



Tracking Stable Isotope Enrichment in Tree Seedlings with Solid-State NMR Spectroscopy

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Enriching plant tissues with ¹³C and ¹⁵N isotopes has provided long-lasting, non-reactive tracers to quantify rates of terrestrial elemental fluxes (e.g., soil organic matter decomposition). However, the molecular location and level of isotope enrichment may differ among plant tissues. This factor is central to the integrity and interpretation of tracer data, but is seldom considered in experiments. We propose a rapid, non-destructive method to quantify molecular isotope allocation using solid-state ¹³C and ¹⁵N nuclear magnetic resonance spectroscopy. With this method, we tracked and quantified the fate of multiple pulses of ¹³CO₂(g) and K ¹⁵NO₃(l) in boreal tree seedling roots and leaves as a function of time. Results show that initial preferential ¹³C carbohydrate enrichment in the leaves was followed by redistribution to more complex compounds after seven days. While ¹³C allocation within the roots was uniform across molecules, ¹⁵N results indicate an initial enrichment of amine molecules after two hours.

Terrestrial ecosystems are changing due to anthropogenic pressures, leading to far-reaching effects on global biogeochemical cycles. Assessing the net CO₂ flux between terrestrial ecosystems and the atmosphere remains one of the largest uncertainties in understanding of the global carbon cycle. Mechanisms controlling the persistence of soil organic matter are complex and reflect the interacting influences of abiotic conditions, nutrient supply, microbial community structure, and organic matter composition¹. Soils globally contain twice as much carbon as the atmosphere and world's vegetation combined, yet how this major carbon pool will respond to global environmental change is not well understood. Carbon storage in boreal forest soils, which alone represents close to 25% of the global soil carbon stocks, is particularly susceptible as northern regions are anticipated to experience warming of up to 5°C by 2100 due to climate change².

Accurate and quantitative rates of soil carbon fluxes are increasingly required to better constrain predictions of soil carbon turnover. Carbon transfer from plant residues to soils occurs both as surface litterfall or below-ground *via* root exudates and turnover. Carbon-13 (natural abundance 1.07%) and ¹⁵N (natural abundance 0.364%) have been used to track both of these elements as they are mineralized from the decomposing enriched plant materials, taken up by the soil microbial biomass, stabilized into organic matter, or alternatively respired back to the atmosphere^{3,4}. Vascular plants, particularly woody perennial species, contain a range of complex tissues made up of varying amounts of different plant biopolymers^{5,6} depending on environmental conditions⁷. In addition these biopolymers display different decay rates, at least initially, with compounds such as lignins and waxes taking longer to decompose in the soil environment compared to proteins, soluble sugars, or cellulose⁸. Consequently, molecular characterization of labeled litter inputs should be combined with isotopic flux measurements if the fluxes are to be accurately interpreted in the broader context of ecosystem biogeochemical cycling.

Historically, isotope labeling of plant material originated with the use of radioactive carbon (¹⁴C)^{9,10}. With increased availability of stable isotopes (notably ¹³C and ¹⁵N) and improved quantitative analytical instrumentation, stable isotopes have become an important tool for tracer studies in terrestrial ecosystems¹¹. Experimentally, enrichment is either achieved by continuous addition of the stable isotope or by applying pulses. Since the introduction of pulse chase labeling⁹, the method has been adopted as the technique of choice compared to the more cumbersome continuous labeling apparatus. Numerous studies have used stable isotope enriched organic matter to examine carbon¹²⁻¹⁴ and nitrogen biogeochemical cycles¹². However, for methodological reasons, most studies have been restricted to non-woody fast growing graminoids that show little complexity in



tissues and their associated biopolymers. Here, we selected trembling aspen (*Populus tremuloides* Michx.), a dominant upland tree species in North-American boreal forests¹⁵, which is closely related to the equally common *Populus tremula* in the northern hemisphere of Eurasia. Aspen leaf tissues display a diverse and complex composition including cellulosic, aromatic and alkyl carbon species⁵. The objective of the present study was to determine how pulse-labeling with $^{13}\text{CO}_2(\text{g})$ and $\text{K } ^{15}\text{NO}_3(\text{l})$ affected isotopic enrichment in aspen seedlings at both the elemental and biopolymer level. To provide the greatest contrast in tissue composition, we present results on both foliage and woody root tissues for isotopic (^{13}C , ^{15}N) abundance in response to three pulses and two chase periods (two hours and seven days). In addition, we quantified and characterized isotope assimilation in plant biopolymers using solid-state ^{13}C and ^{15}N nuclear magnetic resonance (NMR) analysis.

Results

The ^{13}C and ^{15}N concentrations increased in the seedling tissues with each successive pulse label; however, timings and patterns of isotope enrichment were different (Figure 1). Two hours after the first $^{13}\text{CO}_2$ pulse, leaf tissues had doubled in ^{13}C concentrations compared to background levels. Although not all ^{13}C was retained within the leaves, a steady enrichment to 2.7 atom % from a background concentration of 1.1 atom % was observed with successive pulses. Root tissues did not show any detectable ^{13}C enrichment two hours after the first pulse, but a significant increase in ^{13}C concentration was evident seven days later (1.4 atom %). In contrast to ^{13}C enrichment patterns, ^{15}N enrichment was first noted in the roots two hours after the first pulse, with subsequent labels further increasing the concentration from 0.4 to 10.7 atom % ^{15}N . Enrichment of ^{15}N in the leaves could be detected seven days after the first pulse label. Final concentrations of ^{13}C and ^{15}N for unlabeled leaves and roots were similar to the initial ones; i.e., without pulse-labeling the proportion of ^{13}C to ^{12}C and ^{15}N to ^{14}N did not change during the experiment.

Variation in the biopolymer allocation of ^{13}C in aspen seedlings with repeated pulses and multiple chase times depended on the tissue type (Figure 2). As can be seen on the ^{13}C spectra of leaves, the O-alkyl signal (corresponding to oxygen-bonded or carbohydrate type carbons) was strongest two hours following labeling, and corresponded to 41–48% of the total integrated spectral area (Figure 3). In comparison, the alkyl carbon signal (from aliphatic carbons in polymethylene such as cutin) was originally much smaller, but increased with time, so that the O-alkyl and alkyl peaks were closer in intensity with a longer (seven days) chase period (Figure 2). The resolution of peaks attributed to aromatic and phenolic carbons (arising from lignin and tannins) improved with ^{13}C enrichment, and the intensity of both increased in proportion with the longer chase time.

Allocation of ^{15}N to plant biopolymer groups, as with ^{13}C , varied depending on the chase time (Figure 4). Nitrogen characterization was limited to a subset of labeled samples as the signal from nuclei other than amides was indistinguishable from the noise on the control samples with natural abundance ^{15}N levels. Results are qualitative as amine nitrogen is known to be under-represented, and nonprotonated nitrogen is difficult to detect¹⁶. After two pulses and a chase time of 7 days, the ^{15}N NMR spectra for leaves and roots both had NMR peaks characteristic of amide and amine nitrogen well above the background noise (Figure 4).

Discussion

Solid-state NMR spectroscopy may be used as an analytical tool to characterize the environment surrounding nuclei of interest (e.g., ^{13}C or ^{15}N) from which the chemical composition of plant or soil organic matter may be identified^{5,17}. The magic angle spinning technique (MAS) entails spinning samples rapidly about the magic angle (54.7°) relative to the applied magnetic field. The method greatly reduces the effects of chemical shift anisotropy, resulting in significant improvements in resolution compared to what is obtained for solid-state NMR spectra of stationary samples. The method also reduces the effects of homonuclear (e.g., ^{13}C - ^{13}C) dipolar coupling. This technique is often combined with cross polarization (CPMAS) whereby the magnetization is transferred from abundant spins (usually ^1H) to less abundant spins; i.e., ^{13}C or ^{15}N . The latter technique provides a signal enhancement as great as the ratio of the magnetogyric ratios of the nuclei, ≈ 4 for ^{13}C and ≈ 10 for ^{15}N . However, since in most cases the less abundant spins also have a much longer spin-lattice relaxation time (T_1) than do the ^1H nuclei, the greatest benefit of the CP technique is usually that the repetition time of the experiment is controlled by the T_1 value of the latter. Hence, one may acquire many more transients in a given period of time, greatly improving the signal/noise ratios of the spectra¹⁸. However, it is important to realize that spectra obtained with CPMAS are not quantitative, since the signal arising from the nuclei are not all enhanced to the same extent. An alternative to the CPMAS technique is to obtain spectra with MAS using direct polarization (DP), which provides qualitative results provided the recycle time is significantly greater than the greatest T_1 value for the nuclei of interest¹⁹. For the samples in the present study, ^{13}C spectra were obtained by DP; to eliminate the interference from natural abundance ^{13}C in either the probe or the rotor drive tips and end caps, a modified DP spin-echo pulse sequence was used²⁰. The ^{15}N enrichment was qualitatively characterized by the CPMAS technique since obtaining suitable ^{15}N DP spectra was not practical.

Chemistry of seedling roots was different from leaves in that the samples did not exhibit much variation with the different levels of

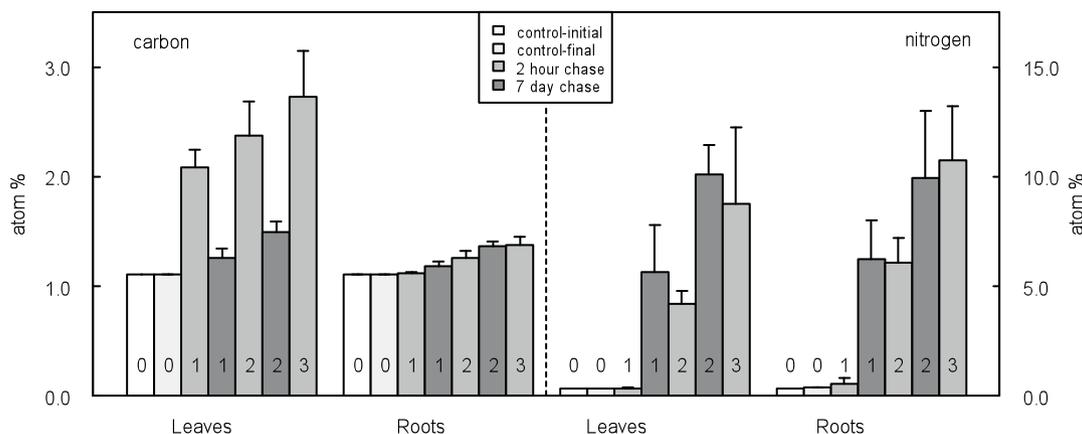


Figure 1 | Atom percent concentration of ^{13}C and ^{15}N in seedling leaves and roots at different pulse and chase times. Chase time of control, 2 hour or 7 day is indicated by shading in the box. The number of pulses (0–3) is indicated by a number at the base of the barplot. Error bars represent one standard deviation ($n=6$).

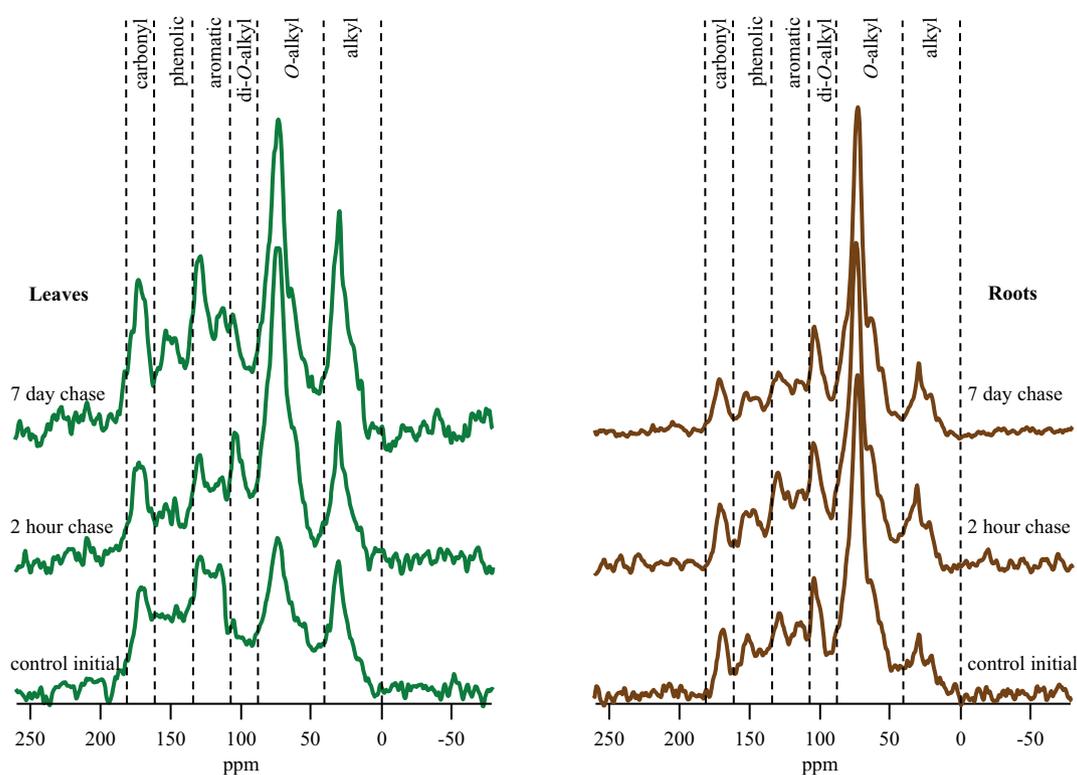


Figure 2 | Direct polarization ^{13}C NMR spectra with control-initial, one pulse and 2 hour chase and one pulse 7 day chase ^{13}C enriched seedling leaves and roots. Common integral regions representing the different magnetic environments of carbon are shown on the spectra.

enrichment (Figures 2 and 3). Hence, the roots showed fairly uniform ^{13}C enrichment across the different biopolymers. For the leaves, the shift in intensity of the *O*-alkyl carbon peak between the two chase times likely reflects the initial fixation of carbon as glucose followed by a partial redistribution to more complex compounds such as cutin or lignin. On the other hand, uniform ^{13}C enrichment within the root biopolymers reflects non-discriminatory incorporation into new structural root tissues, including cellulose and lignin, rather than preferential accumulation into *O*-alkyl C in the form of

soluble sugars or starch reserves. While the ^{15}N NMR spectra reflected the acquisition and allocation of nitrate by the seedling; two hours after the K^{15}NO_3 pulse, roots had taken up and reduced the labelled nitrate into amine groups. Roots contain nitrogen reductase and other enzymes for amino acid and amide formation, which allows the roots to synthesize organic solutes for nitrogen distribution throughout the plant^{21,22}. Our results support this pathway as evidenced by the relatively higher amide/amine peak ratio on the root spectra after a 7 day chase period (Figure 4).

For biogeochemical studies aimed at elucidating organic matter processes and ecosystem fluxes, a distinction between plant-labeled material and native soil organic matter needs to be made. Hence knowledge of the isotope enrichment levels across different tissues and biopolymers is essential. Here we demonstrated that both the allocation of ^{13}C and ^{15}N in leaves and roots was dependent on the chase time (Figure 1). Similar results have been reported during pulse labeling of birch (*Betula papyrifera*) and fir (*Pseudotsuga menziesii*) trees, where the initial increase in foliage ^{13}C concentration decreased while that of roots increased with a longer chase time²³. The level of enrichment also needs to be sufficient for planned biogeochemical tracer studies. Enrichment of ^{13}C at the end of our labeling experiment was 2.7% in leaves and 1.4 atom % in roots. This is comparable to levels previously reported for needles (1.6% ^{13}C) and roots (1.4 atom % ^{13}C) of Douglas-fir seedlings, which were demonstrated to be sufficient to follow tracers during a soil incubation study²⁴. The ^{15}N enrichment levels for leaves (8.7% ^{15}N) and roots (10.7 atom % ^{15}N) also were comparable to enriched ponderosa pine tissues used in a field decomposition study²⁵. The NMR spin-active isotopes of carbon (^{13}C) and nitrogen (^{15}N) make up 1.1 and 0.4%, respectively of the total number of atoms at natural abundance levels in our control samples. With tissue enrichment, there was a subsequent increase in the abundance of both spin-1/2 atoms, which directly translated into an enhanced signal-to-noise ratio and improved characterization. Using this technique, we were able to identify that uniform labeling required a one-week chase period for both the leaves and the roots.

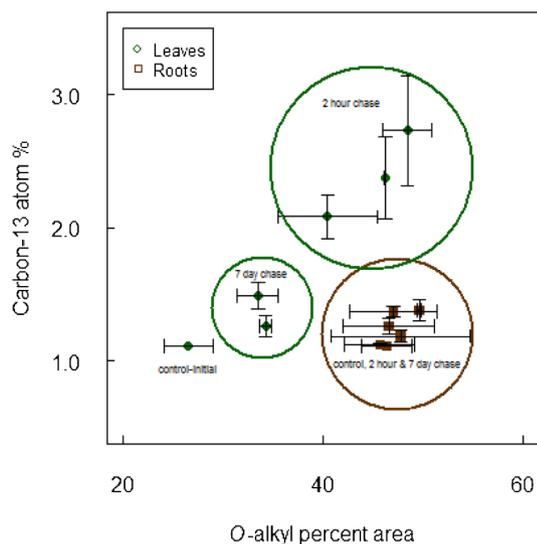


Figure 3 | Relating the proportion of *O*-alkyl carbon to ^{13}C enrichment for the multi-pulse and multi-chase stable isotope labeling of seedling leaves and roots. One standard deviation for *O*-alkyl carbon ($n=2$) and carbon-13 atom % ($n=6$) are indicated by error bars on the graph.

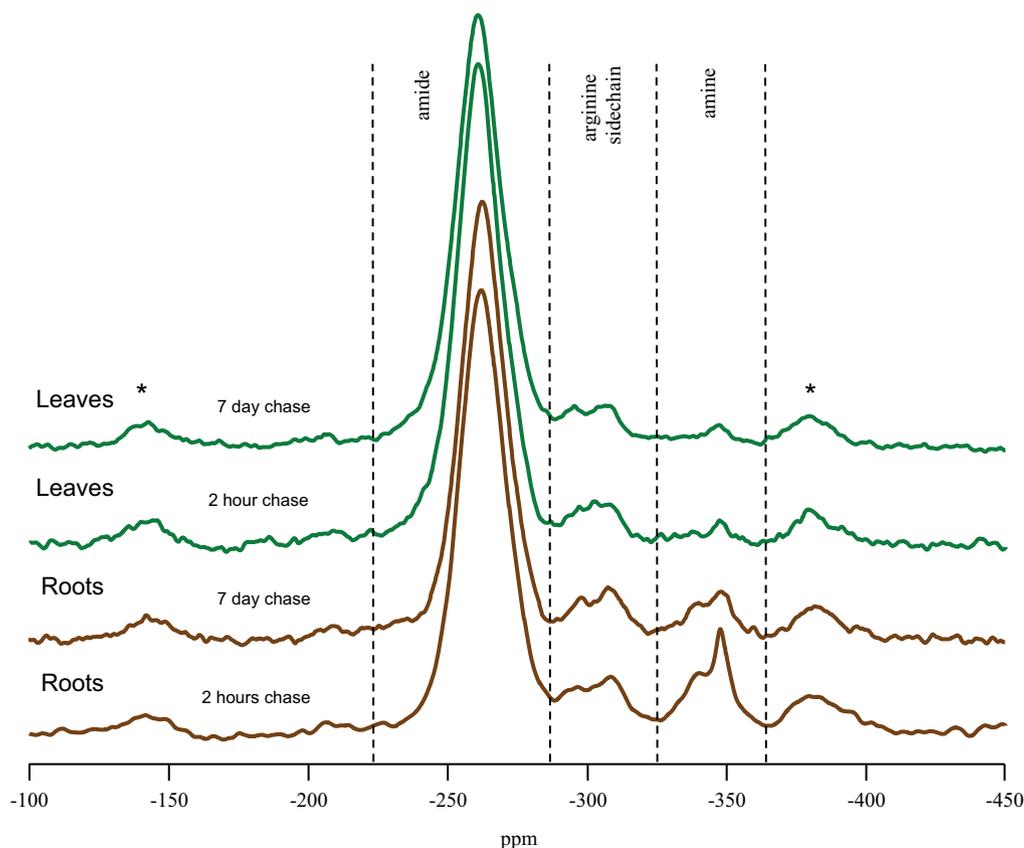


Figure 4 | Cross polarization ^{15}N NMR spectra of seedling leaves and roots indicating qualitative differences between the two chase times (2 hour and 7 day). The 7 day chase samples had experienced 2 pulses while the 2 hour samples had 3 pulse of $^{13}\text{CO}_2$. Common integral regions representing the different magnetic environments of nitrogen are shown on the spectra. The asterisks indicate spinning side bands.

Combining NMR characterization with stable isotopic labeling has been successfully applied in other fields, notably plant metabolomics²⁶, but, to our knowledge, this represents the first use of this rapid, non-destructive method to quantify molecular isotope allocation using DP ^{13}C NMR and ^{15}N CPMAS spectroscopy for stable and non-reactive tracers in biogeochemical studies. Further work using multi-dimensional NMR methods or a combined instrument approach (e.g., solid-state NMR, solution-state NMR, compound specific isotope analysis with isotope ratio mass spectrometry), as in the area of plant metabolomics²⁶, would continue to refine our knowledge on the allocation of the stable isotopes within the tissues. With knowledge of these methods not only could enriched plant organic matter for decomposition in biogeochemical studies be better characterized but also investigations on the abiotic influences (e.g., water stress) or biotic interactions (e.g., insect grazing) on root exudates and plant physiology could be elucidated.

Methods

Seedling growth and enrichment. Thirty three one-year-old aspen seedlings, grown from an open pollinated aspen seed source at a commercial nursery (Smoky Lake Nursery, Smoky Lake, Alberta), were used in this study. Dormant seedlings were planted into pots (15 cm wide and 17 cm high) filled with a mixture of two thirds peat and one third sand. Seedlings were grown in a greenhouse with an average air temperature of 21°C , a photoperiod of 16 hours, and daily watering. Seedlings flushed after 7 days and seedlings were fertilized once with a liquid application of 2.0 g L^{-1} of 20-20-20 N-P-K with chelated micronutrients (Plant Products Co., Brampton, Ontario). After 5 weeks of growth, three seedlings were harvested to establish initial baseline measurements and three additional seedlings were transferred to a separate greenhouse with identical growing conditions to provide the source for the final, unenriched (control) measurements.

Seedlings were labeled once per week for three consecutive weeks. Following each pulse (label), harvesting occurred after two distinct chase periods: two hours and seven days after the pulse (except for the third pulse). Labeled nitrogen (^{15}N) was added to the seedlings via an application of $50\text{ ml K }^{15}\text{NO}_3$ (0.207 M of N, 60 atom %

^{15}N) solution (Sigma-Aldrich Chemicals, St. Louis, MO, USA). Enrichment of carbon (^{13}C) was achieved by adding 100 ml of 99.9% ^{13}C CO_2 (Cambridge Isotope Laboratories, Inc. Andover, MA, USA) to each seedling enveloped in a Mylar tubular bag (36 cm diameter with the length varying depending on the seedling height (VacPac[®], Baltimore MD). A 50 ml gastight syringe (Hamilton, Reno NV) was used to inject $^{13}\text{CO}_2$ gas. Seedlings were allowed to take up the $^{13}\text{CO}_2$ gas for 30 minutes before the bags were removed. At sampling time (two hours and seven days after the pulse), three seedlings were randomly selected for harvesting and separated into three components: leaves, stem, and roots. Plant components were oven dried at 60°C , weighed, coarsely ground and stored until further analysis. The complete aspen seedling enrichment experiment was run twice.

Laboratory analyses. Samples were finely ground using a Retsch MM200 ball mill grinder (Retsch Inc. Newtown, USA) and analysed for total carbon and nitrogen contents on a Costech ECS 4010 Elemental Analyzer (Costech Analytical Technologies Inc. Valencia, USA). The isotopic composition of the samples was measured on a Costech ECS 4010 Elemental Analyzer equipped with a thermocouple detector joined to a Finnigan Deltaplus Advantage Isotopic Ratio Mass Spectrometer (ThermoFinnigan, Bremen, Germany).

Nuclear magnetic resonance. Samples for ^{13}C were analyzed on a Chemagnetics CMX Infinity 200 [magnetic field (B_0) = 4.70 T, Larmor frequency (ν_L) (^{13}C) = 50.3 MHz] spectrometer with a 7.5 mm double-resonance MAS probe capable of high-power ^1H decoupling (Varian, Ft. Collins, CO, USA). Samples were loaded into a 7.5 mm OD zirconium oxide rotor with Kel-F drive tips and end caps, and spacers made of Teflon (DuPont, Circleville, OH, USA). The magic angle was set to 54.74° by maximizing the ^{79}Br NMR signal for spinning sidebands of KBr. Carbon-13 spectra were referenced to tetramethylsilane (0 ppm) by setting the high-frequency adamantane peak to 38.56 ppm^{27} .

Direct polarization ^{13}C with a spin echo pulse ($90 - \tau - 180 - \tau - \text{ACQ} - \tau = 400\text{ s}$) was used to acquire spectra^{18,20}. A total of 500 transients (2000 for the control initial leaves) were acquired for each free induction decay (FID) with: 5.0 kHz spinning rate, $4.5\text{ }\mu\text{s}$ 90° pulse width, and 100 s recycle time. Processing of the resulting FID was performed with WIN-NMR version 6.0 (Bruker, Germany). Processing the spectra included zero filling to 1 K, line broadening of 100 Hz with phase and baseline correction followed by integration of the spectra. Previously published descriptions on peak identification and classification of integral regions were used to separate the following six regions^{5a}: alkyl (0–47 ppm), O-alkyl (47–93 ppm), di-O-alkyl (93–112



ppm), aromatic (112–140 ppm), phenolic (140–165 ppm), and carbonyl (165–185 ppm).

Nitrogen-15 NMR spectra of magic angle spinning samples were acquired on a Bruker Avance 500 NMR spectrometer, operating at 50.69 MHz for ^{15}N . Spectra were acquired with ramped CP, a $4.0\ \mu\text{s}\ ^1\text{H}\ \pi/2$ pulse, a 1 ms contact time and a recycle delay of 3.0 s. Samples were spun at 6 kHz. Spectra were obtained by adding 8 000 to 20 000 transients and were referenced such that $\delta_{\text{iso}} = 0$ for the isotropic ^{15}N resonance of methyl nitrate by setting the ^{15}N isotropic peak of glycine- ^{15}N (98% ^{15}N) to $-347.54\ \text{ppm}^{28}$. The latter sample was used to optimize acquisition parameters. Spectra of ^{15}N enriched samples contained 4 distinct regions attributed to amide (-220 to $-288\ \text{ppm}$), arginine sidechain (-288 to $-324\ \text{ppm}$) and amine (-324 to $-365\ \text{ppm}$) nitrogen sites¹⁶; the much more intense signal from the amide site contained spinning sidebands whose integrated intensities were added to that of the isotropic peak. In addition, to confirm the ^{13}C spectra acquired on the CMX 200, select samples were analyzed with either ramped CP or Bloch decay pulse sequences on the Avance 500 spectrometer operating at a frequency of 125.81 MHz. for ^{13}C .

Statistics. Descriptive statistics, sample means and standard deviations were determined using R version 2.11.0 (The R Foundation for Statistical Computing).

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Author contributions

C.E.N. designed and conducted the experiment, processed the data and prepared the manuscript. S.A.Q. was instrumental in the project design, supervision and manuscript preparation. S.M.L. was involved in project design and provided seedlings, greenhouse space and tree growth expertise. G.M.B. acquired ^{15}N spectra and, with R.E.W., provided both expertise and NMR time.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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