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Does long-term drought or repeated defoliation affect seasonal leaf N cycling in young beech trees?

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Running head: Nitrogen cycle in beech trees under stress

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Abstract

Forest trees adopt effective strategies to optimize nitrogen (N) use through internal N recycling. In the context of more recurrent environmental stresses due to climate change, the question remains whether increased frequency of drought or defoliation threatens this internal nitrogen recycling strategy. We submitted 8-year-old beech trees to two years of either severe drought (Dro) or manual defoliation (Def) to create a state of N starvation. At the end of the 2nd year before leaf senescence, we labeled the foliage of the Dro and Def trees, as well as that of control (Co) trees, with ^{15}N-urea. Leaf N resorption, winter tree N storage (total N, ^{15}N, amino acids, soluble proteins) and N remobilization in spring were evaluated for the three treatments. Defoliation and drought did not significantly impact foliar N resorption or N concentrations in organs in winter. Total N amounts in Def tree remained close to those in Co tree, but winter N was stored more in the branches than in the trunk and roots. Total N amount in Dro trees was drastically reduced (-55%), especially at the trunk level, but soluble protein concentrations increased in the trunk and fine roots compared to Co trees. During spring, ^{15}N was mobilized from the trunk, branches and twigs of both Co and Def trees to support leaf growth. It was only provided through twig ^{15}N remobilization in the Dro trees, thus resulting in extremely reduced Dro leaf N amounts. Our results suggest that stress-induced changes occur in N metabolism but with varying severity depending on the constraints: within-tree ^{15}N transport and storage strategy changed in response to defoliation whereas a soil water deficit induced a drastic reduction of the N amounts in all the tree organs. Consequently, N dysfunction could be involved in drought-induced beech tree mortality under the future climate.

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Introduction

The drought periods and heat waves are expected to increase in severity and frequency in the coming decades (Coumou et al. 2013, Wagner et al. 2013, IPCC 2021). Such climate hazards may alter the functioning of vital processes in trees, and beyond a certain threshold, induce tree dieback or even threaten their survival (Senf et al. 2018, Archambeau et al. 2020, Taccoen et al. 2021). Hydraulic signals and how the tree carbon (C) metabolism respond to soil water deficit have been fairly well studied in recent years (McDowell 2011, Choat et al. 2018, Hartmann et al. 2018), but the responses of tree nitrogen (N) metabolism received less attention, despite its importance for tree functioning (Gessler et al. 2017). The external supply of nitrogen necessary for tree metabolism depends largely on the uptake of mineral N from the soil by the roots (Bazot et al. 2013, Villar-Salvador et al. 2015). A severe soil water deficit limits the soil water and nutrients available to the trees, hinders the roots ability to explore the soil and decreases microbial activity, which strongly influences nutrient concentrations in the soil (Kreuzwieser and Gessler 2010, Cregger et al. 2014). Trees have adopted a dedicated strategy to optimize their N use efficiency under soil N limitations via internal recycling (Vitousek 1982). But any reduced nutrient availability, coupled to tree hydraulic dysfunction during prolonged drought events, may likely damage their internal N cycle and cause severe physiological dysfunction (Gessler et al. 2017). Under a temperate climate, the internal N cycle of deciduous trees is marked by its seasonality (Cooke and Weih, 2005). In spring, the N stored in the perennial organs (trunk, branches and roots) is remobilized towards the new leaves and shoots, and metabolized into proteins, thus playing an essential role in leaf functioning during the summer. In autumn, leaf N resorption is an important process: foliar proteins degrade into amino acids which are transported via the phloem towards the wood parenchyma where they are stored during winter in the form of amino acids and vegetative storage proteins (Sauter et al. 1989, Wetzel et al. 1989, Stepien et al. 1994, Millard 1996). According to the studies, leaf N resorption efficiency has been observed as decreased (Marchin et al. 2010, Estiarte and Peñuelas 2015) or increased (Meier and Leuschner 2014, Touche

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et al. 2024) by drought whereas in response to defoliation, the few studies available in forest trees have shown no effect on leaf N resorption efficiency in the remaining foliage (May and Killingbeck 1995, Gortari et al. 2021). The N reserves in the tree are at their maximum concentrations at the end of winter (El Zein et al. 2011b, Bazot et al. 2013). In the 1990s, use of both ^{15}N stable isotope analysis and N-compound biochemistry made it possible to quantify these seasonal processes and to characterize the nutrient budget of young trees (Millard 1994, Tagliavini et al. 1995). More recently, the N budget has also been characterized in mature forest trees (El Zein et al. 2011ab, Bazot et al. 2013).

European beech (*Fagus sylvatica* L.) is known to be more drought-sensitive than other European broad-leaves (Zang et al. 2014, Zimmermann et al. 2015), but paradoxically, it has also a remarkable potential for recovery after drought stress (Elling et al. 2007). However, in Europe, recent models predict a sharp decrease in the beech distribution range by 2100 (Landmann et al. 2008, Cheaib et al. 2012). A recent illustration occurred after the exceptional drought in 2018 (Schuldt et al. 2020, Rohner et al. 2021): a large-scale die-back was observed in beech forests, accompanied by a degradation in crown condition, and an early leaf fall before the senescence could occur. Early defoliation may worsen tree nitrogen deprivation in beech because an important amount of total tree N (about 38%) is located in the foliage in summer (El Zein 2011). How stresses such as drought or defoliation might impact the seasonal nitrogen cycle is not well understood (Babst and Coleman 2018). Consequently, we undertook to study in detail the consequences of repeated defoliation and a prolonged drought during two years on the N cycle of beech trees. Both defoliation and drought can induce dysfunction in certain physiological processes that are essential to overall tree metabolism. In the present paper, we present the consequences of experimental N deprivation on the internal N cycle and N stock rebuilding in 8-year-old beech trees. We compared the impact of two methods of N deprivation for trees: 1) a two-year severe water shortage, which decreased the uptake of nutrients, their use and their transport within the tree to the leaves; and 2) manual defoliation (removing 75 % of the leaves)

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repeated for two successive years, which reduced the internal N pool in the trees. We compared the impact of these two constraints on tree N stocks and on tree seasonal internal N cycling. To track internal N changes, we labeled all the foliage of control, defoliated and water-stressed beech trees with ^{15}N-urea in September (before leaf senescence), at the end of the 2nd stress period. We followed the fate of the ^{15}N from the senescent leaves toward the perennial storage organs and estimated the N returning to the soil by analyzing litter N. Finally, we tracked ^{15}N remobilization and N allocation among organs for spring growth. We also analyzed seasonal changes in the concentrations of non-structural N compounds (amino acids, soluble proteins) in the different tree organs.

We evaluated the following hypotheses:

(H1) A drastic reduction in N induced by recurrent yearly defoliation is likely and no additional N uptake will be possible due to prolonged drought over several seasons. Therefore, since nitrogen is the main driver of growth and subsequently of biomass accumulation, a significant reduction in the total amounts of C and N in the tree is expected; (H2) Under defoliation and drought, at leaf fall, leaf N resorption should increase and less N return to the soil through the litter; (H3) Regardless of their potential effect on seasonal growth, the treatments should also decrease winter N storage per dry mass unit in perennial organs, especially under drought; (H4) In spring, changes in stored N compounds as well as in ^{15}N remobilization from the perennial organs should be source driven and unaffected by the current N supply, as proposed by Millard and Grelet (2010).

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Materials and methods

Plant material and experimental design

Fagus sylvatica seedlings were grown in an open ground nursery at the INRAE Grand-Est site (Champenoux, France, 48°75′N, 6°34′E, 229 m asl) for seven years (2007-2013). In 2014, a transparent roof built of polycarbonate sheets was installed 5m above the seven-year-old trees to intercept rainfall. Three treatments were imposed on the trees (n=336 per treatment) for three years (2014-2016): (i) a control treatment (Co) in which the trees were kept intact and regularly irrigated; (ii) a defoliation (Def) treatment in which the trees were submitted to yearly manual defoliation (removal of 75% of the foliage in June) and regularly irrigated; and (iii) a drought treatment (Dro) where the trees were not irrigated, thus inducing a predawn twig water potential down to -2.0 MPa (Chuste et al. 2019, 2020). The root systems of Dro trees were isolated with a rigid waterproof plastic sheet (DELTA®-MS) buried to a depth of around 1.80 m. The Dro trees were slightly irrigated (about 40 mm) only once a year in November, every year. An automatic drip watering system delivered between two and four liters of water per tree to the Co and Def trees two to three times a week. Irrigation was adjusted to avoid any water shortage in these two treatments.

Soil characteristics and soil water content measurements

The experimental site is characterized by a homogeneous silty–clay loam soil 60 cm deep (silt: 61±1.28%; clay: 27±0.98%; sand: 12±0.66%), a pH ranging from 7.5 to 8 and an organic matter content between 12.1 and 14.9 $g \cdot kg^{-1}$ (E Silva 2010). Below 60 cm, the grey marl of the Jurassic inferior (Lotharingian) is characterized by a swelling heavy clay soil with a high bulk density. We used a neutron probe (TROXLER TX 4301, Research Triangle Park, NC, USA) to measure the volumetric soil water content. Three neutron probe access tubes were installed in each treatment to quantify water content at different depths: two tubes measured from 0 to 1 m in depth and the other one measured from 0 to 1.6 m. Relative Extractable Water (REW, in %) was calculated according to

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Bréda et al. (1995). In the Co and Def treatments, the REW was maintained above 40%, the threshold below which stomatal closure reduces transpiration (Granier et al. 1999). In the Dro treatment, the REW remained below 40% throughout the experiment and dropped below 15% at the end of each growing season (Chuste 2018, Chuste et al. 2020).

Foliar ^{15}N labeling procedure

The labeling experiment was performed at the end of September 2015 before leaf fall according to the procedure described by Zeller et al. (1998). Zeller et al (1998) showed that beech trees efficiently metabolize urea when it is applied to the leaves. Metabolized N (^{15}N) is transformed into amino acids and proteins and behaves in the same way as the rest of the unlabeled leaf N, then leaf N as a whole (a mixture of ^{14}N and ^{15}N) is transferred to the perennial parts of the tree (see also Chuste et al. 2019). In the context of the present study, we used ^{15}N as a tracer of leaf N. For this, it is crucial that the applied urea N enters the leaf N protein pool, previously shown by Zeller et al. (1998). The timing for the labeling is summarized in Figure 1. Forty-four trees randomly distributed in the different treatments (14 Co trees, 16 Dro trees and 14 Def trees) were chosen for labeling. A crown bag made of polyethylene was placed over the total foliage of each tree to isolate it from its local environment. In the late afternoon, an aqueous solution of ^{15}N urea (10.4 atom%, 5.0 $g.L^{-1}$) was sprayed inside the bag onto the leaves with a hand sprayer. The urea solution was sprayed in a fine mist to limit the formation of drops and ensure a homogeneous labeling of the leaves. After labeling, the plastic bag was kept on the tree for the night, then very carefully removed the next morning to avoid any contamination of the soil and among trees. In October 2015, a net was installed around each tree to collect the falling ^{15}N-labeled litter during late autumn.

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Sampling protocol

We harvested two Co, two Def and four Dro labeled trees one month after labeling (October 2015) to evaluate the incorporation of ^{15}N in the different organs of the tree. We chose to double the number of Dro trees to sample to ensure that the transport system was still functional after almost two years without watering. We wanted to verify that ^{15}N could still be exported from labeled leaves to other organs of Dro trees. In each tree, the following main organs were sampled: leaves, twigs, branches, trunk top, trunk and coarse roots. In each treatment, the ^{15}N label was recovered in the leaves (source of the ^{15}N) and in the other organs, indicating that the internal process of leaf N redistribution inside the tree was underway (Figure S1). The leaves and branches were still the most enriched in ^{15}N at that time, compared to the trunk and roots (Figure S1). Nine unlabeled trees (3 Co; 3 Def; 3 Dro) were also harvested in autumn to assess the natural abundance of ^{15}N in each tree organ.

The other labeled trees were harvested at two key phenological dates for estimating within-tree N winter store and ^{15}N distribution on the one hand, and spring ^{15}N allocation to new growth on the other: 1) in February 2016 (winter), 5 months after labeling at the theoretically highest N storage level in perennial organs; and 2) in June 2016 (spring), 9 months after labeling at the theoretical end of spring N remobilization (El Zein 2011b), once leaf expansion was completed. The timing of the harvests is summarized in Figure 1. We harvested six trees per treatment and per date. In each tree, the total biomass of the following main organs was sampled: leaves, twigs, branches, trunk top and main trunk. Some wilted, browned and leafless branches, identified as dead branches, were collected separately. A subsample of coarse (d>2mm) and fine roots (d<2mm) was also taken. In February 2016, we also collected the litter in the net on the trees of the three treatments. A subsample of each aerial and subterranean organ was immediately frozen in liquid nitrogen, stored at -80°C, then freeze-dried (Dura-Top (r), Dura-Dry (r), FTS Systems (r), Stone Ridge, NY, USA). The freeze-dried subsamples were weighed, ground into a fine powder with a ball mill (CEPI SODEMI CB2200, Cergy, France) then stored in vials in the dark until the isotopic and biochemical analyses. The rest of the aerial

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organs was also kept, dried three days at 80°C and weighed. The total dry matter (DM) of each aerial organ was obtained by summing the weight of the dry matter (DM) of the subsample and the remainder of each organ. Once the experiment was fully completed (2018), the whole aerial system of 18 additional trees (n = 6 per treatment) was harvested and their whole root system was excavated and dried for three days at 80°C. The root biomass of all the trees sampled in 2015 and 2016 was then estimated a posteriori with the allometric relationship between total aboveground and belowground biomass (total coarse roots, d>2mm), as shown in Figure S2. For the same age class, our root biomass results were close to those in a study that had investigated the biomass production of beech trees in the North-East of France (Le Goff and Ottorini 2022). We were not able to fully recover the fine root system with an excavator. However, with regard to the total root system, coarse roots appear to account for 95% of the total belowground biomass increment while fine roots account for only 5%, independently of tree age (Le Goff and Ottorini 2022). Therefore, we took fine roots into account for our concentration calculations but assumed they could be neglected when calculating amounts. Therefore, we based each calculated amount (C, N and soluble N compounds) on total roots minus fine roots and referred to this result as ROOT in our figures and tables.

Leaf measurements

We assessed several leaf characteristics on 100 randomly-sampled mature leaves per tree on both unlabeled trees in 2015 (June 2015) and labeled trees in 2016 (June 2016) for each treatment. The individual leaf area was measured with a portable area meter (LI 3000 A) connected to a belt conveyer (LI-3050A, both LI-COR, Lincoln Nebraska, USA), then the leaves were dried for 48h at 80°C and weighed. The mean individual leaf area and the mean leaf mass per area (LMA) were calculated for each tree. For harvested trees, the leaves remaining after sampling were also dried for 48h at 80°C and weighed to determine the total leaf mass of each tree. We calculated the total number of leaves

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and the total leaf area of the trees based on the allometric relationship between the leaf biomass and the leaf area of the 100 sampled leaves.

Nutrient resorption efficiency

Nutrient resorption efficiency was calculated as described by Killingbeck (1996) and more recently by Zhang et al. (2018):

$$Nur = \frac{(N_{green} - N_{sen})}{N_{green}} * MLCF * 100 \quad (1)$$

where N_{green} and N_{sen} are the N concentrations in green leaves (sampled in June 2015) and in the litter (sampled in February 2016), respectively. The Mass Loss Correction Factor (MLCF) corresponds to the percentage of leaf mass remaining in the litter compared to the mass of the green leaves (Vergutz et al. 2012). European beech is a deciduous temperate species; therefore, we used a MLCF value of 0.784 as recommended in Vergutz et al. (2012).

Isotopic analyses and calculations

Total C and N concentrations (% of dry matter) and ^{15}N isotopic abundance (atom%) of each organ were measured with an elemental analyzer (Eurovector, Redavalle, Italy) coupled to an Isoprime (Elementar UK) at the isotopic platform "Plant Biochemistry and Molecular Physiology" (INRAE, Montpellier, France).

The isotopic abundance for N expressed in atom% (A_N %) was defined as:

$$A_N = \frac{^{15}N}{^{14}N + ^{15}N} * 100 \quad (2)$$

The ^{15}N enrichment (atom %) in each organ after tree labeling was defined as:

$$^{15}N_{excess} = A_{N\ labeled\ organ} - A_{N\ unlabeled\ organ} \quad (3)$$

where $A_{N\ labeled\ organ}$ is the ^{15}N abundance of the labeled tree organ and $A_{N\ unlabeled\ organ}$ is the natural ^{15}N abundance of the unlabeled tree organ, with an $A_{N\ unlabeled\ organ}$ of about 0.3683 ± 0.0031 atom% to 0.3709 ± 0.0013 atom% depending on the considered organ.

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The concentration of ^{15}N (mg.100g^{-1} DM) incorporated by labeling in the dry matter (DM) of a given organ was calculated as:

$$^{15}N_{concentration} = \frac{^{15}N_{excess} * [N]}{100} * 1000 \quad (4)$$

where [N] is the N concentration (g.100g^{-1} DM) in the organ. The ^{15}N amount (mg.organ^{-1}) incorporated by labeling into each organ was calculated as:

$$^{15}N_{amount} = \frac{^{15}N_{concentration}}{1000} * \frac{\mathrm{DM}}{100} \quad (5)$$

where DM is the dry matter (g) of the organ.

N partitioning and ^{15}N allocation represent the ratio (%) of the amount of N or ^{15}N, respectively, incorporated into a given organ relative to the total amount of N or ^{15}N incorporated into the whole tree.

$$N_{partitioning} = \frac{N_{amount\ of\ the\ organ}}{N_{amount\ of\ the\ tree}} * 100 \quad (6)$$

and

$$^{15}N_{allocation} = \frac{^{15}N_{amount\ of\ the\ organ}}{^{15}N_{amount\ of\ the\ tree}} * 100 \quad (7)$$

Amino acid concentration, amount by organ and within-tree partitioning

For each organ, the amino acids (AA) were extracted from 20 mg of dry matter at 4°C in 1.5 mL of 70% methanol. After shaking for 30 minutes and centrifuging for five minutes at 17,000g at 5°C, the Eppendorf tubes were immediately placed on ice. Total AA were assayed by colorimetry at a wavelength of 570 nm with a ninhydrin reagent, following Yemm and Cocking (1955). AA content was determined with reference to a standard curve established from a stock solution of leucine (25 mM). Results were expressed as AA concentrations (g.100g^{-1} DM *i.e.,* %DM); AA amount per organ (g.organ^{-1}) was calculated by multiplying AA concentrations by the biomass of the considered organ in the tree. AA partitioning was expressed as the ratio (%) of the amount of amino acids incorporated into a given organ relative to the total amount of amino acids in the whole tree.

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Soluble protein concentration, amount by organ and within-tree partitioning

All soluble proteins (PROT) were extracted from 10 mg of dry matter at 4°C in 1.5 mL of extraction buffer [Na_2/KH_2PO_4 0.1M at pH 7.38; Dithiothreitol (DTT) 5mM 0.8 mg/mL; Polyvinylpyrrolidone (PVP 40,000) 19.5 mg/mL; Polyethylene glycol (PEG 20,000) 4.5 mg/mL] mixed in a ball mill (Vibro-mill MM400-RETSCH) two times for 45 seconds. The samples were centrifuged for 15 minutes at 12,000 g at 4°C and kept on ice. Total soluble proteins were assayed by colorimetry at 595 nm with Coomassie blue (Bio-Rad Protein Assay Dye Reagent Concentrate, 500-0006), as in Bradford (1976). The PROT content was determined with reference to a standard curve established from a stock solution of Bovine Serum Albumin (Bio-Rad Protein Assay Standard II, 500-0007). Results were expressed as PROT concentrations ($g.100g^{-1}DM$ *i.e.,* %DM); PROT amount per organ ($g.organ^{-1}$) was calculated by multiplying PROT concentrations by the biomass of the considered organ. PROT partitioning was expressed as the ratio (%) of the amount of soluble proteins incorporated into a given organ in the tree relative to the total amount of soluble proteins in the whole tree.

Statistical analyses

All statistical analyses were performed in R version 4.1.2 (2021-11-01). Before statistical analysis, all data were checked for normality (Shapiro-Wilk test) and homoscedasticity (Levene's test). We used ANOVA models to compare leaf properties, C, N and ^{15}N content and concentration, amino acids and proteins content and concentration between dates and treatments. Differences are considered significant when $p<0.05$. Statistically significant differences among groups were further tested with Tukey's post hoc test. Values are presented as mean ± standard error.

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Results

Impact of defoliation and water stress on leaf growth variables and on leaf N resorption efficiency

After only one year of constraints (2015), no treatment effect was noted on the LMA of the trees (Figure 2A) whereas in 2016, LMA was significantly higher in Def than in Co trees (Figure 2B). Individual leaf area (+63%), total leaf area (+307%) and leaf number (+169%) markedly increased in Co trees between 2015 and 2016 (Figures 2C-2H). The total leaf area and leaf number of the Def trees (+174% and +133%, respectively) also increased over time but to a lesser extent than for the Co trees (Figures 2E-, 2B-2H). In contrast, leaf growth in the Dro trees was markedly reduced over the two years (2015, 2016) with significantly smaller leaves (-39% and -61%, respectively), lower total leaf area (-52% and -68%) and a lower number of leaves (-28% and -38%) than for the Co trees (Figures 2C-2H).

In 2015, summer leaf N concentrations ranged from 1.83 to 2.28 % (Table 1), without any significant effect of treatment at $p<0.05$. The following winter, litter N concentrations ranged from 0.44% (Dro) to 0.65% (Def) resulting in a similar N resorption efficiency [comprised between 55% (Def) and 61% (Dro)].

C and N seasonal changes and their partitioning within the tree in relation to the constraints

After two years of drought, the total C amount in the above-ground and root systems was strongly reduced (-62%) compared to the Co trees (Table 2A, 2B). N amounts were also reduced by drought, more so in the above-ground (-59%) than in the root system (-43%). In particular, drought significantly reduced the C and N amounts in the leaves in spring (Table 2A). However, N concentrations in spring leaves were remarkably stable (2 to 2.3%) regardless of treatment (Figure S3B). Similar reductions in C and N amounts in response to drought were also observed in winter but statistical differences with the Co trees were only noted for the top of the trunk at that time (Table 2A). C and N amounts in the organs of the Def and Co trees were not significantly different, except

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in spring when a marked decrease in the amounts of C and N was noted in the trunk top along with a decrease in N in the trunk of the Def trees (Table 2A). A season x treatment interaction effect was noted on the amounts of C and N in the leaves i.e. litter in winter and mature leaves in spring (Table 2B). Globally, season and treatment effects were significant for C and N amounts in the total above-ground system whereas in the root system, they were only significant for C, without any season x treatment interaction. Effect of season was much more marked on N concentrations: spring N concentrations were significantly reduced in the trunks and fine roots of the Co trees, but also reduced in the trunk, branches and coarse roots in def trees (Figure S3A, S3B). In Dro trees, spring N concentrations decreased only in the branches but increased in fine root (Figure S3A, S3B).

Carbon partitioning among organs was preserved regardless of the treatment and the season: trunk (41 to 54%), roots (23 to 24%) and branches (14 to 19%) (Figure 3A, 3B). Carbon partitioning to Def litter was less than to Co litter due to the defoliation in 2015. In spring, before defoliation, leaf C partitioning was significantly higher for the Def than the Co trees in connection with increased LMA (Figure 2B). In winter, tree N was mainly partitioned to the trunk (39 to 41%), branches (23 to 25%) and roots (22 to 29%). N partitioning to the roots was significantly increased by drought (Figure 3C). Whatever treatment, litter N always represented less than 6% of total tree N. Spring growth induced marked changes in N partitioning with a decrease in N in the trunk and roots of the Co and Def trees (Figure 3D). About 40% of tree N appeared in Co leaves, even more (51%) in Def leaves (Figure 3C). However, under drought, N partitioning to the roots and trunk remained high, and was significantly higher than in the other treatments. In the Dro treatment, the leaves accounted for only 18% of tree total N in spring and N partitioning in the trunk top was reduced compared to Co trees (Figure 3D).

Amounts, concentrations and within-tree allocation of ^{15}N

In winter, ^{15}N exported from the leaves during autumnal N resorption was mainly allocated to the trunk (42%), branches (25%) and roots (20%) in the Co trees (Figure 3E). In response to defoliation

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(Def), ^{15}N was allocated more to the branches (48%) and less to the trunk (23%) and roots (8%). Drought (Dro) also modified ^{15}N allocation in the trees with less going to the trunk (30%) and more to the branches (38%).

In spring, ^{15}N was remobilized from the perennial organs (mainly the branches, trunk and roots) of the Co trees to fuel new growth and was allocated mainly to the leaves, where it accounted for about 49% of total tree ^{15}N (Table 2A, 2B, Figure 3). By comparison, more ^{15}N was allocated to leaves (62%) in the Def treatment and less (36%) in the Dro treatment (Figure 3F). In terms of amount, leaf ^{15}N did not significantly change in response to defoliation (5.75mg in Def vs 6.99 mg in Co) but was strongly reduced (-77%) in response to drought, as was the N amount (Table 2A). The ^{15}N amount was significantly lower in the trunk, trunk top and roots of Def and Dro trees than in the Co trees (Table 2A), and the most important decrease in ^{15}N concentrations between winter and spring occurred in twigs for all treatments and in the branches and trunks for the Dro and Def trees (Figure S3C, S3D).

Amounts, concentrations and within-tree partitioning of AA and PROT

In winter, AA and PROT amounts in the Co trees (Figure 4A, 4D) were mainly present in the trunk (AA: 41%, PROT: 49%), roots (AA: 27%, PROT: 27%) and branches (AA: 28%, PROT: 17%). In terms of concentration, AA in the trunk, trunk top and coarse roots of the Def trees were significantly higher than those of the Co trees (Figure S4A, S4B). PROT branch concentrations were also higher than those of the Co trees while PROT concentrations in the trunk and coarse roots were lower (Figure S4D, S4E). Significantly fewer AA (13%) and more PROT (32%) were partitioned to the branches in the Def trees (Figure 4B, 4E). In the Dro trees, AA partitioning to the trunk (7.39%) was drastically reduced and most of the AA in the tree were found in the roots (73.62%) (Figure 4C, 4F). PROT amount was strongly reduced in Dro trees with lower PROT concentrations in the trunk (Figure S4)

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but the PROT distribution among the organs remained broadly similar to that of the Co trees (Figure 4D, 4E, 4F).

In spring, both the amounts, concentrations and the partitioning of AA to the trunk, branches and roots of the Co trees decreased markedly compared to winter (Figure 4A). PROT concentration decreased also in twigs, the trunk and roots (Figure S4D). We noted that PROT partitioning in the Co trees was also slightly less to the roots and trunk but not to branches (Figure 4D). Leaf AA accounted for 45% (Figure 4A) and leaf PROT for about 20% of the total amount in the Co trees (Figure 4D). In response to defoliation, the main changes were that AA amount increased in the branches (Figure 4B), and PROT amount decreased in the trunk top (Figure 4E). A reduction in AA partitioning (Figures 4A, 4B) and an increase in PROT partitioning to the leaves was also noted (Figures 4D, 4E). In terms of concentration and compared to Co trees, AA increased in the trunk, the trunk top and dead branches of Def trees (Figures S4A, S4B). In spring, most of the AA and PROT amounts in the Dro trees stayed in the trunk and roots (Figures 4C, 4F), and PROT amount in Dro branches was lower than in the Co branches (Figure 4F). AA concentration increased in coarse and fine roots of Dro trees (Figure S4C) and PROT concentrations in the trunk and fine roots of the Dro trees were higher than in the Co trees but lower in twigs (Figure S4D, S4E, S4F). The amount, concentration and partitioning of AA (Figure S4C, Figure 4C), as well as PROT amount and partitioning (Figure 4D, 4F) to Dro leaves were lower than for Co and Def leaves (Figures 4A, 4B). However, PROT leaf concentration was not affected by drought (Figure 4SD, 4SF).

Discussion

Delayed impacts on C and N stocks of repeated defoliation compared to a prolonged soil water deficit

After one year of defoliation (-75 %), the following growing season, the Def beech trees were able to maintain leaf growth, and C and N levels were similar to those of the Co trees. However, after two successive years of defoliation, a decrease in total leaf area was observed during the third growing

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season of the experiment, mainly due to a reduction in individual leaf area in Def trees. Such a decrease in foliage growth following a defoliation event has also been observed on several deciduous and evergreen tree species and at various stages of development: on *Quercus velutina* saplings (Wiley et al. 2013), *Quercus petraea* and *Quercus ilex* saplings (Schmid et al. 2017) or on mature *Quercus robur* (Marçais and Bréda 2006) and *Northofagus pumilio* trees (Piper et al. 2015). Finally, after the two-successive years of defoliation that we applied, the quantities of C and N in beech trees were also impacted. Indeed, C and N amounts in beech trees were significantly reduced in the youngest part of the trunk (trunk top) as was the N amount in the rest of the trunk compared to controls (Table 2). In addition to the effect of repeated defoliation on *Fagus sylvatica* C and N amounts, underlying changes in growth are possible, especially at the anatomical level of the transport system; Future research should include this aspect. In fact, a recent study on *Fagus crenata* (Ueda et al. 2024) showed that repeated defoliation for 4 years reduced hydraulic transport safety in beech because the total area of inter-vessel pits with thin pit membranes increased per unit of vessel wall area. Ueda et al. (2024) hypothesize that repeated defoliation can increase drought stress and the risk of drought-induced tree mortality by increasing susceptibility to xylem cavitation and embolism.

In our study, drought affected the beech trees earlier than defoliation did. In fact, drastic reductions in foliage growth were observed from the growing season of the second year of soil water deficit. Leaf area reduction in response to drought is commonly observed in beech trees to reduce water loss through transpiration (Bréda et al. 2006). In our study, total N amount in the trees was also drastically reduced in response to drought, especially at the trunk level. This reduction in N was mainly due to a strong reduction in primary and secondary growth in response to drought (Chuste et al. 2020), as evidenced by the strong lessening of C accumulation in the water-stressed trees (Table 2). Reduced cambial growth in beech trees following drought events are common and have been shown at various sites (van der Werf et al. 2007, Charru et al. 2010, Leuschner 2020). However, depending on soil conditions and other local factors, when the precipitation pattern becomes favorable again, beech

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trees are able to regain pre-drought cambial growth rates only three years after drought onset; the most resistant individuals even show improved post-drought growth (Camamero et al. 2018). The potential for growth recovery after defoliation depends on the species and is generally greater in deciduous than in evergreens (Krause and Raffa 1996). Growth recovery also depends on the level of defoliation (Anttonen et al. 2002). Following defoliation, new leaf production later in the same growing season is sometimes observed, depending on the species, defoliation intensity and the period. In our study, despite severe defoliation (75% in 2014 and 2015), none of our defoliated beech trees produced new leaves in the same season, nor did any die or show signs of dieback (Chuste 2018). However, prolonged drought or repeated defoliation created a significant reduction in the total C and N amounts in the trees. Our hypothesis (H1) was therefore verified, though with a delayed effect and at a lesser magnitude than expected for the defoliated beech trees, whose organs seemed able to resist repeated N loss longer and better than Dro trees. The reason probably lies in the fact that the defoliated beech trees were able to take up more N from the soil to compensate for the loss of N through defoliation, especially in our fertile soil conditions. On the contrary, limited access to water and minerals induced by our experimental severe water stress may have forced the water-deprived trees to depend almost exclusively on their reserves for survival. Mobilizing stored compounds to recover from stress and help maintain C and N homeostasis and growth is certainly key in beech, which, like other hardwood species, store their reserves in the woody parts of the tree, which are generally protected from herbivory (Delaporte et al. 2016, Chuste et al. 2020). In contrast, evergreen species store a significant part of their reserves in the foliage and are therefore less tolerant to defoliation (Krause and Raffa 1996, Millard et al. 2001, Chuste et al. 2019), as well as to drought (DeSoto et al. 2020). However, it has been shown that young beech trees (aged 8 to 10 years) take up more N from the soil than do older trees, due to their lower internal N storage capacity (Simon et al. 2021). Consequently, the tipping point at which the availability of C and N becomes insufficient to support

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growth and storage may occur earlier in 10-year-old beech trees than in older ones with larger stocks, particularly in response to drought and a limited access to soil N resources.

Autumnal foliar N resumption: a way to optimize N recycling

Our study shows that the foliage of trees submitted to defoliation or soil water deficit for two years managed to maintain high leaf N concentrations, similar to the control trees (about 2%, and even a little more in the Def trees). These results are in line with a previous study with the same experimental design, which demonstrated that N metabolism was still active in the leaves of both Def and Dro trees during the growing season of the 2nd year of treatment (Chuste et al. 2019). The lack of access to N soil under water stress or the major loss of N caused by spring defoliation forced the trees to rely at least partly on internal N remobilization from storage in perennial organs (branch, trunk, root) to maintain high leaf N concentrations. This leaf N concentration homeostasis in beech tree match for example results of Ognjenović et al. (2023) who did not find significant foliar N concentration changes in beech trees (ICP forests) in response to defoliation events. A recent meta-analysis on tree nutritional changes during drought found inconsistent relationships between tree nutritional status and drought survival. Certain nutrients (P, K, Fe and Cu) pointed out by these authors could, however, serve as a potential early warning signal of decline in tree vitality (He et al. 2024). The beech trees, subjected for two years to internal N restrictions, had to maintain high N levels in their leaves for two reasons:

i) to ensure and stimulate leaf metabolic activity and C acquisition in order to limit the risk of C starvation (McDowell 2011) – indeed, a large amount of leaf N in plants is invested in Rubisco proteins (Makino 2003, Evans and Clarke 2019), and

ii) amino acids such as proline, can act as osmoprotectors under water stress some tree species (Peuke et al. 2002, Gessler et al. 2017, Chuste et al. 2019). High N partitioning to the leaves could

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452 also be a strategy for local nitrogen storage near the growing organs, as hypothesized in other studies
453 (Ourry et al. 2001, Millard et al. 2007).
454 In autumn, prior to dormancy, nutrient resorption by deciduous tree species is a fundamental process
455 through which the tree can withdraw nutrients from senescing tissues prior to abscission (Hagen-
456 Thorn et al. 2006). Previous studies have shown that this process allows trees to recover up to 31%
457 of their nitrogen (Cleveland et al. 2013), and that temperate deciduous trees may exhibit high N
458 resorption (Aert 1996). In our study, about 60% of the leaf N was recycled (Table 1), which is
459 consistent with the values found in the meta-analysis by Zhang et al. (2018). In our study, 3 to 6% of
460 C, 4 to 6% of N and 4 to 9% of ^{15}N (corresponding to mobile non-structural N compounds) of the
461 beech trees returned to the soil through the litter and so were not able to be internally recycled. The
462 low N concentrations we found in the beech litter for all treatments indicate that N resorption was
463 very efficient and that N levels were maintained despite the constraints (Table 2). This result
464 contradicts our hypothesis (H2), which was that, under stress, the trees would intensify their recycling
465 of leaf N. Our results therefore suggest that leaf N resorption in beech is efficient and that its
466 efficiency depends little on the environment also observed on mature beech trees exposed to drought
467 (Touche et al. 2022, 2024). But they also identified that the resorption efficiency of other mineral
468 nutrients such as potassium and magnesium could be impacted by drought in beech. Even though
469 beech trees are capable of efficient cycling for major nutrients, the tree response to water and nutrient
470 deficiencies is likely to depend also on complex interactions between tree roots, micro-organisms,
471 soil nutrients (Calvaruso et al. 2017) and soil type. These interactions should be the subject of future
472 research.
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476 *Prolonged water deficit decreased winter N storage whilst repeated defoliation modified within-tree*
477 *N storage location*

478 Amino acid and soluble protein amounts were reduced in the aerial organs of our water-deprived trees
479 compared to the controls, mainly because of the severe reduction in growth of the beech trees in
480 response to drought. In the roots, which also exhibited a strong reduction in growth in response to
481 drought, soluble proteins were also reduced whereas amino acids increased. We assume that this
482 accumulation of amino acids in the roots was the result of proteolysis triggered to protect them from
483 dehydration according to Wargo (1972) and Parker and Patton (1975). How drought events affect tree
484 metabolite concentrations, can be indicative of underlying biochemical regulation processes, as
485 underlined by authors like Jia et al. (2020). In our study, winter concentrations of amino acids and
486 proteins in beech organs were not drastically affected by drought except for the trunk, where soluble
487 protein concentrations significantly decreased. This result suggests that, despite efficient leaf N
488 resorption, the internal nitrogen storage function of water-deprived beech trees was impaired due to
489 decreased protein synthesis in the trunk. Our hypothesis (H3), in which we supposed that winter N
490 storage would decrease under drought, was validated by these results.

491 Olmo et al. (2014) showed that, for ten tree species including *Fagaceae* like oak, in general, the root
492 systems responded to drought through a decrease in the proportion of fine roots (2 to 0.5 mm) and an
493 increase in the proportion of very fine roots (<0.5mm) in the deepest soil levels (20-40 cm deep).
494 Even after a few weeks of drought, saplings from six deciduous and evergreen tree species showed
495 common responses, for example a reduction in soil N uptake and in C allocation to roots as well as a
496 reduction in root biomass (Joseph et al. 2021). Using minirhizotrons, Zwetsloot and Bauerle (2021)
497 found that dry summers in mature beech stands stunted the growth of fine roots and even shortened
498 their lifespan. In our study, given the total absence of irrigation for two consecutive growing seasons,
499 no root growth was possible at all (Joseph Levillain, personal communication). It is therefore
500 reasonable to hypothesize that the extreme 2-year drought we imposed on the beeches drastically

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altered root absorption of N, in particular by stopping the growth of fine roots and, possibly, by increasing dieback among the fine roots as also reported by Leuschner (2020) in beech trees exposed to extreme drought. The dead branches, which were more numerous under drought, kept a small, definitively sequestrated, part of the N, ^{15}N and soluble N compounds of the tree thus also contributing, along with the dead roots in the soil, to N loss in the living components of the trees under drought. These combined effects could be the cause of the reduction in protein storage we observed, mainly in the trunk. Therefore, in the event of prolonged drought for more than two consecutive years, beech trees may no longer be able to fully meet their N requirements for basic functions, and a large reduction in N storage can potentially reduce their stress acclimatization and survivability.

We also observed reductions in both amino acid and soluble protein concentrations in the trunk and roots in response to defoliation, although this was counterbalanced by a significant increase in soluble protein concentrations in the branches. In terms of amounts, defoliation resulted in fewer amino acids and more soluble proteins in the branches than in the control trees; inversely, the roots presented more amino acids and fewer soluble proteins. Total N was unchanged. These results suggest that defoliation induced a change in storage location and organ functioning rather than a decrease in winter N storage.

Tracking the mobile ^{15}N pool from autumnal leaf N resorption within the tree brought additional information to these results. The percentage of ^{15}N allocated to the trunk was reduced in favor of the branches in defoliated trees, and a significant decrease in ^{15}N concentrations in the trunk and fine roots was noted. The same tendency was observed in the water-stressed trees compared to the controls. These original results suggest within-tree changes in mobile N transport from the senescent leaves to other organs that reveal a tree storage strategy modified in response to stress. Defoliation episodes impair root nutrient uptake (Piper et al. 2015) and consequently the ^{15}N in the defoliated trees stayed more in the branches near growth spots (twigs, leaves), ready to be remobilized and meet the demands for new leaf growth. Interestingly, the ^{15}N label was still transported from leaves to roots under drought and this indicates that the internal transport of metabolites was still ensured despite probable

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526 hydraulic dysfunctions such as increased viscosity and reduced transport velocity in the phloem, as
527 shown by Dannoura et al. (2019) in trees from the same experiment, and by Hesse et al. (2019) on
528 mature beech trees submitted to repeated dry summers.

529

530 *N compound remobilization and allocation of ^{15}N to new twigs and leaves in the spring are source*
531 *driven*

532 Total beech N in the spring was not significantly different from that in winter regardless of treatment,
533 thus suggesting that there was no significant N uptake at that time of the year. This confirms El Zein
534 et al.'s (2011b) conclusions on young beeches; the authors showed that the spring growth of mature
535 beech trees mainly depends on the remobilization of stored N. Indeed, the internal N cycle in
536 deciduous trees makes it possible to decouple growth from N absorption (Millard 1996), and the
537 internal N changes that we observed in spring were likely due to remobilized N exchanges between
538 actively growing and storage organs, regardless of growing conditions. In spring, we noted a marked
539 decrease in amino acid, soluble protein and ^{15}N concentrations in the twigs, trunks and roots of both
540 control and defoliated beech trees. Above- and below-ground organs contributed to spring growth but,
541 in response to defoliation, the N soluble compounds and ^{15}N stored in the branches were also
542 remobilized. We assume that the extra winter N storage in the branches of the defoliated trees then its
543 spring remobilization helped them to recover a total leaf area and a leaf count similar to the unstressed
544 trees. This also suggest source-driven N remobilization. Compared to the control trees, the observed
545 elevated concentrations of amino acids in the trunks and trunk tops of the defoliated trees in spring
546 could be due to higher amino acid requirements for protein synthesis in the new shoots (Chuste et al.
547 2019). Under drought, even though a decrease in ^{15}N concentrations was noted in the twigs, trunk top,
548 trunk and branches, indicating the occurrence of N remobilization, nothing indicates that proteins in
549 these organs were remobilized for new leaf growth, which was drastically reduced. In fact, the soluble
550 protein concentrations in the drought-stressed trees remained equal to the concentrations in the

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perennial organs (trunks and roots) of the unstressed trees. These results suggest that the strongly reduced leaf growth we observed during the third year of drought stress was probably more due to internal hydraulic limitations preventing N remobilization and transportation than to limited N stored resources. We found also higher concentrations of amino acids in the fine and coarse roots of the drought-stressed trees in spring than for the control trees, thus suggesting stress-induced changes in N metabolism. Drought-stressed trees may need more N osmoprotectants to guarantee cell integrity and prevent osmotic stress from killing the root system. In fact, severe drought is known to increase amino-acids, which play an important role in protecting organs like leaves from dehydration in tree species such as apple (Sircelj et al. 2005) or beech (Fotelli et al. 2002). However, in our drought-stressed beech trees, the amino-acid concentrations in the few newly formed spring leaves decreased significantly, thus suggesting impaired leaf metabolism and problems with osmotic adjustments during the third year of extreme drought. We therefore assume that a threshold, after which survival is jeopardized for 10-year-old beeches, is reached after 3 years of extreme drought. Even after implementing survival mechanisms such as a strong reduction in growth, maintaining C storage (Chuste et al. 2020), or an induced early senescence of the foliage (Massonnet et al. 2021), such a tipping point has been also observed in the 3d year of severe water deficit for nonstructural carbohydrates in the same trees (Chuste et al. 2020), suggesting that three successive years of drought alter C and N metabolisms enough to threaten beech survival. Studies in the 1970s showed that defoliation and drought can cause a marked increase in amino acids in tree species such as maple and oak, especially in the coarse roots (Wargo 1972, Parker and Patton 1975). These authors showed that specific amino acids, threonine, cysteine, tyrosine, proline and asparagine, were involved in the response to defoliation and drought. Changes in these amino acids, in particular the increase in asparagine, (Parker and Patton 1975) and the increase in sugars often observed in response to stress are known to increase tree survival (Chuste et al. 2020, Leuschner 2020). However, according to Wargo (1972), the accumulation of amino acids in the roots could also jeopardize the survival of

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stressed trees by making them more palatable to certain fungi like *Armillaria mellea*. In fact, the negative impact of extreme weather events on the health of tree root systems and the increasing occurrence of armillaria root diseases have been regularly reported and could become serious threats to trees under future climatic conditions (Kim et al. 2022). Our results showed that changes in stored N compounds as well as ^{15}N remobilization from perennial organs in the spring were source driven and dependent on N stores regardless of treatment, thus confirming our hypothesis H4. However, remobilization itself was impaired in the trees submitted to three years of severe water deficit, even when they had high N stores. Water is strongly involved in the process of N remobilization and N distribution to growing organs; therefore, water deficit is a strong limiting factor under prolonged drought and may alter these processes as well as phloem transport (Dannoura et al. 2019).

Conclusion

Our results suggest stress-induced changes in N metabolism in response to recurrent constraints such as soil water deficit and defoliation, but the degree of severity depends on the constraints. Defoliation had a relatively small impact on N stocks and this, only in the growing season following the second year of defoliation. The impact was mainly expressed through modifications in the within-tree distribution of stored N compounds and the N lost by defoliation was potentially and partially compensated for by soil N uptake. Soil water deficit had a more severe and faster impact on beech trees both in terms of storage and remobilization, which is mainly source-dependent. As our drought-stressed beech trees seemed to have already optimized leaf N resorption as much as possible in the N cycle, no more compensation was possible under drought when soil N was not accessible. Our results suggest that the within-tree N storage capacity and the remobilization of N stores could be threatened under future climatic conditions where soil water deficits will become more frequent and intense. The N cycle should be further studied as it is a possible process involved in drought-induced tree mortality.

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Data Availability statement

The data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Data

Supplemental figures S1, S2, S3 and S4.

Conflict of Interest

The authors declare no conflict of interest.

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Authors contributions

P.M. C.M. and N.B. conceived the idea and acquired the funds; C.M., P.M., P.-A.C. and B.Z. designed the methodology and collected samples. P.-A. C. P.T., C.M. and P.M. collected and analyzed the C, N and ^{15}N data; B.G. and L.C. collected the biochemistry data, P.M. and C.M. analyzed all the data; P.M. and C.M. drafted the manuscript. All the authors revised the manuscript and gave approval of the final manuscript.

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Legend captions for tables

Table 1. Nitrogen concentrations in green leaves (Ngreen, %DM) in summer 2015 and leaves in the litter (Nsen, %DM) in winter 2016, and nitrogen resorption efficiency (NuR, %) of the European beech submitted to the three treatments: control (Co), defoliation (Def) and drought (Dro). Mean ± SE with n = 12, 11 and 24 for Ngreen in the control, defoliation and drought treatments, respectively, and n = 6 for Nsen for all treatments in winter 2016. There were no significant differences ($p<0.05$) among treatments.

Treatment	Ngreen (%DM)	Nsen (%DM)	NuR (%)
Co	1.83 + 0.32	0.47 + 0.16	58
Def	2.28 + 0.24	0.65 + 0.22	55
Dro	1.97 + 0.36	0.44 + 0.15	61

Table 2A. Changes between winter 2015 and spring 2016 in C, N and ^{15}N amounts in the main organs of European beech trees (leaf, twigs, dead branches, branches, trunk top, trunk and roots) in the three treatments: control (Co), defoliation (Def) and drought (Dro). Mean ± SE (n = 6) for each organ except dead branches (winter: n= 2 Co; n= 4 Def; n= 5 Dro; spring: n= 5 Co; n=5 Def, n=5 Dro). Lowercase letters indicate significant differences ($p<0.05$) among treatments for a given date and organ. Differences between seasons for a given organ and treatment are presented as *($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). Note: leaf is litter in winter and mature leaves in spring.

Organ	**Treatment**	**Carbon amount (g)**		**Nitrogen amount (g)**		**^{15}N amount (mg)**	
		winter	*spring*	*winter*	*spring*	*winter*	*spring*
Leaf	Co	21.49 ± 6.35	80.25 ± 14.68**b****	0.25 ± 0.08	3.81± 0.63**b*****	0.61 ± 0.19	6.99 ± 1.33**b*****
	Def	8.98 ± 2.12	72.82 ± 11.87**b*****	0.18 ± 0.05	3.64 ± 0.63**b****	0.63 ± 0.19	5.75 ± 1.35**b****
	Dro	5.99 ± 3.07	14.91 ± 4.18**a**	0.06 ± 0.03	0.63 ± 0.03**a****	0.15 ± 0.05	1.62 ± 0.32**a*****
Twig	Co	2.16 ± 0.54	5.35 ± 1.79	0.05 ± 0.03	0.09 ± 0.01	0.14 ± 0.02	0.15 ± 0.04
	Def	5.00 ± 1.65	4.08 ± 0.89	0.10 ± 0.03	0.07 ± 0.01	0.53 ± 0.21	0.13 ± 0.01
	Dro	1.41 ± 0.59	2.16 ± 0.49	0.03 ±	0.05 ± 0.01	0.12 ±	0.12 ± 0.02

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				0.01		0.03	
Branch	Co	84.90 ± 39.21	126.71 ± 31.33**b**	1.36 ± 0.72	1.41 ± 0.32**b**	2.55 ± 0.95	1.79 ± 0.49
	Def	57.27 ± 16.72	80.91 ± 15.22**ab**	1.09 ± 0.40	0.93 ± 0.18**ab**	3.65 ± 1.18	1.29 ± 0.11
	Dro	31.55 ± 9.28	34.67 ± 5.98**a**	0.47 ± 0.14	0.43 ± 0.07**a**	1.52 ± 0.49	0.89 ± 0.23
Dead branch	Co	2.87 ± 1.66	1.16 ± 0.90	0.03 ± 0.01	0.01 ± 0.01	0.03 ± 0.02	0.01 ± 0.01
	Def	0.49 ± 0.28	1.12 ± 0.82	0.01 ± 0.00	0.01 ± 0.01	0.03 ± 0.03	0.04 ± 0.02
	Dro	2.91 ± 2.39	2.04 ± 1.10	0.03 ± 0.02	0.02 ± 0.01	0.05 ± 0.03	0.01 ± 0.01
Trunk Top	Co	16.24 ± 5.03**b**	12.44 ± 0.94**b**	0.23 ± 0.07**b**	0.21 ± 0.02**b**	0.36 ± 0.09**b**	0.34 ± 0.06**b**
	Def	7.21 ± 1.31**ab**	3.46 ± 0.99**a***	0.10 ± 0.01**ab**	0.08 ± 0.02**a**	0.19 ± 0.05**ab**	0.11 ± 0.03**a**
	Dro	2.68 ± 0.85**a**	0.80 ± 0.53**a**	0.04 ± 0.01**a**	0.02 ± 0.01**a**	0.08 ± 0.02**a**	0.02 ± 0.01**a**
Trunk	Co	164.39 ± 34.40	303.05± 59.57**b**	1.51 ± 0.31	2.26 ± 0.46**b**	3.49 ± 0.72**b**	2.69 ± 0.31**b**
	Def	153.59 ± 45.90	178.24 ± 21.47**ab**	1.57 ± 0.45	1.39 ± 0.16**a**	1.45 ± 0.41**a**	1.02 ± 0.13**a**
	Dro	92.49 ± 25.93	147.92 ± 29.15**a**	0.75 ± 0.21	1.23 ± 0.27**a**	1.12 ± 0.28**a**	1.37± 0.36**a**
Root	Co	90.63 ± 23.98	163.33 ± 32.53**b**	0.99 ± 0.30	1.56 ± 0.33	2.33 ± 0.98	1.36 ± 0.20**b**
	Def	71.90 ± 19.10	105.83 ± 13.84**ab**	0.87 ± 0.24	0.98 ± 0.15	0.57 ± 0.20	0.27 ± 0.06**a**
	Dro	41.26 ± 11.36	62.76 ± 11.23**a**	0.56 ± 0.18	0.89 ± 0.12	0.70 ± 0.21	0.65 ± 0.15**a**
Total above-ground	Co	290.12 ± 75.15	528.76 ± 102.83**b**	3.39 ± 1.10	7.78 ± 1.44**b***	7.15 ± 1.61	11.96 ± 2.15**b**
	Def	232.39 ± 66.25	340.44 ± 45.83**ab**	3.04 ± 0.91	6.11 ± 0.88**b***	6.47 ± 1.70	8.02 ± 1.36**ab**
	Dro	136.55 ± 37.08	202.16 ± 37.08**a**	1.38 ± 0.39	2.37 ± 0.45**a**	3.03 ± 0.80	4.03 ± 1.69**a**
Total tree	Co	380.75 ± 99.08	692.09 ± 135.35	4.38 ± 1.39**b**	9.34 ± 1.76**b**	9.49 ± 2.50	13.32 ± 2.25**b**
	Def	304.29 ± 85.33	446.27 ± 59.63	3.91 ± 1.15**ab**	7.09 ± 1.02**ab**	7.04 ± 1.82	8.29 ± 1.38**ab**
	Dro	177.80 ± 48.43	264.93± 47,70	1.96 ± 0.56**a**	3.26 ± 0.53**a**	3.72 ± 0.96	4.67 ± 0.76**a**

Table 2 B. Changes between winter 2015 and spring 2016 in C, N and ^{15}N amounts in the main organs of European beech trees (leaf, twigs, dead branches, branches, trunk top, trunk and roots) in the three treatments: control (Co), defoliation (Def) and drought (Dro). Mean ± SE (n = 6) for each organ except dead branches (winter: n= 2 Co; n= 4 Def; n= 5 Dro; spring: n= 5 Co; n=5 Def, n=5 Dro).

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943 Statistical values (represented as F and P values) for season and treatment effect and their interactions
944 are given for each organ. Note: leaf is litter in winter and mature leaves in spring.

945

Organ	Amount	Season		Treatment		Season x Treatment	
		F_{value}	P_{value}	F_{value}	P_{value}	F_{value}	P_{value}
Leaf	C	40.419	**<0.001**	12.435	**<0.001**	6.4553	**0.005**
	N	62.343	**<0.001**	11.464	**<0.001**	9.4172	**<0.001**
	^{15}N	38.470	**<0.001**	6.592	**0.004**	4.496	**0.020**
Twig	C	1.198	0.283	3.157	0.057	1.695	0.201
	N	0.357	0.555	2.934	0.069	1.394	0.264
	^{15}N	3.208	0.083	3.219	0.054	3.162	0.057
Branch	C	1.491	0.232	5.029	**0.013**	0.357	0.703
	N	0.023	0.882	3.161	0.057.	0.042	0.959
	^{15}N	4.924	**0.034**	1.842	0.176	0.973	0.390
Dead branch	C	0.194	0.665	0.703	0.507	0.303	0.742
	N	0.224	0.641	0.769	0.477	0.226	0.800
	^{15}N	0.726	0.404	0.128	0.880	0.397	0.678
Trunk Top	C	2.986	0.094	16.937	**<0.001**	0.121	0.886
	N	0.947	0.338	19.027	**<0.001**	0.003	0.997
	^{15}N	1.836	0.186	17.708	**<0.001**	0.237	0.791
Trunk	C	5.424	**0.027**	4.438	**0.021**	1.183	0.320
	N	1.720	0.200	3.689	**0.037**	1.068	0.356
	^{15}N	0.955	0.336	13.448	**<0.001**	0.835	0.444
Root	C	6.716	**0.015**	6.897	**0.003**	0.875	0.427
	N	3.062	0.090	2.839	0.074	0.515	0.603
	^{15}N	1.577	0.219	6.269	**0.005**	0.606	0.552
Total above-ground	C	6.688	**0.015**	6.805	**0.004**	0.960	0.394
	N	13.574	**<0.001**	8.396	**0.001**	1.677	0.204
	^{15}N	4.155	**0.050**	8.510	**0.001**	0.966	0.392
Total tree	C	6.700	**0.015**	6.832	**0.004**	0.940	0.402
	N	11.129	**0.002**	7.073	**0.003**	1.258	0.299
	^{15}N	2.025	0.165	8.653	**0.001**	0.416	0.663

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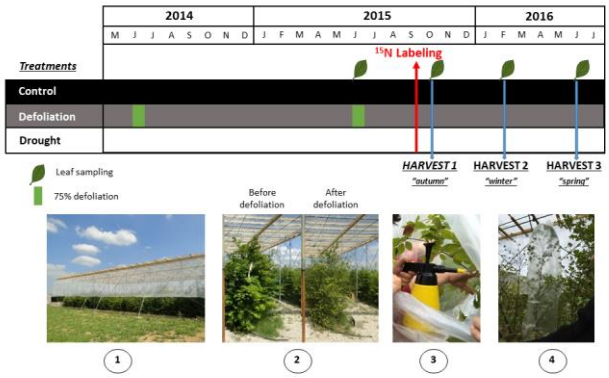
949 **Legend captions for figures**



950

951 **Figure 1.** Schematic representation of the ^{15}N labeling experiment: three treatments were applied
952 from 2014 to 2016: control (Co), defoliation (Def) and drought (Dro). Schedule of the experiment
953 since the onset in 2014 (Photograph 1). The Co and Dro treatments lasted from May 2014 to June
954 2016; 75% of the foliage (green box) was removed once a year, in June 2014 and June 2015
955 (Photograph 2). The foliage was labeled in September 2015 by spraying a ^{15}N-urea solution on the
956 leaves of the crown (Photograph 3). A polyethylene bag was installed over the tree before labeling
957 and remained in place after labeling for a full night; it was then carefully removed the morning after
958 labeling (Photograph 4). We took a first sample one month after labeling (Harvest 1, autumn) to
959 confirm that the tracer had been incorporated into the perennial organs via leaf N resorption. Then,
960 trees were harvested at two key phenological dates, in winter (Harvest 2, in February and in spring
961 (Harvest 3, in June 2016). Leaf sampling was done in June 2015 and at each harvest date.

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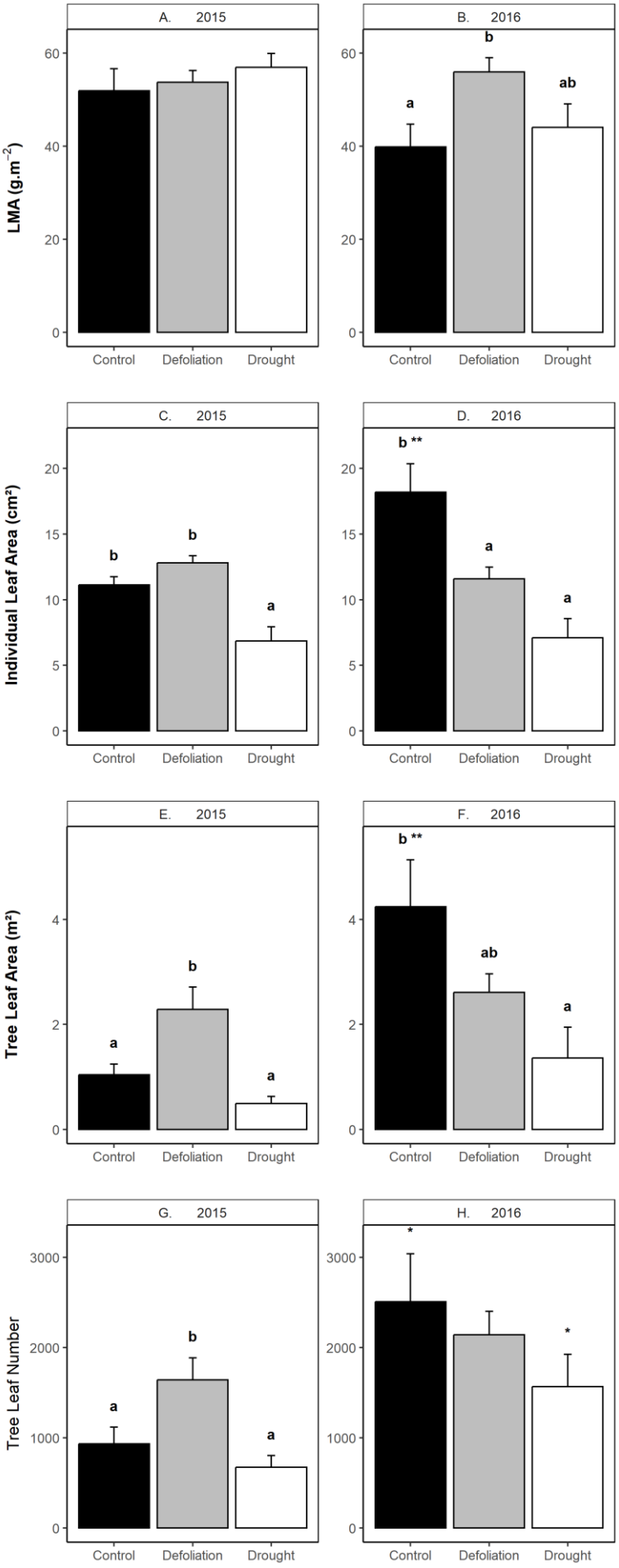

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962

963 **Figure 2.** Leaf characteristics in June 2015 and June 2016 of the European beech trees submitted to
964 the three treatments: Control (Co), defoliation (Def) and drought (Dro). Leaf mass area (LMA; A),
965 individual leaf area (B), total leaf area (C) and number of leaves per tree (D). "Year effect" is indicated
966 by an asterisk if a significant difference was found between years: *($p<0.05$), ** ($p<0.01$) or ***
967 ($p<0.001$). Mean ± SE. n=6. Different letters indicate a significant difference ($p<0.05$) among
968 treatments for each year.
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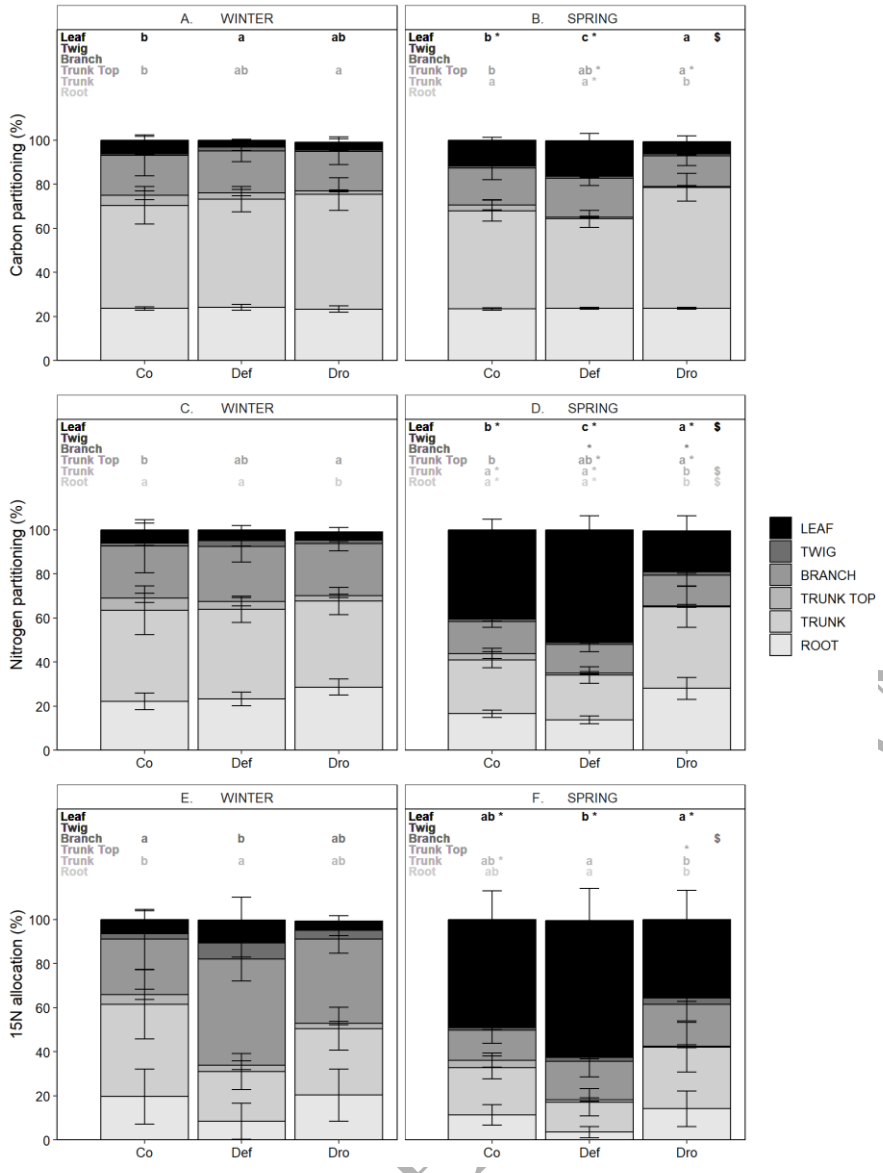

Figure 3. Changes in C and N partitioning (%) and ^{15}N allocation (%) among organs of European beech trees between winter (A, C, E) and spring (B, D, F). Each line of letters indicates significant differences between treatments for a given organ: lower light grey letters for the roots, trunk and trunk top; middle grey letters for branches and twigs; and upper black letters for leaves. Mean ± SE, n = 6 for each organ. Note that leaves were in the litter in winter and were mature leaves removed from the tree in spring.

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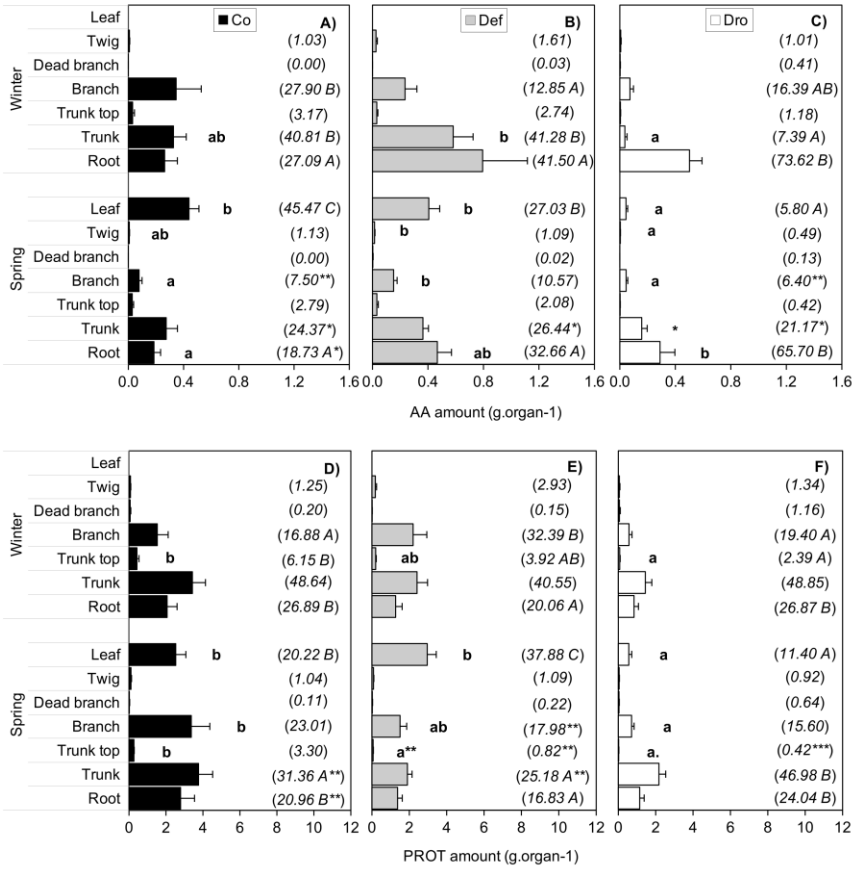

977

978 **Figure 4.** Changes between winter and spring amino acid (A to C) and soluble protein (D to F)
979 amounts (mg. organ^{-1}) and in their partitioning (%) among organs for European beech trees submitted
980 to three treatments: Control (Co), defoliation (Def) and drought (Dro). Note that for the leaf organ,
981 leaves were in the litter in winter and were mature leaves removed from the tree in spring. The
982 numbers in italics and brackets correspond to the % of amino acids or soluble proteins partitioned to
983 each organ. Uppercase letters indicate significant differences ($p<0.05$) in partitioning among
984 treatments for a given date and organ. Lowercase letters indicate significant differences ($p<0.05$) in
985 amounts among treatments for a given date and organ. "Season effect" is indicated by an asterisk if a
986 significant difference was found between winter and spring: *($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$).
987 Values are mean ± SE, n = 6 for each organ except dead branches (winter: n= 2 Co; n= 4 Def; n= 5
988 Dro; spring: n= 5 Co; n=5 Def; n=5 Dro).

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