

Uptake of soil mineral nitrogen by *Acacia mangium* and *Eucalyptus urophylla* × *grandis*: No difference in N form preference

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Abstract

The introduction of N₂-fixing tree species in fast growing tree plantations is a sustainable management option aiming to reduce the risk of nitrogen (N) deficiency due to a large and frequent exportation of nutrients at harvest. Differences in soil mineral N preferences between *Eucalyptus urophylla* × *grandis* and *Acacia mangium* may, in addition to facilitation related to atmospheric N₂ fixation, contribute to the success of mixed-species plantations of the two species on nutrient-poor soils of the coastal Congolese plains. We tested whether these two species differ in their preference for nitrate or ammonium by supplying either ammonium or nitrate enriched in ¹⁵N to six-month-old potted trees growing in an open-air nursery. Although the uptake of nitrate tended to be higher than that of ammonium by both species, the difference was not significant and there was no significant difference between the species regarding their preferred form of soil mineral N. Despite much lower N contents in foliage, stems, and roots of eucalypt compared to acacia, the specific rates of N uptake were up to three times higher for eucalypt than acacia, which suggests that atmospheric N₂ was the major source of N in the six-month-old acacias. We conclude that N₂ fixation rather than complementarity for soil mineral nitrogen alleviates the competition between species in successful mixed eucalypt and acacia plantations.

Key words: acacia / eucalypt / ferralic arenosols / ¹⁵N labeling / N preferences / N uptake

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1 Introduction

Nitrogen-fixing tree species (NFTS) are common in natural forests worldwide and acacias are sympatric with eucalypts in several Australian forests (Pfautsch et al., 2009; Adams et al., 2010). The introduction of acacia in eucalypt plantations is considered as a promising sustainable management option to improve nutrient cycling and soil fertility (Forrester et al., 2005a; Bouillet et al., 2013), alleviating the risk of nitrogen (N) deficiency due to the frequent exportation of large quantities of harvested biomass (Laclau et al., 2010). Three types of ecological interactions may be found in mixed-species stands that include an NFTS (Keltly, 2006). Facilitation, defined as the positive influence of one species on the growth of another (Fridley, 2001), is expected if N limits growth and if N₂ fixation by NFTS increases nitrogen availability (Binkley, 1992; Khanna, 1997; Kaye et al., 2000; Koutika et al., 2014). Competition between individuals of two species occurs when they are foraging for the same limiting resources and results in slower growth in at least one of the two competing species (Forrester et al., 2005b). For example, the failure of a mixed-species plantation of acacia and eucalypt has been ascribed to exacerbated competition for either water (Nouvellon et al., 2012) or phosphorus (Boyden et al., 2005; Forrester et al., 2006).

However, in some cases, two species in a mixed plantation may exploit a limiting resource more efficiently than either of them in a monoculture (Fridley, 2001; Cardinale et al., 2007; Hinsinger et al., 2011). This complementarity, which may be due to niche differentiation and which reduces competition, may result from temporal or spatial partitioning. For example, the canopy stratification observed in mixed-species stands of eucalypt and acacia leads to a greater absorption of the photosynthetically active radiation (Bauhus et al., 2004; le Maire et al., 2013). Spatial partitioning of below-ground resource acquisition is also possible (Lehmann, 2003; Allen et al., 2004; Mulia and Dupraz, 2006) but is yet poorly documented (Jose et al., 2006). In addition to spatial partitioning, competition for a resource may be reduced when each species of a mixed stand uses a different form of the resource (McKane et al., 2002). Regarding mineral nitrogen, one species may rely more on ammonium and the other more on nitrate (Miller and Bowman, 2002; Weigelt et al., 2005; Pfautsch et al., 2009).

An increase in N availability related to higher amounts of N in above-ground litterfall was observed in mixed-species stands of *Eucalyptus urophylla* × *grandis* and *Acacia mangium* in coastal Congo where the mixed-species stands gave a great-



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er wood yield than monospecific stands (Epron et al., 2013; Koutika et al., 2014). At this site (Tchichelle et al., 2016) as in other sites in Congo (Bernhard-Reversat, 1996) and in Brazil (Voigtlaender et al., 2012) N mineralization was higher under acacia than under eucalypt. However, while nitrate represented about 55% of the total mineralized N under both eucalypt and acacia, ammonium was the dominant form of mineral nitrogen in the soil under eucalypt, whereas it was nitrate under acacia (Tchichelle et al., 2016). This has also been observed in southern China for *E. citriodora*, *A. mangium*, and *A. auriculaeformis* (Li et al., 2001). This may suggest that eucalypts take up nitrate preferentially, depleting soil nitrate, and that acacia and eucalypt do not use the same form of soil mineral nitrogen. If such a partitioning of mineral N forms occurred, it would contribute to an efficient utilization of available soil N in mixed-species plantations of acacia and eucalypt, which, in addition to the facilitation related to N_2 fixation, could explain the success of mixed-species plantations on the nutrient-poor soils of the coastal Congolese plains. However, previous studies have shown that several eucalypt species (Turnbull et al., 1996; Garnett and Smethurst, 1999; Garnett et al., 2003; Warren, 2006; Warren and Adams, 2007) preferentially take up ammonium, and one study reports ammonium preference for *E. regnans* and for the co-occurring *A. dealbata* and *A. melanoxylon* (Pfausch et al., 2009).

While our field observations reported above suggested that *E. urophylla* × *grandis* preferentially takes up nitrate rather than ammonium whereas *A. mangium* does not exhibit any particular preference for either of the two sources of soil mineral N, this hypothesis is weakly supported by the literature. The aim of this study was therefore to test whether the hybrid *E. urophylla* S.T. Blake × *E. grandis* Hill ex Maid (*E. urophylla* × *grandis*) and *A. mangium* Wild differ in their respective preference for nitrate or ammonium. With this in mind, we supplied either ^{15}N -enriched ammonium or ^{15}N -enriched nitrate to six-month-old potted trees growing in an open-air nursery.

2 Material and methods

2.1 Experimental design

The experiment was conducted in a tree nursery close to Tchissoko village in the Republic of the Congo (4°44'41''S, 12°01'51''E, 100 m asl). The mean annual air temperature is 25°C with low seasonal variations and the annual precipitation averages 1200 mm. Soil was collected (0–10 cm deep) in a nearby savannah, similar to those that were afforested with eucalypts over the last 40 years, and used to fill 10-L plastic pots (10 kg of dry soil). The soil in this area is classified as a Ferralic Arenosol (WRB) and contains more than 90% of sand and less than 3% of clay (Nouvellon et al., 2008). The pH is acidic (5.2) and the concentration of total N is low (0.4 g kg⁻¹). Seeds of *A. mangium* were sown and cuttings of *E. urophylla* × *grandis* (clone 18-147) were planted in May 2013 and were grown for six months outside. A multi-nutrient fertilizer (5 g, N-P-K, 12-13-21, $\delta^{15}\text{N} = 1.5\text{‰}$) was added two months after planting. The pots were watered daily.

2.2 ^{15}N labeling

Two ^{15}N -labeled solutions and one ^{15}N -unlabeled solution containing each 38 mM of N (19 mM as nitrate and 19 mM as ammonium) were prepared by mixing KNO_3 and $(\text{NH}_4)_2\text{SO}_4$, either labeled at 99% (Cambridge Isotope Laboratories Inc., Andover, MA, USA) or unlabeled ($\delta^{15}\text{N}$ of 8.1‰ for NO_3^- and 3.9‰ for NH_4^+). At six months, the trees were divided into six batches of eight plants of each species (16 pots per batch). One batch received 80 mL of water per pot, one received 80 mL of unlabeled NO_3^- and NH_4^+ (3 mmol N per pot), two batches received labeled NO_3^- (unlabeled NH_4^+) and two batches received labeled NH_4^+ (unlabeled NO_3^-). Using a syringe, 20 mL of solution were slowly and evenly sprayed on the surface of the pot. The same amount was given four times within 1 h (totaling 80 mL per pot). The amount of added N was twice the expected amount in the native soil in order to override a potential different pool size of nitrate and ammonium in the native soil. Irrigation was stopped two days before labeling to avoid drainage of the N-labeled solution. Pots were randomly arranged under a transparent plastic film stretched 2 m above the plants in case of rain. The plants were irrigated twice with 160 mL of water, 6 h and 30 h after labeling.

2.3 Plant harvest and analyses

Half of the plants (*i.e.*, the batch that had received water only, one that had received $^{15}\text{NO}_3^-$, and one that had received $^{15}\text{NH}_4^+$) were harvested 24 h after the N-labeled solutions had been administered. The other half was harvested 48 h after labeling. The shoots were excised and separated into foliage and stems. The roots, including attached nodules for acacia, were removed from the soil using a 1-mm mesh screen. They had colonized the entire soil volume in all pots. Roots were carefully washed in water, immersed in a 0.1 M KCl solution to remove labeled ammonium or nitrate adsorbed on the surface and rinsed again in water once more. Soil samples from pots with acacia and eucalypt that did not receive any N solution were kept in a chilled box and then stored in a refrigerator for less than 24 h to determine the amount of mineral N in the soil just before labeling. Soil subsamples (20 g) from these 16 pots were suspended in 50 mL of 1 M KCl solution and the extracts were analyzed for NO_3^- and NH_4^+ using colorimetry (SKALAR-SAN++).

Foliage, stems and roots were oven-dried at 65°C for a week, weighed, finely ground, and analyzed for total N concentration and ^{15}N composition using an elemental analyzer (CE, Milan, Italy) coupled to a mass spectrometer (Delta-S, Thermo-Finnigan, Bremen, Germany). The precision of the analyses (standard deviation of atom fraction expressed in ‰) was 0.001% (for 23 measurements of an internal standard at 0.366%, $\delta^{15}\text{N}$ of -3.3‰) and 0.013% (for 35 measurement of an internal standard at 0.390%, $\delta^{15}\text{N}$ of 49.5‰), respectively. Three more enriched internal standards (0.527%, 0.689% and 1.248%), that were run only three times each, showed SD below 0.005.

2.4 Data analyses

The ^{15}N results are expressed as atom fraction, $x(^{15}\text{N})$, according to the nomenclature recommended by the Commission on Isotopic Abundances and Atomic Weights of the International Union of Pure and Applied Chemistry (Coplen, 2011). Excess ^{15}N in foliage, stems and roots, $x^E(^{15}\text{N})$, was calculated after taking into account the background ^{15}N atom fraction measured on the same organs on the trees growing in pots receiving no ^{15}N -labeled solution, $x(^{15}\text{N}_{UN})$, and expressed per unit of dry mass knowing tissue N concentration:

$$x^E(^{15}\text{N}) = N \times [x(^{15}\text{N}) - x(^{15}\text{N}_{UN})], \quad (1)$$

$x^E(^{15}\text{N})$ in the whole plant was calculated as the weighted average of the $x^E(^{15}\text{N})$ in leaves, stems and roots, taking into account the mass distribution between these three compartments, and was used to estimate the rates of uptake of nitrate or ammonium (NUR) as

$$NUR = \frac{x^E(^{15}\text{N}) \times \left(1 + \frac{T_{UN}}{T_U}\right)}{t}, \quad (2)$$

with t , the time elapsed during labeling and sampling, T_U the amount of added ^{15}N per kg of soil, in the form of either nitrate or ammonium, and T_{UN} the amount of nitrate or ammonium in the soil just before labeling to account for the dilution of the tracer. The specific rate of uptake was calculated by dividing NUR by the mass of the whole plant.

Statistical analyses were performed with the R software version 3.2.4 (R Core Team, 2016). One-way ANOVA was used to estimate the effect of the species on the concentration of mineral N in the soil collected in pots that did not receive any N solution, and plant biomass and N concentration. Three-way ANOVA was used to test the effect of species, N form, and labeling-to-harvest time and their interactions on excess ^{15}N in the plant compartments and on N uptake rates. Data were log-transformed to meet the requirement of homoscedasticity.

3 Results

The concentration of mineral N of soil collected from pots that did not receive any N solution was lower in pots planted with eucalypts than in pots planted with acacias (Table 1). At the age of 6 months, the biomass of acacias was higher than that of eucalypts, and tissue and whole-tree N concentrations were greater in acacia than in eucalypt (Table 2). Acacia's roots were well nodulated.

All tree compartments were labeled 24 h after ^{15}N application to soil (Fig. 1), and the ^{15}N excess in all compartments increased significantly between 24 and 48 h (Table 3). While roots were more labeled than either stems or foliage after 24 h, the increase in $x^E(^{15}\text{N})$ with time was more pronounced in the foliage. The foliage of eucalypts was almost one order of magnitude more labeled than the foliage of acacias after 48 h, and significantly higher ^{15}N enrichment in eucalypt was observed in all compartments (Table 3). Except for roots, there was a significant trend towards greater ^{15}N excess when the plants had been labeled with $^{15}\text{NO}_3^-$.

Table 1: Mean concentrations (\pm standard errors, $n = 8$) of ammonium (NH_4^+), nitrate (NO_3^-) and total mineral N ($\text{NH}_4^+ + \text{NO}_3^-$) in soil sampled from pots that were not sprayed with N solution and in which *Acacia mangium* and *Eucalyptus urophylla* \times *grandis* plants had grown during 6 months. Differences between species were tested using ANOVA (P -values shown). The amount of N added to the other pots was $0.15 \text{ mmol kg}^{-1}$ of soil for both NH_4^+ and NO_3^- ($0.30 \text{ mmol kg}^{-1}$ of soil in total).

	NH_4^+	NO_3^-	$\text{NH}_4^+ + \text{NO}_3^-$
	/ mmol kg^{-1} of soil		
Acacia	0.12 ± 0.01	0.04 ± 0.01	0.17 ± 0.02
Eucalypt	0.08 ± 0.01	0.03 ± 0.01	0.11 ± 0.01
P -value	0.05	0.12	0.03

Table 2: Mean biomass (foliage, stems, roots, and total) and N concentrations of six-month-old *Acacia mangium* and *Eucalyptus urophylla* \times *grandis* (\pm standard errors, $n = 48$). Total N concentration was calculated as the weighted average of N concentration in leaves, stems, and roots taking into account the mass of each of the three compartments. Differences between species were tested using ANOVA (P -values shown).

	Foliage	Stem	Root	Total
Biomass / g DM				
Acacia	30.0 ± 1.9	13.1 ± 0.9	9.1 ± 0.5	52.3 ± 3.1
Eucalypt	7.2 ± 0.5	4.3 ± 0.5	6.2 ± 0.3	17.7 ± 0.9
P -value	< 0.001	< 0.001	< 0.001	< 0.001
N concentration / g kg^{-1} DM				
Acacia	21.7 ± 0.4	7.8 ± 0.2	16.5 ± 0.5	17.4 ± 0.3
Eucalypt	10.2 ± 0.3	3.6 ± 0.1	5.2 ± 0.3	6.9 ± 0.2
P -value	< 0.001	< 0.001	< 0.001	< 0.001

Table 3: Probabilities (P -values) for the main effects of species (S), labeled N form (either ammonium or nitrate, N), labeling-to-harvest time (T) and their interactions on excess ^{15}N in foliage, stem, and roots, and on the rate of N uptake, tested with three-way ANOVA based on 64 individuals ($2 \text{ S} \times 2 \text{ N} \times 2 \text{ T} \times 8$ replicates).

	Excess ^{15}N in			N uptake
	Foliage	Stem	Root	
Species (S)	< 0.001	< 0.001	< 0.001	< 0.001
N form (N)	0.047	0.021	0.12	0.06
Time (T)	< 0.001	< 0.001	< 0.001	0.21
S \times N	0.25	0.06	0.32	0.21
S \times T	0.52	0.29	0.14	0.44
N \times T	0.98	0.69	0.67	0.75
S \times N \times T	0.27	0.58	0.92	0.79

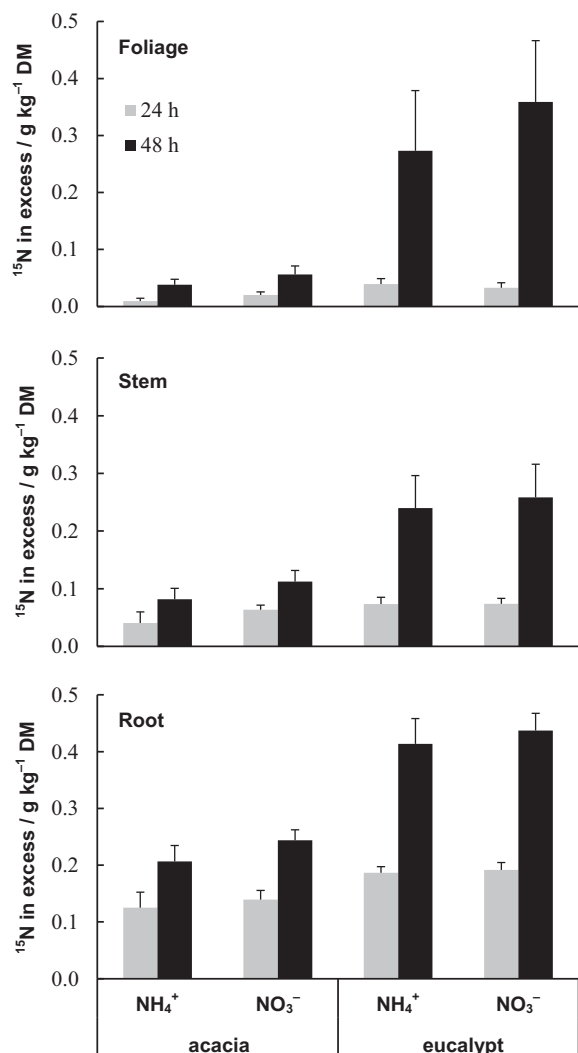


Figure 1: Mean ¹⁵N in excess in foliage, stem, and root of *Acacia mangium* and *Eucalyptus urophylla* × *grandis* 24 h (grey bars) and 48 h (black bars) after injecting ¹⁵N-labeled NH₄⁺ or ¹⁵N-labeled NO₃⁻ into the soil (± standard errors, *n* = 8).

Because there was more ammonium than nitrate in the soil before labeling (Table 1), the rates of ammonium and nitrate uptake were calculated by taking into account the difference in dilution of the labeled N sources in their respective pool [Eq. (2)]. The specific rates of nitrate or ammonium uptake (Fig. 2) calculated after 48 h were higher than those calculated after 24 h, especially for eucalypt, but the difference was not significant (Table 3). The specific rates of N uptake were significantly higher for eucalypt than acacia (Table 3), up to three times higher when calculated over 48 h. In both species, the uptake rate tended to be greater for nitrate than for ammonium, but not significantly (Table 3). When calculated over 48 h, nitrate accounted for 58% and 54% of the total soil mineral N uptake in eucalypt and acacia, respectively, but there was no significant difference in the preferential form of N uptake between the two species (no significant interaction between N form and species; Table 3). This lack of interaction between species and N form was also found when the specific rate of uptake was expressed per unit of root mass rather

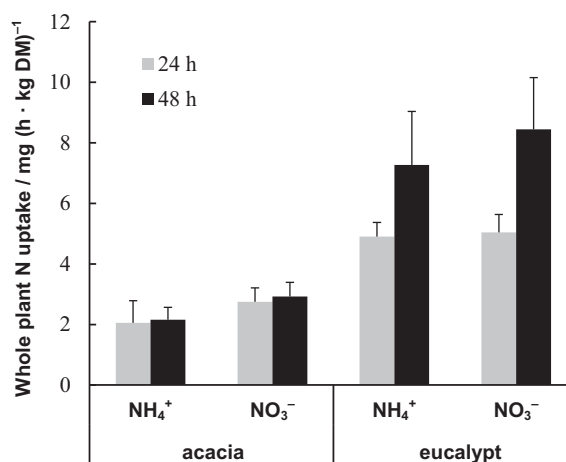


Figure 2: Mean whole-plant ¹⁵N specific uptake rate by *Acacia mangium* and *Eucalyptus urophylla* × *grandis* 24 h (grey bars) and 48 h (black bars) after injecting ¹⁵N-labeled NH₄⁺ or ¹⁵N-labeled NO₃⁻ into the soil (± standard errors, *n* = 8).

than per unit of whole plant mass, as well as when the rate was not divided by mass.

4 Discussion

Both species took up both nitrate and ammonium, with a slight preference for nitrate over ammonium. The rate of ammonium uptake may even have been overestimated if high rates of nitrification occurred during our experiment, transforming part of the ¹⁵N-labeled ammonium into ¹⁵N-labeled nitrate over time. However, we consider this unlikely because, although the uptake rates of mineral N were higher when calculated on a 48 h than on a 24 h basis, the difference between the two was not significant nor was the interaction between time and N form. The concentration of ammonium just before labeling was two to three times higher than that of nitrate depending on the species (Table 1). Although this difference in concentration just before labeling was taken into account to calculate the rates of uptake, the addition of nitrate had a greater effect than the addition of ammonium (0.15 mmol kg⁻¹ of soil for both) on their respective pool size (Table 1). This differential increase in substrate availability may have influenced the rate of uptake of the two N forms and altered the balance between them.

Our results contrast with several reports of preferential ammonium uptake in various eucalypt species (Turnbull et al., 1996; Warren, 2006; Warren and Adams, 2007; Pfautsch et al., 2009). However, preference for nitrate has also been reported in a few studies regarding some of these species when grown in soil rather than in a hydroponic solution, suggesting that the roots may be out-competed for ammonium by soil microbes (Warren, 2009). In addition, the uptake rates of nitrate and ammonium vary according to their respective mobility in the soil (this theoretically favoring nitrate uptake; Nordin et al., 2001) although nitrate utilization is more costly for the plant because nitrate has to be reduced to ammonia (Pfautsch et al., 2009). Because root biomass of acacias was higher than that of eucalypts, the difference in mobility be-

tween ammonium and nitrate combined with a potential difference in root length density may have masked differences in uptake capacity. In addition, the difference in foliage biomass between our two species may have caused differences in water consumption, resulting in lower hydraulic conductivity in pots with acacia than in pots with eucalypt. However, because pots were maintained at all times close to field capacity, the effects of differences in water consumption should have been negligible.

E. urophylla × *grandis* and *A. mangium* did not show any difference in their preference for nitrate or ammonium. It is commonly assumed that the N-uptake preference of plants reflects the ammonium : nitrate ratio found in their native habitat (Nordin et al., 2001; Wang and Macko, 2011) with no evidence of acclimation to differences in the relative availability of the different N forms (Warren, 2006). In tropical trees and grasses, species from drier sites show a preference for nitrate, whereas species from wetter sites tend to prefer ammonium (Houlton et al., 2007; Wang and Macko, 2011). The eucalypt species used in our experiment is a hybrid between two non-sympatric species, i.e., *E. urophylla* from the Flores Island and *E. grandis* from northern Queensland. The seeds of *A. mangium* we used were from the provenance PH482 from Papua New Guinea grown in a trial in Congo. While we are unable to relate our results to the native environment of our plant materials, the fact that both *E. urophylla* × *grandis* and *A. mangium* perform well in forest plantations in coastal Congo (Bouillet et al., 2013) suggests similar pedo-climatic requirements that may explain their similar preferences.

An overlap in the use of the two different mineral forms of soil N may occur in plantations in which these two species are mixed because they do not show any significant difference as to preference for nitrate or ammonium. Our experiment was conducted in pots in which the two species were not in interaction. Although it has been shown that interspecific competition between two alpine meadow species showing similar N-form preferences in the absence of competition did not change their preferences (Ashton et al., 2008), we cannot rule out the possibility of an adaptive shift in preference with our two species.

The present study focused on soil mineral N but some tree species are able to take up amino acids in sufficient quantities to significantly contribute to their nitrogen budget. While this was evident in boreal forests where amino acid concentration in the soil exceeds that of both ammonium and nitrate (Näsholm and Persson, 2001; Nordin et al., 2001; Persson and Näsholm, 2001), amino acids from the soil solution were not a significant source of N for trees in a Puerto Rican tropical forest (Houlton et al., 2007). Uptake of glycine or glutamine was reported for several eucalypt species, but did not seem to be a significant component of their N nutrition (Warren, 2006, 2009; Warren and Adams, 2007; Pfautsch et al., 2009). The high rates of microbial mineralization of organic N in the warm and wet climate conditions encountered in sub-equatorial areas are supposed to limit the availability of organic N for tree nutrition, trees being supposedly out-competed by soil microbes (Hodge et al., 2000; Jones et al., 2005). It would, however, be necessary to measure the pool

of amino acids in the soil alongside that of nitrate and ammonium to definitively exclude amino acids as a potential source of N nutrition. In addition, the role of ectomycorrhizal fungi giving access to a larger pool of organic N needs to be considered (Turnbull et al., 1995; Chalot and Brun, 1998). While our soil-grown plants were probably mycorrhized, our data do not permit disentangling of plant-driven from mycorrhiza-driven uptake.

The rate constant of mineral N uptake, estimated by dividing the sum of the specific rates of nitrate and ammonium uptake by the tree N concentration, was one order of magnitude higher for eucalypt (0.002 h^{-1}) than for acacia (0.0003 h^{-1}). This suggests that atmospheric N_2 was the main N source of these six-month-old acacias. Nitrogen fixation is an important part of the N budget of acacia trees in mixed-species plantations in coastal Congo, with $340 \text{ kg of fixed N ha}^{-1}$ after 3 years of a first rotation (Bouillet et al., 2013). In a second rotation of the same plot, the fraction of N derived from atmospheric fixation estimated using the isotope dilution method was 60% in pure acacia plots and 80% in the mixed-species plots after 2 years (unpublished data). The higher percentage found in the mixed-species plots may contribute to alleviate the competition between eucalypts and acacias for soil mineral N.

5 Conclusion

Contrary to what we expected given the difference in the dominant form of mineral N in the soil of eucalypt and acacia stands in coastal Congo, *E. urophylla* × *grandis* and *A. mangium* did not differ in their preference for nitrate or ammonium. Both species showed a slight preference for nitrate over ammonium. However, because uptake rates were assessed on individually potted young trees that were not interacting with each other, the actual rates for field-grown trees in mixed-species stands may be different and may depend on their mycorrhizal status. It follows that niche differentiation regarding soil N under more realistic field conditions cannot be definitively ruled out. Nevertheless, N_2 fixation by acacia remains the main process alleviating competition for mineral N in mixed-species stands of these two species.

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