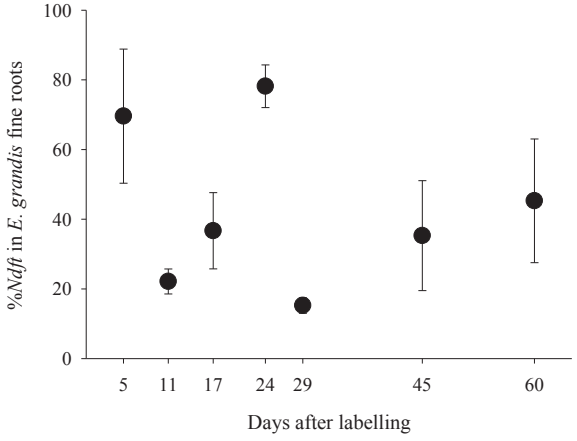

Fig. 7. Estimates of the percentage of *Eucalyptus grandis* nitrogen derived from *Acacia mangium* (%NDFT) for 5, 11, 17, 24, 29, 45 and 60 days after *Acacia* labeling. Vertical bars indicate standard errors between individual estimates (n = 6). The mean %NDFT value during the 60 day study period was 43.2%.

4. Discussion

4.1. A suitable method for ^{15}N labeling donor trees

^{15}N pulse labeling aims to monitor the fate of ^{15}N without modifying the N status of the target species (He et al., 2006). ≈0.9 g $^{15}N{-}NO_3$ was injected into the *Acacia* stem representing 0.85%, 0.74% and 0.94% of the crown N (estimated from destructive sampling of leaves and branches 2 months after labeling: see Table 1 in Supplementary material). These percentages were close to the 1% concentration of crown N proposed by Swanston and Myrold (1998). The low amount of N injected did not significantly increase the N concentration of the leaves and fine roots of the labeled *Acacia* trees over the study period (see Table 2 in Supplementary material). However, high $\delta^{15}N$ values in the rhizosphere 5 days after labeling may have resulted from an initial increase in N root exudates, consistent with the slight enrichment of the soil N after foliar ^{15}N-labeling of *Medicago sativa* and *Trifolium subterraneum* plants (Gardener et al., 2012). An increase in N exudates owing to the damage to the tree cannot be excluded (Hale and More, 1979). In our experiment, ^{15}N-enriched nitrate exchanges between xylem and phloem saps may also have occurred after injection in the stem (Pate et al., 1998; Peuke et al., 2013), which would explain the rapid increase in ^{15}N in the rhizosphere.

The stem injection method made it possible to label *Acacia* leaves rapidly. The same technique was used to label 20-year-old *Picea sitchensis* (Nair et al., 2014) where the average $\delta^{15}N$ within the needles was 4850‰, 4.5 months after injection. In our experiment, there was a high ^{15}N enrichment of young *Acacia* leaves from 5 days after labeling (Fig. 4) which suggests a rapid transport of $^{15}N{-}NO_3^-$ to the foliage by mass flow (Millard et al., 2006; Augusto et al., 2011). However, the rise in $\delta^{15}N$ within the *Acacia* leaves up to 17 days after labeling suggests that other processes were involved in the accumulation of ^{15}N in the leaves. A reduction of injected nitrate in the stem (Black et al., 2002; Miller and Cramer, 2005) leading to the production of ^{15}N-enriched amino acids (Pfautsch et al., 2009) transported in the xylem sap to the leaves could explain why ^{15}N concentrations continued to increase after the rapid mass flow of injected $^{15}N{-}NO_3^-$. The limited number of sampled leaves and a dilution effect depending on the size of *Acacia* trees (Swanston and Myrold, 1998) may have accounted for the high $\delta^{15}N$ variability in the young *Acacia* leaves between blocks.

4.2. Evidence of short-term N transfer belowground

In agreement with our first hypothesis, N was rapidly transferred belowground since ^{15}N enrichment was already detectable in fine *Eucalyptus* roots 5 days after labeling. Large differences in $\delta^{15}N$ values in the young *Eucalyptus* leaves sampled probably reflected high $\delta^{15}N$ variability in the crown. ^{15}N dilution during translocation of N compounds from roots to leaves (He et al., 2006) could explain the sharp decrease in $\delta^{15}N$ values observed from the stem to the canopy of *Eucalyptus* trees (Table 1). This variability and the low number of replicates (6 per distance from labeled *Acacia* trees for a given date) led to non-significant ^{15}N enrichment of the young *Eucalyptus* leaves on some sampling dates (Fig. 6). However, the $\delta^{15}N$ in young *Eucalyptus* leaves was higher in most of the samples than the maximum $\delta^{15}N$ background value (2.19‰). The increase in $\delta^{15}N$ values during the study period was proportionally higher in fine *Eucalyptus* roots than in young *Eucalyptus* leaves. This pattern might be linked to ^{15}N enrichment in fine roots relative to leaves (Pardo et al., 2012; Craine et al., 2015), consistent with $\delta^{15}N$ background values of 2.38‰ in the fine roots and 1.53‰ in the young leaves of the *Eucalyptus* trees sampled in our study. Despite a high variability within the crown, the effect of the distance from the

labeled trees tended to be more pronounced for young leaves than for fine roots. The lack of precipitation between the ^{15}N labeling and the sampling of fine roots and leaves 5 days after labeling shows that the short-term N transfer cannot be explained by an uptake of ^{15}N-rich compounds dissolved in *Acacia* throughfall and stemflow.

Direct and indirect routes might account for the short-term N transfer observed belowground between *A. mangium* and *E. grandis* trees. As found for carbon (Simard et al., 1997; Fitter et al., 1998) and for phosphorus (Wilson et al., 2006; Teste et al., 2014), N transfer may occur when plants are directly connected through common mycorrhizal networks (CMN) (e.g. Ingleby et al., 2007). Such a transfer was observed in a greenhouse experiment between Nitrogen Fixing Trees (NFT) and non-NFT (He et al., 2004, 2005), and in the field between *Pinus sabiniana* and *Quercus douglasii* (He et al., 2006), and *Pseudotsuga menziesii* tree and seedlings (Teste et al., 2009). In our experiment, N may have been transferred between *Acacia* and *Eucalyptus* via CMNs since both *A. mangium* and *E. grandis* roots can be colonized by ectomycorrhizal *Pisolithus* sp. and *Scleroderma* sp. (Founoune et al., 2002; Duponnois and Plenchette, 2003; Ducousso et al., 2012). As in other subtropical forests (Toju et al., 2014), *Acacia* and *Eucalyptus* roots in our experiment were colonized by both arbuscular mycorrhizas and ectomycorrhizas in the 0–10 cm soil layer (Bini, 2012; Pereira, 2014).

Root exudation of N compounds could also be a major pathway for direct N transfer from *Acacia* to *Eucalyptus* trees. N exudates, mainly ammonium, amino acids and ureides, may account for up to 70% of the total N of annual legumes, and constitute a source of N that can be rapidly taken up by neighboring plants (Wichern et al., 2008; Fustec et al., 2010). In our experiment, N exuded by *Acacia* trees might be expected to be taken up by *Eucalyptus* trees. Significant ^{15}N enrichment of the rhizosphere of both species was observed in our experiment from 5 days after labeling. $\delta^{15}N$ values were of the same magnitude in the *Acacia* and *Eucalyptus* rhizospheres (Fig. 5c, d), despite much higher ^{15}N enrichment of fine *Acacia* roots than of fine *Eucalyptus* roots (Fig. 5a, b). This pattern could be related to the dense entanglement of the roots of the two species, as observed when separating the roots in the laboratory, causing rapid N exchange between the rhizospheres. Soil N inputs in the soil derived from exudation decrease drastically in the first few millimeters from the plant fine roots (Merbach et al., 1999; Schenck zu Schweinsberg-Mickan et al., 2010, 2012). The capacity of fine *Eucalyptus* roots to take up rapidly large amounts of N released in the vicinity of fine *Acacia* roots could, therefore, help to explain the dense intermingling of roots of the two species in the upper soil layer which helps to increase N transfer (Xiao et al., 2004). The similar values of $\delta^{15}N$ in the rhizosphere at a distance of 0.75 m and 2.25 m from the labeled *Acacia* trees, despite generally higher ^{15}N enrichment of fine *Acacia* roots at a distance of 0.75 m than at 2.25 m could reflect the high N uptake by *Eucalyptus* roots in the first years after planting (Laclau et al., 2010). Time series analysis of changes in the soil solution have clearly shown a very fast uptake of nutrients by fine *Eucalyptus* roots in the top soil (Laclau et al., 2004, 2010; Mareschal et al., 2013), which might prevent ^{15}N accumulation in the rhizosphere. A similar ^{15}N enrichment of the rhizosphere at the 2 distances from the labeled *Acacia* trees sampled during periods with low rainfall and low soil moisture (24–29 days after labeling) as well as during periods with high rainfall and high soil moisture (11–17 days after labeling) suggest that the amounts of N released by the *Acacia* roots leached below the 0–10 cm soil layer were low relative to the uptake by neighboring trees.

Rapid transfer of N might also occur indirectly through quick turn-over of very fine roots or of mycorrhizal hyphae of *Acacia* trees (Staddon et al., 2003). Furthermore, very rapid decomposition of nodules cannot be excluded: the half-life of *Erythrina variegata* and *G. sepium* nodules decomposing in litterbags varied from 3 to 5 days in tropical conditions (Nygren et al., 2000).

A mass balance approach was used to give a rough estimate that approx. 3% of the amount of ^{15}N injected into the stem of each labeled *Acacia* tree was transferred within 2 months to the neighboring *Eucalyptus* trees within a radius of 6.2 m. This probably under-estimated the amount of ^{15}N transferred since account was not taken of a possible increase in *Eucalyptus* tree N content during the study period (eq. (2)), as well as the ^{15}N transfer to big and medium-sized roots, and very deep fine roots (at depths > 2 m). This rapid ^{15}N transfer would be consistent with significant short-term belowground N transfer to *Eucalyptus* trees. High percentages of N derived from transfer (%NDFT) in this study also suggest that N transfer from *Acacia* to *Eucalyptus* trees is an important facilitation process in the mixed species stand. However, %NDFT was probably overestimated using equation (8), with a mean %NDFT value of 78% at 24 days, and some individual %NDFT estimates of 100%. While the ^{15}N compounds transferred from *Acacia* to *Eucalyptus* trees were considered to have the $\delta^{15}N$ signature of *A. mangium* fine roots in equation (8), large uncertainties in the patterns of belowground *Acacia* N transfer (from root exudates, mycorrhizal hyphae…) and in ^{15}N discrimination during this process (Craine et al., 2015) prevented any reliable estimate of %NDFT in our study. %NDFT values in pot experiments usually range between 1 and 15% (Chalk et al., 2014). However, up to 32% of N derived from transfer was reported in *E. maculata* seedlings associated with *C. cunninghamia* in two-chambered containers (He et al., 2005). Our study was conducted on 2-year-old trees that might give significantly different results from young seedlings as shown by experiments on *E. maculata* and *C. cunninghamia* where the %NDFT at 12 months was double that at 6 months of age (He et al., 2004, 2005).

4.3. Short-term N transfer at long distance from Acacia trees

N was mainly released belowground close to the trunk of the labeled *Acacia* trees. Fine *Acacia* roots were ^{15}N enriched at a distance of 0.75 m and 2.25 m, with mean $\delta^{15}N$ values of 178‰ and 41‰, respectively. By contrast, fine *Acacia* roots had $\delta^{15}N$ values of only 4‰ at 3.8–5.3 m 60 days after labeling (Fig. 5a), showing that the horizontal growth of fine *Acacia* roots was likely restricted to the first few meters from the *Acacia* stem.

Some compartments of the *Eucalyptus* trees as far as 6.2 m from the labeled *Acacia* trees showed signs of ^{15}N enrichment (Table 1 and Fig. 6) indicating that short-term N transfer was not restricted to the *Eucalyptus* trees located in the immediate vicinity of the labeled *Acacia* trees (hypothesis 2). The capacity of 2-year-old *Eucalyptus* trees to take up N released by *Acacia* roots at a distance of 5–6 m is consistent with the rapid growth of *E. grandis* roots shown by Christina et al. (2011). Rapid transfer of N within fine *Eucalyptus* roots was shown by the $\delta^{15}N$ values at 3.8–5.3 m from the labeled trees being close to those at 0.75 and 2.25 m (Fig. 5b).

5. Conclusion

Direct injection of ^{15}N into the xylem as used in our study was shown to be suitable for pulse ^{15}N labeling of *Acacia* trees. This method could be useful for tracing belowground N transfer between tree species in other mixed forests. We showed, for the first time in the field, the short-term belowground transfer of nitrogen from nitrogen fixing trees to neighboring trees. Nitrogen was transferred belowground to *Eucalyptus* trees within a radius of 6 m around *Acacia* trees, from 5 days after labeling. This suggests that this facilitation process may promote N nutrition of non-NFTs growing in unfertile tropical soils when the fine roots of NFTs and

non-NFTs are intermingled or are directly connected through common mycorrhizal networks.

Conflict of interests

No conflict of interests declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2015.08.017.

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