Estimating symbiotic N_2 fixation in *Robinia pseudoacacia*

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Abstract

Estimating symbiotic di-nitrogen (N_2) fixation is challenging, especially when working with woody N_2 fixers in field trials. Fortunately, isotope methods based on ^{15}N natural abundance or on ^{15}N artificial enrichment (dilution method) make it possible to estimate the proportion of nitrogen derived from the atmosphere (Ndfa) in N_2-fixing species. These methods have been extensively used in the field for herbaceous species, much less for tree species such as alder and acacia, and rarely for black locust (*Robinia pseudoacacia*). The objectives of this study were to characterize the fixation potential of black locust in a plantation by using the two ^{15}N isotope methods in the field, and to document values of isotope fractionation occurring during N_2 fixation (the *B* value). *B* values were estimated both by growing trees on an N-free medium in controlled conditions (B_{lab}) and by making Ndfa calculated with the natural abundance method converge with Ndfa calculated with the ^{15}N dilution method in the field (B_{field}). The two methods gave consistent estimates of the *B* value. *B* values ranging between –1.4 and –3.2‰ were found, varying with the age of the plant material. Up to 76% of the N in the black locust trees came from the atmosphere, representing more than 45 kg N ha^{-1} over five years and confirming that black locust may be well adapted to N-poor soils.

Key words: black locust / ^{15}N labelling / nitrogen derived from atmosphere (Ndfa) / dilution method / natural abundance

Accepted January 08, 2018

1 Introduction

In intensive tree plantations dedicated to biomass production, soil nitrogen content (N) often becomes a limiting factor for tree growth due to frequent wood exportation (*Ericsson*, 1994). As a result, N fertilizers have to be added to sustain productivity. However, due to the environmental impact they create and their financial cost for forest plantation owners, fertilizers may not be desirable. An alternative way to sustain productivity in an intensive tree plantation could be to use N_2-fixing trees as N fertilization substitutes, trees such as alder (*Alnus* spp.) under temperate latitudes or acacia (*Acacia* spp.) under both temperate and tropical latitudes (*Binkley* et al., 1992; *Bernhard-Reversat*, 1996). The high capacity of alder and acacia to fix atmospheric N_2 through their symbiosis with *Frankia* and *Rhizobium*, respectively, has been widely described. Atmospheric N_2 captured by those N_2-fixing trees is potentially interesting for soil fertility because fixation rates range from 40 to 320 kg N ha^{-1} y^{-1} for alder and from 1 to 200 kg N ha^{-1} y^{-1} for acacia (*Forrester* et al., 2006; *Tobita* et al., 2016). Black locust (*Robinia pseudoacacia*) is another N_2-fixing tree species associated with *Rhizobium* in temperate latitudes, but its N_2-fixing potential has been much less described and documented than that of alder or acacia (*Danso* et al., 1995; *Mantovani* et al., 2015). Black locust is one of the most widely planted woody species in the world, including recently for bioenergy purposes (*Vitková* et al., 2017). It is the second most common broadleaved tree (after *Quercus rubra*) introduced for wood production in Europe. Because of its invasiveness, it is now a common part of the Central European landscape.

There is no single easy way to measure N_2 fixation, and since all current methodologies have limitations, measuring the exact amount of N_2 fixed is still challenging (*Munroe* and *Isaac*, 2014). Ideally, several different methods should be used simultaneously, particularly if they are complementary, *i.e.*, do not rely on the same underlying assumptions (*Unkovich* et al., 2008). The methodologies available today can be roughly classified into three broad approaches: (1) estimating N_2 fixation as the net increase in total N of a plant–soil system using the N balance method, (2) separating plant N into the fraction taken up from the soil and the fraction derived from N_2 fixation (N difference comparing total N of the N_2-fixing species with that of a neighbouring non N_2-fixing species, ^{15}N natural abundance or ^{15}N isotope dilution methods, and ureide nodule analyses), and (3) measuring the activity of nitrogenase, the enzyme responsible for N_2 fixation (acetylene reduction and hydrogen evolution methods). For field experiments on leguminous and actinorhizal tree species, the isotope methods seem to be the most suitable and precise (*Domenach* et al., 1989; *Unkovich* et al., 2008).

The ^{15}N isotope dilution method used to estimate the N_2 fixation dependency of legumes is based on the ^{15}N enrichment

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of the soil with a labelled fertilizer and the use of paired plots, one containing the legume and the other a non-N_2-fixing plant as a baseline reference [initially described in *McAuliffe* et al. (1958)]. The ^{15}N isotope dilution technique has been extensively used to estimate biological N_2 fixation in crop, pastoral, forestry, and agroforestry systems including legumes and actinorhizal species (reviewed in *Chalk* and *Ladha*, 1999; *Tchichelle* et al., 2017). The use of ^{15}N natural abundance to estimate legume biological N_2 fixation is a more recent development [first soil-based experiments by *Amarger* et al. (1979) and *Kohl* et al. (1980)]. This method also requires the use of a non-N_2-fixing reference plant, and in addition, the isotope fractionation occurring during biological fixation (the *B* value) must be determined. This method has been widely applied in annual crops (grains and forage legumes; *e.g.*, *Li* et al., 2009), but also in woody perennials that include leguminous and actinorhizal plants (*Shearer* and *Kohl*, 1986). The choice of methodology often depends on practical considerations such as the initial ^{15}N abundance of the soil, the cost of the ^{15}N-enriched fertilizer, the scale of the experiment, the analytical and instrumental facilities available and the work required to determine the *B* value. *Pauferro* et al. (2010) and *Oberson* et al. (2007) consider that the natural abundance method is the easiest to apply in the field.

A recent review suggests that the isotope methods used to determine N_2 fixation do not provide consistent estimates of the dependence of N_2-fixing species on biological N_2 fixation over a wide range of species, scales, and settings (*Chalk* et al., 2016a). In woody perennials, biological N_2 fixation estimated by the ^{15}N dilution method gave higher values than the natural abundance method in more than 80% of the reviewed cases, with an average difference between the two methods of about 30% (*Chalk* et al., 2016a). The reasons for this discrepancy can be of varying nature, for example, an asynchrony of mineral N uptake between legume and reference plants may exist (*Witty*, 1983), or the reasons may be specific to one of the two methods. For instance, in the dilution method, labelling may be non-uniform in the N_2-fixing and reference species plantations; in the natural abundance method, errors may occur in the estimation of the *B* value, often taken from the literature, without new experimental measurements (*Peoples* et al., 2009; *Chalk* et al., 2016a). Large errors in the calculation of N_2 fixation can be generated by using incorrect *B* values, especially when the proportion of N derived from the atmosphere (Ndfa) is higher than 85% (*Unkovich* and *Pate*, 2000). Moreover, current estimates of *B* values found in the literature are often biased for two reasons: (1) values are typically calculated based on aerial tissues due to ease of sampling, without taking into account the non-uniform distribution of ^{15}N among plant organs, and (2) an adjustment for seed N is often lacking in the calculation (*Nebiyu* et al., 2014).

To our knowledge, our study is the first to assess N_2 fixation by *Robinia pseudoacacia* by combining approaches in the field and under controlled conditions and by using both natural abundance and isotope dilution methods. The objectives of the present study were (1) to evaluate the N_2 fixation potential of *Robinia pseudoacacia* in symbiotic association with *Rhizobium* by estimating the percentage of N fixed through the association (Ndfa) with the ^{15}N isotope dilution methods, and (2) to document values of isotope fractionation occurring during biological N_2 fixation (*B* values) in black locust trees. *B* values were estimated by making Ndfa calculated with the natural abundance method converge with Ndfa calculated with the ^{15}N dilution method in the field (B_{field}). These values were then compared with *B* values obtained experimentally by growing trees on an N-free medium under controlled conditions (B_{lab}).

2 Material and methods

2.1 Plantation site and soil description

The experimental site covered 0.7 ha in central France (Centre Val de Loire; 47°48'25.5"N 1°58'36.1"E) at Saint-Cyr-en-Val. The climate was temperate with a mean annual temperature of 11°C. The average annual rainfall was 620 mm. Soil characteristics were determined for the 0–45 cm top layer. The soil was a Gleyic Luvisol (World Reference Base for Soil Resources classification) composed of 668 ± 61 g kg^{-1} sand, 217 ± 41 g kg^{-1} loam and 94 ± 25 g kg^{-1} clay. Average soil pH was 6.0 ± 0.4. This site had previously been an agricultural fallow for more than 15 years. The plantation was established in March 2011. No fertilization or irrigation was applied. Herbicide was spread once a year and mechanical weeding was done regularly. The plantation was composed of two monocultural blocks: one with poplar (*Populus* × *euramericana*, clone Dorskamp) and one with black locust (*Robinia pseudoacacia*, provenance Nyirseg). Poplar was used as reference species because of growth habits close to black locust. They both are fast growing and pioneer species with shallow, invasive roots (*Burns* and *Honkala*, 1990). The density of the plantation was 1428 trees ha^{-1} (2 m between the trees in a given row and 3.5 m between rows).

2.2 ^{15}N labelling of soil

Ammonium sulfate [$(^{15}NH_4)_2SO_4$ 99 atom % ^{15}N, Cambridge Isotope Laboratories, Inc.] was diluted in deionized water (24.8 mg L^{-1}). In June 2012, 15 months after planting, the labelled solution was manually spread over half of the plantation in each block at a rate of 0.08 kg N ha^{-1} (10 L of labelled solution per tree). To avoid contamination between the labelled and un-labelled zones, two buffer tree rows were kept between the two zones, representing about 0.1 ha, which were not used in the experiment.

2.3 Soil, tree, and litter fall sampling

Soil samples were collected 3 months (month 19) and almost 3 years after labelling (month 50). Samples were taken using a soil auger in the centre of the labelled and un-labelled zones in each block of the two mono-specific plantations (four locations per species and per date), at depths of 2.5, 7.5, 12.5, and 22.5 cm.

Sampled trees covering the range of basal areas in the stand were harvested in January 2013 (month 23 after planting), June 2013 (month 28), June 2014 (month 40), and June 2015

(month 52). In the labelled zone, four trees per species were harvested at age 23 months, eight at age 28 months, six at age 40 months, and six at age 52 months. In the un-labelled zone, four trees per species were harvested at age 23 months, two at age 40 months, and two at age 52 months. The root systems (stump and coarse, medium and fine roots) were excavated in the Voronoï polygon, the elementary space defined by the half distances between the sampled tree and its neighbours (*Levillain* et al., 2011). A mechanical mini-shovel was used to dig out the stump and the coarse roots (diameter above 10 mm), then the medium and fine roots (diameters between 5 and 10 mm, and below 5 mm, respectively) were manually sorted. Tree organs (leaves, branches, stem, and roots) were also sampled.

Litter fall was collected every 4 weeks in seven litter-traps (50 cm × 50 cm) per species and per block. The traps were located on the Voronoï polygon area at 30 cm, 1.15 m, and 2 m from randomly selected trees in order to cover spatial variability. Samples were then pooled per year (2012, 2013, and 2014), per species, and per block. Tree organs, litter fall, and soil samples were dried at 65°C for 48 h, then weighed, ground to a fine powder (ring crusher, SODEMI, Saint-Ouen, France), and stored in air-tight vials until analyses were carried out.

2.4 Chemical analyses

Total N concentration (mg g^{-1} of dry weight) and ^{15}N isotope composition ($\delta^{15}N$, ‰) in the dry matter of each organ (leaves, branches, stem, roots), the litter fall, and the soil samples were measured with an elemental analyzer (NA-1500, Carlo Erba, Milan, Italy) coupled with an isotope ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany). $\delta^{15}N$ was calculated as the relative difference of the sample isotope ratio (^{15}N : ^{14}N) compared to that of the international standard, the atmospheric N_2. The precision of the $\delta^{15}N$ measurement was ± 0.4‰. A weighted whole-tree $\delta^{15}N$ was calculated for each tree as follows (*Bouillet* et al., 2008):

$$\delta^{15}N_{tree} = \frac{\delta^{15}N_{leaves}N_{leaves} + \delta^{15}N_{branches}N_{branches} + \delta^{15}N_{stem}N_{stem} + \delta^{15}N_{roots}N_{roots}}{N_{leaves} + N_{branches} + N_{stem} + N_{roots}}, \quad (1)$$

with N, the N content of each tree component, calculated by multiplying its biomass and its N concentration.

In soil samples collected 3 months after labelling, extractable phosphorus (P; g kg^{-1} dry soil) was measured with the Duchaufour method (*Duchaufour* and *Bonneau*, 1959). Mineral N (N in NO_3^- and NH_4^+; mg kg^{-1} dry soil) was measured in KCl extracts, using continuous-flow colorimetric spectroscopy (*Krom*, 1980).

2.5 Estimation of N_2 fixation

The percentage of N derived from atmospheric N_2 fixation (Ndfa, %) was calculated with the ^{15}N dilution method from the following equation (*Fried* and *Middelboe*, 1977):

$$Ndfa = \left(x^E(^{15}N)_{NFS} - x^E(^{15}N)_{FS}\right) / x^E(^{15}N)_{NFS}, \quad (2)$$

where $x^E(^{15}N)_{FS}$ was the excess atom fraction of *R. pseudoacacia* and $x^E(^{15}N)_{NFS}$ was the excess atom fraction of *P.* × *euramericana*, giving:

$$x^E(^{15}N) = (x_{labelled} - x_{unlabelled}), \quad (3)$$

where $x_{labelled}$ and $x_{unlabelled}$, the ^{15}N atom fractions of trees growing in the labelled and un-labelled zones, were calculated in % from $\delta^{15}N$ as:

$$x = \frac{\left(\frac{\delta^{15}N}{1000} + 1\right) \times R_{st}}{\left[\left(\frac{\delta^{15}N}{1000} + 1\right) \times R_{st}\right] + 1} \times 100, \quad (4)$$

where R_{st} corresponds to the ratio between the isotopes ^{14}N and ^{15}N of air (R_{st} = 0.003676). Because un-labelled trees were not sampled at age 28 months, the $x_{unlabelled}$ values for age 40 months were used. Excess ^{15}N atom fraction was then expressed in mg kg^{-1} dry soil.

A weighted average of $x^E(^{15}N)$ was estimated for the N_2-fixing and non-fixing trees as above [Eq. (1)], using the $x^E(^{15}N)$ of each tree component instead of $\delta^{15}N$.

2.6 Estimations of the *B* value

2.6.1 Convergence of the isotope methods in the field (B_{field})

The *Ndfa* was calculated with the ^{15}N natural abundance method according to the following equation (*Shearer* and *Kohl*, 1986):

$$Ndfa = \frac{\delta^{15}N_{REF} - \delta^{15}N_F}{\delta^{15}N_{REF} - B}, \quad (5)$$

where $\delta^{15}N_{REF}$ was the isotope composition of *P.* × *euramericana*, chosen as the reference non-fixing tree, $\delta^{15}N_F$ was the isotope composition of *R. pseudoacacia*, and *B* was the weighted whole plant ^{15}N fractionation occurring during N_2 fixation. The B_{field} values were those which allowed a convergence of the Ndfa estimated from ^{15}N natural abundance method with the Ndfa estimated from the ^{15}N isotope dilution method (*Doughton* et al., 1992).

2.6.2 Isotope composition of *R. pseudoacacia* grown in N-free medium under controlled conditions (B_{lab})

Seeds were collected from the *R. pseudoacacia* on the experimental site and were placed for a few seconds in a grinder to facilitate germination. They were then placed on moist filter paper and maintained for 2 weeks with daily temperature cycles (16 h at 20°C and 8 h at 30°C). The individual seed-

lings were then transplanted into 1-L pots containing 80% sterilized sand and 20% perlite, and transferred to a growth chamber with a 14/10 h light/dark cycle, 400 μmol photons m^{-2} s^{-1} of photon flux density, 22/18°C day/night temperatures, and 75% relative humidity. The seedlings were inoculated with 25 mL of a bacterial suspension (added into each pot) obtained from crushed nodules collected at the experimental site and solubilized in an N-free nutrient solution. The N-free nutritive solution consisted of (mM): 1.0 $CaCl_2$, 5.0 KCl, 1.0 KH_2PO_4, 1.0 $MgSO_4$, 0.05 H_3BO_3, 0.02 $MnSO_4$, 0.0008 $ZnSO_4$, 0.0003 $CuSO_4$, 0.0006 Na_2MoO_4, 0.0002 $CoSO_4$, and 0.1 FeNa-EDTA. Fifty mL of N-free nutrient solution were supplied to each seedling every 3 d for 19 weeks. After 12, 16, and 19 weeks of growth, three plants were sampled and analysed for biomass, nitrogen concentration, and $\delta^{15}N$ measurements. The plants were separated into leaves, stems, roots and nodules. These organs, plus some un-germinated seeds, were dried at 65°C for 48 h, then ground to a fine powder for isotope analyses. Whole-plant $\delta^{15}N$ was calculated from the weighted means of $\delta^{15}N$ in the various organs [modified from Eq. (1)] and was then corrected ($\delta^{15}N_{cor}$) with the isotope composition in the seeds as follows (*Högberg* et al., 1994):

$$\delta^{15}N_{cor} = \frac{\left(\delta^{15}N_{whole\ plant} \times N_{whole\ plant}\right) - \left(\delta^{15}N_{seed} \times N_{seed}\right)}{N_{whole\ plant} - N_{seed}}. \quad (6)$$

Fractionation occurring during N_2 fixation (the *B* value) was the difference in isotope composition between the substrate (atmospheric N_2) and the corrected whole-plant ^{15}N composition ($\delta^{15}N_{cor}$). Because atmospheric N_2 was the international reference for N isotope composition ($\delta^{15}N_2 = 0$), then B_{lab} was equal to $\delta^{15}N_{cor}$ (*Vincent*, 1970).

2.7 Statistical analyses

Means were expressed with their standard errors. They were compared between species and among sampling dates for the field experiment, or among organs and sampling dates for the pot experiment with two-way ANOVA tests from the R software (*R Core Team*, 2016). The statistical tests were considered significant at $^{*}P \leq 5\%$, $^{**}P \leq 1\%$, or $^{***}P \leq 0.1\%$.

3 Results

3.1 Soil composition

Because total soil N did not differ between labelled and un-labelled zones, values averaged for the two zones are presented in Fig. 1. Total soil N decreased slightly with soil depth (P < 5%), irrespective of species and date (Fig. 1). Total soil N was significantly lower 50 months after planting (3 years after labelling) than 19 months after planting (three months after labelling) for poplar only (P < 0.1%). In the un-labelled zone, soil ^{15}N isotope composition ($\delta^{15}N$) ranged from +3.5 to +5.0‰, irrespective of soil depth, tree species or sampling time. In the labelled zone, 3 months after labelling, the ^{15}N enrichment was between 15 and 20‰ for the shallowest horizon and it decreased rapidly with soil depth (data not shown). In the labelled zone, 3 months after labelling (month 19), $x^E(^{15}N)$ in the most superficial soil horizons reached 0.53 and 0.84 mg kg^{-1} for the black locust and poplar plantations, respectively. These values were not significantly different

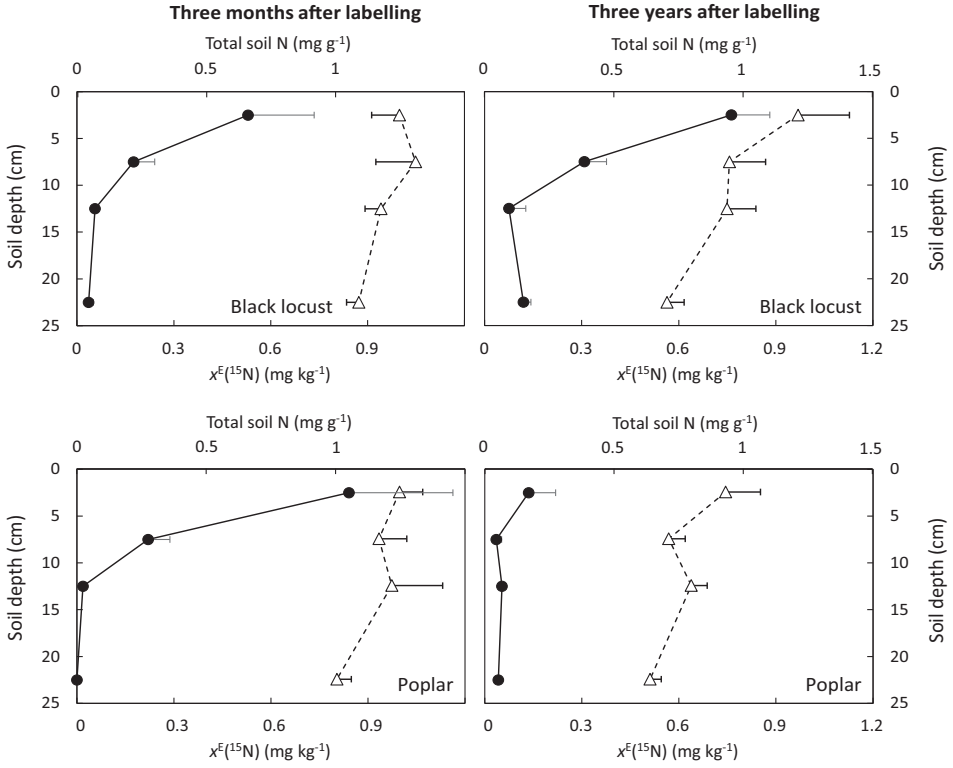

Figure 1: Soil profiles of total N (mg g^{-1} $_{dry\ soil}$, triangles, dotted lines) and ^{15}N excess atom fraction [$x^E(^{15}N)$, mg kg^{-1} $_{dry\ soil}$, circles, continuous lines] in the labelled zone 3 months (left panels) and 3 years (right panels) after labelling. Black locust = upper panels and poplar = lower panels. Means ± standard errors, *n* = 4 for total N and *n* = 2 for $x^E(^{15}N)$.

between species, irrespective of the soil layer. $x^E(^{15}N)$ values decreased rapidly with soil depth. A significant date × species interaction was observed (P < 1%). Three years after labelling (month 50), $x^E(^{15}N)$ values remained high in the upper soil layer of the black locust plantation (0.76 mg kg^{-1}), not significantly different from the values obtained 3 months after labelling, while in the poplar plantation, $x^E(^{15}N)$ values were much lower (0.14 mg kg^{-1} for the upper layer) regardless of soil depth.

Soil mineral N and extractable P did not differ between labelled and un-labelled zones, values averaged for the two zones are presented in Tab. 1. Three months after labelling, soil NO_3^- was significantly higher in the black locust plots as compared to the poplar plots (Tab. 1). On the contrary, soil P was higher in the polar plots as compared to the black locust plots. A significant soil-depth effect was observed for NH_4^+, with a decrease with depth.

3.2 Time course of tree and litter fall $\delta^{15}N$

Significant date and species effects were observed for tree and litter fall ^{15}N isotope composition ($\delta^{15}N$) in the un-labelled zones (Fig. 2). Lower tree $\delta^{15}N$ values were observed at 52 months compared to the two previous sampling dates for both species, though there was no difference for litter fall. Both tree and litter fall $\delta^{15}N$ were much higher for poplar than for black locust throughout the experiment. Similarly, significant date and species effects were observed for tree and litter fall ^{15}N excess atom fraction [$x^E(^{15}N)$] in the labelled zone (Fig. 2). A general decrease in $x^E(^{15}N)$ was observed with time for both species, with much higher values for poplar than for black locust throughout the experiment.

Table 1: Mean (± standard error) soil extractable phosphorus (P, g $kg^{-1}_{dry\ soil}$) and mineral N (N-NO_3^- and N-NH_4^+, mg $kg^{-1}_{dry\ soil}$) 3 months after labelling (month 19 after planting) in the black locust and poplar plantations and for the different soil depths (n = 2).

	N-NO_3^- (mg kg^{-1})	N-NH_4^+ (mg kg^{-1})	P (g kg^{-1})
Black locust			
0–5 cm	0.99 ± 0.33	0.48 ± 0.13	0.03 ± 0.00
5–10 cm	0.99 ± 0.22	0.69 ± 0.16	0.05 ± 0.01
10–15 cm	0.92 ± 0.56	0.22 ± 0.07	0.04 ± 0.01
15–30 cm	0.95 ± 0.51	0.32 ± 0.08	0.04 ± 0.00
Poplar			
0–5 cm	0.35 ± 0.08	0.63 ± 0.07	0.09 ± 0.01
5–10 cm	0.26 ± 0.16	0.32 ± 0.05	0.06 ± 0.02
10–15 cm	0.17 ± 0.02	0.13 ± 0.03	0.08 ± 0.01
15–30 cm	0.30 ± 0.09	0.20 ± 0.14	0.07 ± 0.00

3.3 Percentage of N derived from atmospheric fixation

For all ages, the percentage of N derived from atmospheric N_2 (Ndfa) represented more than half of the N assimilated by the trees, with values ranging from 58.6 to 76.4% and corresponding to 19.1 up to 35.3 kg N_2 fixed per ha (Tab. 2). The percentage decreased slightly between ages 23 and 40 months, from 76.3 to 58.6% (–23%), then, increased up to 71.5% at month 52. At the end of the experiment, total N_2 fixed since planting, taking into account cumulated litter falls, was 45.4 kg ha^{-1}.The B_{field} values, obtained from the convergence of the Ndfa calculated by the natural abundance method with the Ndfa estimated by the isotope dilution method, ranged between –1.4 and –3.2‰ (Tab. 2).

3.4 Isotope composition of black locust seedlings grown on an N-free medium

Significant date and organ effects were observed on the ^{15}N isotope composition of the black locust seedlings grown in the N-free medium (P ≤ 0.1%). Stems showed the most negative values, ranging between –3.0 and –4.1‰, while values were positive for nodules and ranged between 6.6 and 9.0‰. Both stem and weighted whole-plant isotope composition significantly increased with time between 12 and 19 weeks of growth, and the same, but not significant, trend was observed for the other organs (Tab. 3, Fig. 3). The B_{lab} values estimated for the black locust seedlings grown on an N-free medium ranged between –1.4 and –3.0‰ when seeds were taken into account (Fig. 3). In spite of large differences in the age of the plant material (3–5 months *vs.* 2–5 years), these estimated B_{lab} values encompassed the B_{field} values needed for the convergence of the Ndfa values estimated by the natural abundance method with the ones estimated by the ^{15}N dilution method (–1.4 to –3.2‰).

Table 2: Percentage of N derived from atmospheric N_2 (Ndfa ± standard error, SE) estimated with the isotope dilution method in black locust at 23, 28, 40, and 52 months after plantation; *B* values used in the natural abundance method for convergence with Ndfa estimated by isotope dilution (B_{field}), and N derived from atmospheric N_2 in the standing biomass (Natm) for each age and at the end of the experiment, taking into account litter fall.

Months since planting	Ndfa (%)	B_{field} value (‰)	Natm (kg N ha^{-1})
23	76 (± 14[a])	–1.4	24.0
28	67 (± 6)	–	19.5
40	59 (± 6)	–3.2	19.1
52	71 (± 9)	–2.3	35.3
Total N fixed since planting			45.4

[a]The propagation of errors used to compute SE assumes that all covariances are null. The given SE are therefore their upper limit.

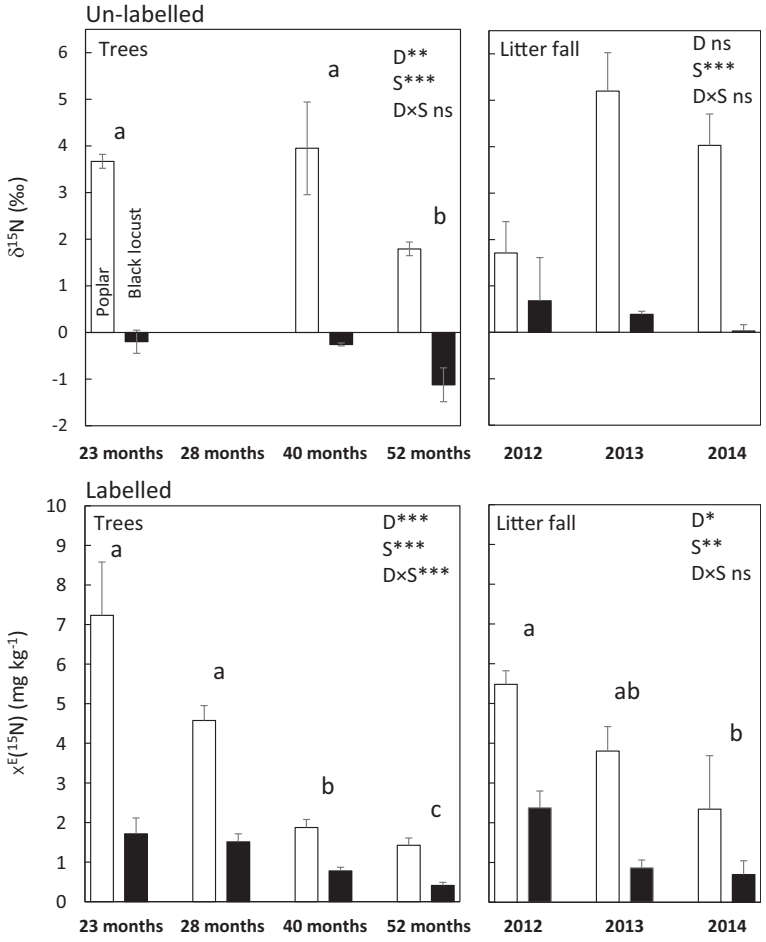

Figure 2: Time course of whole-tree (left panels) and litter-fall (right panels) ^{15}N isotope composition ($\delta^{15}N$, ‰) in the un-labelled zone (upper panels), and excess ^{15}N atom fraction [$x^E(^{15}N)$, mg kg^{-1} $_{dry\ soil}$] in the labelled zone (lower panels), for poplar (white) and black locust (black). Means ± standard errors; $n = 2$ for litter fall. The significance of the date (D) and species (S) effects and their interaction (D × S) in the two-way ANOVA is presented: $^{*}P \leq 5\%$, $^{**}P \leq 1\%$, $^{***}P \leq 0.1\%$, ns for non-significant. Significant differences among dates are denoted by different letters.

4 Discussion

In our study, up to 76% of the N in the black locust standing biomass came from atmospheric N_2 fixation, representing N inputs ranging between 5.7 and 12.5 kg N ha^{-1} y^{-1}. Similar Ndfa values were found by *Mantovani* et al. (2015) for young black locust trees. This reflects a total N amount fixed of 45.4 kg N ha^{-1} almost 5 years after planting, when litter fall is taken into account. Even if these fluxes are likely to be underestimated because root turnover was not taken into account, the quantities of fixed N remained low compared to the (rare) values reported in the literature for black locust: 220 kg N ha^{-1} in a four-time denser 2-year old plantation with an average Ndfa reaching 80% in *Danso* et al. (1995).

Several studies exist in the literature that compare the two isotope methods used to estimate the N quantities derived from atmospheric N_2 fixation (for instance, *Stevenson* et al., 1995; *Burchill* et al., 2014). However, most of these studies involved annual herbaceous species whilst very few investigated woody N_2 fixers [*Domenach* et al. (1989) on alder; *Bouillet* et al. (2008) on acacia]. Only one study concerns black locust (*Domenach*, 1985). Indeed, for N_2 fixing trees, field labelling experiments as well as experimental estimations of the *B* value remain challenging and rare in the literature.

Determining *B* is an important step in the estimation of atmospheric N_2 fixation through the ^{15}N natural abundance method. If the difference in ^{15}N isotope composition between the reference and fixing species is higher than 5‰ or if Ndfa is low, errors in the estimation of the *B* value do not have a significant influence on the calculation of Ndfa (*Boddey* et al., 2000; *Unkovich* et al., 2008). On the other hand, if the ^{15}N isotope composition dif-

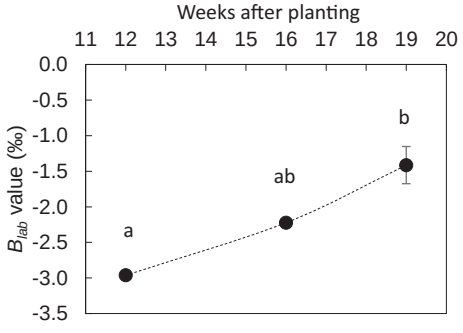

Figure 3: Change in *B* values in black locust seedlings growing in N-free medium [B_{lab} calculated from entire plant isotope composition and taking into account N content in seeds; Eq. (6)] from 21 May (12 weeks) to 20 July (19 weeks). Means ± standard errors, $n = 3$. Different letters indicate significant differences between dates in the one-way ANOVA ($P \leq 5\%$).

Table 3: Mean isotope composition, ($\delta^{15}N \pm$ standard error, $n = 3$) in black locust seedlings growing in an N-free medium. Different capital letters denote significant differences among organs for each date, while different small letters indicate significant differences between dates for each organ, according to the two-way ANOVA tests ($P \leq 5\%$).

	Isotope composition ($\delta^{15}N$, ‰)		
Seeds	1.81 (± 0.41)		
	12 weeks	**16 weeks**	**19 weeks**
Leaves	–2.11 (± 0.05) A	–1.81 (± 0.42) B	–1.13 (± 0.12) AB
Stem	–4.12 (± 0.06) Aa	–4.15 (± 0.06) Aa	–3.01 (± 0.11) Ab
Roots	–2.25 (± 0.20) A	–0.93 (± 0.24) B	+0.06 (± 0.65) B
Nodules	+6.58 (± 1.07) B	+7.85 (± 0.29) C	+8.95 (± 0.70) C

ference between species is small, as is the case in this study between black locust and poplar, or if Ndfa is high, *B* must be very precisely determined to minimize errors in the estimation of Ndfa. Precautions must be taken for the estimation of *B*, notably: (1) plants must be inoculated with the same bacterial strain as those at the experimental site and (2) not too juvenile plants must be used since *B* values vary with time, reaching an equilibrium only after several weeks of growth (*Boddey* et al., 2000). The second recommendation is particularly challenging to follow when working with tree species. In our study, we used the method originally designed by *Doughton* et al. (1992) and used more recently in pot experiments by *Okito* et al. (2004) and *Pauferro* et al. (2010). This method consists in determining which *B* values in the natural abundance method allow a convergence with the Ndfa values calculated with the dilution method (B_{field}). These values are then compared with the *B* values estimated experimentally with the N-free medium method [B_{lab}, method initially proposed by *Vincent* (1970)]. The plants we used for the estimation of B_{lab} were only a few months old, but it was not feasible to grow them for a longer time in pots. They may not yet have been at the equilibrium stage, as defined by *Boddey* et al. (2000), and their *B* value may have increased further with time. It is obvious that seedlings grown in pots in a climate chamber do not resemble field-grown trees. However, our estimated B_{lab} values were of the same order of magnitude (–1.4 to –3.0‰) as the ones needed to make the Ndfa values estimated with the natural abundance method converge with the Ndfa values estimated with the ^{15}N dilution method (B_{field} ranging from –1.4 to –3.2‰). This suggests that the *B* values estimated in both cases are consistent, irrespective of the age of the plant material. As suggested by *Unkovich* et al. (2008), the ideal way to estimate N_2 fixation is to simultaneously use several methods and to reconcile the results obtained.

In the literature, *Domenach* (1985) found a *B* value for black locust of –2.2‰, consistent with the values in the present study. To our knowledge, this is the only previously referenced *B* value for black locust. For alder (*Alnus glutinosa, A. incana*), another tree N_2 fixer from temperate latitudes, *B* values of –1.9‰ are commonly used, and this value is considered to be stable over time and only slightly dependent on *Frankia* strains and alder species (*Domenach* et al., 1988). This *B* value of –1.9‰ has been extensively used in other studies, without additional experiments to verify it (*e.g.*, *Domenach* et al., 1989; *Chalk* et al., 2016b). For *Acacia mangium*, a *B* value of –0.3‰ was determined by *Galiana* et al. (2002) and used thereafter in other studies (*e.g.*, *Bouillet* et al., 2008). For other N_2-fixing woody perennials, including *Calliandra, Gliricidia, Flemingia*, and *Caragana, B* values ranging between 0 and –1.5‰ have been experimentally determined with the N-free medium method (*Peoples* et al., 1996; *Chalk* et al., 2016a), while for diverse herbaceous species, including clover, pea, soybean, lupine, and alfalfa, *B* values ranging between +1.3 and –3.0‰ have been measured [reviewed in *Chalk* et al. (2016a)].

The B_{lab} values measured in our study with seedlings grown in an N-free medium are of the same order of magnitude as the ones from the literature, even for very different species. However, our values vary quite widely (–1.4 to –3.0‰) over time. We observed an increase in B_{lab} values with time, which reflected an increase in the isotope composition in all tree organs. As observed for herbaceous species (*Unkovich*, 2013), this isotope composition differed among plant organs, with, in decreasing order: nodules, roots, and stems. Such results suggest that isotope fractionation may not only occur during N_2 assimilation in nodules, but also during N transport toward the aerial components of the tree. Consequently, the *B* value does not only correspond to the isotope fractionation occurring during N_2 fixation; it is also the result of fractionation occurring inside the plant. At the whole-plant level, *B* is an integrative value, not specific to a particular enzymatic reaction (*Unkovich*, 2013). That is the reason why the *B* value varies among species and over time, and is dependent on site conditions (*Unkovich* and *Pate*, 2000). Such differences in $\delta^{15}N$ among organs for N_2 fixers growing with N entirely obtained through N_2 fixation (including positive $\delta^{15}N$ values for nodules) have been observed for amide-transporting plants (such as black locust), but not for ureide-transporting plants (*Yoneyama* et al., 1986). Higher ^{15}N enrichment in nodules than in other plant organs is a common phenomenon in a wide range of legumes. This phenomenon has been related to nodule metabolism, and increases with nodule age (*Wanek* and *Arndt*, 2002).

At the end of our field experiment, the ^{15}N signal in the soil for poplar was almost identical in the labelled zone and in the unlabelled zone. Quite some time ago, *Witty* (1983) had already stressed that the decrease in the enrichment of plant available soil N over time was a major cause of error in Ndfa measurements. This means that the Ndfa estimated for our last date (3 years after labelling) may be incorrect. However, in spite of a continuous plant $\delta^{15}N$ decline over time, the ^{15}N signal remained much higher in the labelled poplars than in the un-labelled ones. This result suggests that the decrease in soil ^{15}N signal had a limited impact on the plant ^{15}N signal because of high N recycling within the trees. Therefore, our Ndfa calculation for the latter date is likely to be correct. The drastic decrease in the soil ^{15}N signal 3 years after labelling found in the labelled poplar zone but not in the labelled black locust zone, can be related to the fact that poplar depends entirely on soil N, while black locust obtains N from the atmosphere through symbiotic fixation. This is consistent with the decrease in total soil N observed 3 years after labelling in the poplar plots only.

5 Conclusion

To our knowledge, our study is the first one to combine approaches in the field and under controlled conditions to assess N_2 fixation by *Robinia pseudoacacia* using both the natural abundance and isotope dilution methods. Combining the two methods appeared to be the best way to circumvent possible uncertainties in estimated N_2 fixation associated to each one alone. In our two- to five-year old black locust trees, up to more than the three quarters of the nitrogen came from biological N_2 fixation, confirming that this species may be well adapted to N-poor soils.

Acknowledgments

We are particularly grateful to *Erwin Dallé* for his active contribution to data acquisition in the field and for sample and dataset preparation. We also thank the numerous colleagues and students, as well as the GBFOr experimental Unit in Orléans, for their help during labelling and harvesting. Financial support was provided by the *Intens&fix project* (*ANR-2010-STRA-004-03*). The experimental site is part of the *SOERE F-ORE-T network* supported by *GIP ECOFOR, AllEnvi*, and *AnaEE France*. The UMR Silva is supported by the *Laboratory of Excellence ARBRE* (*ANR-11-LABX-0002-01*). We also would like to thank *Victoria Moore* for English editing of the manuscript.

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