SHORT-TERM NITROGEN DYNAMICS ARE IMPACTED BY DEFOLIATION AND DROUGHT IN FAGUS SYLVATICA L. BRANCHES

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6. SHORT-TERM NITROGEN DYNAMICS ARE IMPACTED BY DEFOLIATION AND DROUGHT IN *FAGUS SYLVATICA* L. BRANCHES

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Running head: Nitrogen dynamics in beech branches under defoliation and drought.

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ABSTRACT

The predicted recurrence of adverse climatic events, like droughts, which disrupt nutrient accessibility for trees, could jeopardize the nitrogen (N) metabolism in forest trees. Internal tree N cycling capacities are crucial to ensuring tree survival but how the N metabolism of forest trees responds to intense, repeated environmental stress is not well known. For two years, we submitted 9-year-old beech (*Fagus Sylvatica* L.) trees to either a moderate or a severe prolonged drought or a yearly removal of 75% of the foliage to induce internal N cycling changes. During the 2nd year of stress, in spring and summer, we sprayed ^{15}N-urea on the leaves (one branch per tree). Then, for 14 days we traced the ^{15}N dynamics through the leaves, into foliar proteins and into the branch compartments (leaves and stems segments), as well as its long-distance transfer from the labeled branches to the tree apical twigs. Defoliation caused a short- and mid-term N increase in the leaves, which remained the main sink for N. Whatever the treatment and the date, most of the leaf ^{15}N stayed in the leaves and was invested in soluble proteins (60 to 68 % of total leaf N). ^{15}N stayed more in the proximal part of the branch in response to drought compared to other treatments. The long-distance transport of N was maintained even under harsh drought, highlighting efficient internal N recycling in beech trees. Under extreme constraints creating an N and water imbalance, compensation mechanisms operated at the branch level in beech trees and allowed them (1) to maintain leaf N metabolism and protein synthesis and (2) to ensure the seasonal short- and long-distance transfer of recycled leaf N even under drastic water shortage conditions.

Keywords: soil water deficit, defoliation, *Fagus sylvatica* L., stable isotope labeling**,** nitrogen cycle, protein

6.1. Introduction

Nitrogen (N) is a fundamental macronutrient, the availability of which determines growth, development and productivity in plants (Finzi *et al.*, 2007, Zhang *et al.*, 2016). The influence of soil N availability on tree growth has been widely studied (Canham *et al.*, 1999, He *et al.*, 2009, Simon *et al.*, 2011 and 2017). Trees acquire N from the soil through their roots and associated ectomycorrhizae in both inorganic (nitrate and ammonium) and organic (amino acids, peptides) forms, and also from the atmosphere through N deposition on leaves (Masclaux-Daubresse *et al.*, 2010, Nair *et al.*, 2015). Trees are able to store and remobilize N along the seasons, and this internal N recycling is also an important source of N for their development (Staswick 1994, Neilsen *et al.*, 1997). In spring, in deciduous trees, root N uptake is still limited and winter stored N sustains the growth of the new foliage (Millard and Grelet, 2010). As examples, in young apple trees, Cheng and Fuchigami (2002) showed that about 50% of a tree's N content was remobilized to support new shoot and leaf growth, whatever the tree's N status; in mature sessile oak, El Zein (2011) showed that, during the two weeks following budburst, remobilized N contributed to more than 90% of the total N in twigs and growing leaves. This high investment of stored N in new tissues is key to supporting photosynthetic processes. On one hand, leaf photosynthetic capacity has been shown in many plant species to be highly correlated with leaf N content, since the N invested in the Calvin cycle and thylakoid proteins represents up to 75% of total leaf N (Evans and Seemann, 1989). On the other hand, the primary assimilation of inorganic N requires carbon skeletons and an energy supply (Dong *et al.*, 2002), which reflects the close interrelation between carbon and nitrogen metabolisms in plants.

In the context of climate change, various studies have pointed out the threat of adverse climatic events on forest vulnerability (Anderegg *et al.*, 2015); in particular, extreme drought events have been identified as a major cause of forest dysfunction (Bréda *et al.*, 2006, Bréda and Peiffer, 2014). Water stress has complex and interrelated consequences on water and carbon metabolisms (McDowell *et al.*, 2011). Mineral nutrients, especially N, are also likely to be impacted by recurrent droughts, predicted in climate-change scenarios, which will decrease both nutrient availability in the soil (notably by decreasing microbial activity) and nutrient/water uptake by the fine roots (Kreuzwieser and Gessler, 2010). Fotelli *et al.*, (2002, 2004) used stable isotopic methods such as ^{15}N labeling, to study the physiology of N uptake from the soil and its transport through trees from roots to shoots; they found a net reduction in

N uptake by trees in the case of soil water shortage. In temperate forests, a direct loss of available N can be also observed in the case of tree defoliation (Lovett *et al.*, 2002), which may be caused by various biotic and abiotic disturbances such as insects, frost, wind or hail (Ozolincius and Stakenas, 1996, Lorenz and Becher, 2012).

In the present study, we investigated the effects of repeated extreme drought and defoliation events on metabolism and the export of N within the leafy branches of 9-year-old beech trees (*Fagus sylvatica* L.), a deciduous broadleaf species widespread in Europe, and which is known to be sensitive to severe drought (Peuke *et al.*, 2002). We hypothesized that the N metabolism in the beech branches would be affected under defoliation, as shown for some forest tree species (Millard *et al.*, 2001), and under extreme drought, which has rarely been investigated (Jordan, 2015, Gessler *et al.*, 2016). Gessler *et al.*, (2016) suggested that reduced nutrient availability during drought induces a negative feedback on the carbon balance (by reducing N supply for the photosynthetic apparatus). Any reduction in N uptake induced by drought during the vegetative season would also affect tree N metabolism, more particularly, N assimilation in the leaves. Defoliation could also create a carbon and N limitation that would affect N assimilation in the remaining leaves.

In our experiment, planted beech trees were submitted for two years to extreme stresses (yearly manual defoliation or prolonged droughts) likely to induce changes in the trees' internal N cycle and eventually lead to an N shortage. The second year of stress, a short-term ^{15}N labeling pulse was applied on the leaves of one branch per tree i) at the end of spring N remobilization and ii) in summer, after the 2nd manual defoliation was done. The choice of these two periods was made to reveal i) whether a constraint applied for one year (drought or defoliation) would modify the N functioning in a given branch in the spring of the second year, and ii) how prolonged or repeated constraints impact summer N functioning. We evaluated whether the ^{15}N allocation between the leaves on the one hand and the bearing branch (short-distance transport) on the other hand was disturbed. In addition, we investigated whether ^{15}N allocation was reflected in the local metabolism priorities of source leaves, and how much stress conditions (i) altered soluble protein synthesis (protein concentrations are thought to increase under harsh conditions), and/or (ii) modified the N export from labeled leaves toward the apical shoot.

6.2. Materials and methods

6.2.1. Experimental design and growth conditions

The study was conducted on European beech trees. In 2006, beech seeds were collected in several forests in the Lorraine region of France and sown in 2007 in biodegradable horticultural pots made of wood fiber and filled with a peat and sand mixture. The seedlings were grown for one year in a nursery (INRA Grand-Est Nancy, France). In 2008, about 1000 of the seedlings were transplanted and grown for 7 more years in open ground at the INRA Grand-Est nursery (Champenoux, France, 48°75'N, 6°34'E, 229m asl). In 2014, a rain exclusion system was built above the 8-year-old trees: a semi-rigid structure supporting a transparent roof built with polycarbonate sheets and nets installed around the roof to intercept lateral rain. Light intensity, air temperature and vapor pressure deficit were monitored below the roof. The trees under the roof were subjected to four different treatments for two years (2014, 2015): (1) control (C) in which the trees were regularly irrigated; (2) defoliation (D) in which the trees were submitted to a yearly defoliation and regularly irrigated: manual defoliation of the trees in treatment D was done each year in June; 75% of the total foliage was removed and the removal was homogeneously distributed throughout the tree crown; (3) moderate drought (MD) and (4) severe drought (SD), where the trees were submitted to two levels of soil water deficit. The two drought stress levels were not designed to realistically simulate a climate change scenario, but rather to create drought conditions that were so unfavorable that they would likely cause beech mortality. In fact, lateral rain entering under the roof created some variability in soil water status in the drought treatment at the time of labeling and this allowed us to select trees with contrasting levels of water stress. The soil in the drought treatments was isolated by a rigid waterproof plastic sheet 1.80 meters depth buried vertically around the area. The water status of the seasonal sub-sets of trees in each treatment (24 trees per treatment) was checked by measuring pre-dawn water potential in twigs (ψ_{pd}) at day of year (DOY) 150, 176 and 198 in 2015. We sampled the twigs (one per tree) before sunrise and performed the ψ_{pd} measurement with a pressure chamber (PMS Instruments, Albany, OR, USA).

6.2.2. Soil characteristics and soil water measurements

The studied site was characterized by 60cm-deep homogenous soil with an average texture (Silt: 61 ± 1.28%; Clay: 27 ± 0.98%; Sand: 12 ± 0.66%), a pH comprised between 7.5 and 8, an

organic matter content between 12.1 and 14.9 $g.kg^{-1}$ (E Silva 2010) and a total N comprised between 0.54 to 0.87 $g.kg^{-1}$. Below 60cm, the grey marl of the Jurassic inferior (Lotharingian) era was characterized by a swelling heavy clay soil with a relatively high bulk density.

We used neutron probes (TROXLER TX 4301, Research Triangle Park, NC, USA) to measure the volumetric water content of the soil. Three neutron probe access tubes (aluminum, closed at their base) were installed in each of the four treatment areas in order to quantify water content at different depths: two ranged from 0-1m in depth and one ranged from 0-1.6m. During the growing season, measurements were carried out every two weeks. Counts were logged every 10 cm for the upper 100 cm, and every 20cm below that.

For each depth i (thickness t_i), Total Available Water soil Content (TAWC in mm) was calculated by estimating the characteristic points from pedotransfer classes for gravimetric soil moisture at field capacity (θ_{fc}) and gravimetric soil moisture at wilting point (θ_{wp}). The characteristic points were checked and adjusted with probe measurements, during winter for volumetric soil moisture at field capacity and during summer for volumetric soil moisture at wilting point. Soil bulk density was assessed with the cylinder method. Relative Extractable soil Water (REW in %) was calculated according to Bréda *et al.,* (1995) as follows:

$$REW=100* \frac{TAWC-R}{TAWC} \quad (12)$$

where R is the actual volumetric soil water content in mm, and total soil extractable water content down to 1.60m is estimated to 310 mm.

The soil in the C and D treatments was irrigated regularly throughout the experiment with an automatic drip watering system which delivered between two and four liters per tree two to three times a week. We adjusted the amount of the water according to the REW measurements in order to avoid any water shortage (REW >0.4), with 40% of the REW corresponding to the critical threshold where trees start to avoid water loss by closing their stomata (Granier *et al.,* 1999).

6.2.3. Choice of the branch

One branch per tree was selected at chest height for the ^{15}N labeling experiment. Branch diameter and the distance from the base of the branch to the apex of the tree were measured as were the number of leaves on the branch and individual and total leaf area. A significant global treatment effect was observed on the number of leaves and individual leaf area. Total leaf area

was influenced by season ($p<0.01$) with a significant treatment x season interaction ($p<0.01$). Defoliation did not decrease branch growth in 2014, but in 2015, branch diameter was significantly reduced in the defoliated trees. In the spring 2015, after the 2014 defoliation, the number of leaves on defoliated branches was similar to the number on the water-stressed and control branches. In the summer 2015, the selected branches in the D trees were thicker (diameter significantly larger) than the ones in the C trees and also contained more leaves than controls before 75% of defoliation.

6.2.4. Foliar ^{15}N labeling procedure

Labeling experiments were performed at two dates (**Figure VI.1**): 1) in spring, at the end of May 2015 (DOY: 148) when the leaves of all the trees were fully expanded, and 2) in summer, in July 2015 (DOY: 187) one month after defoliation had been performed on the D trees and when radial growth was maximal. At each date, spring and summer, a set of 12 trees per treatment (C, D, MD and SD) were randomly chosen for labeling (giving 48 trees for each date and 24 trees per treatment for the whole labeling experiment).

On each tree, a branch bag made of polyethylene was placed over the labeled branch to isolate it from its local environment. In the late afternoon, an aqueous solution of ^{15}N-urea was sprayed inside the bag onto the leaves with a hand sprayer (Zeller *et al.,* 1998). Urea is considered to be the most suitable form of N for foliar application due to its non-polarity, rapid absorption, low phyto-toxicity and high solubility (Knoche *et al.,* 1994, Zeller *et al.,* 1998). Leaves absorb N in urea much faster than N in mineral form (Zeller *et al.,* 1998) and urea N is rapidly converted into amino acids used for leaf protein synthesis or for N export (Dilley *et al.*,1961, Dong *et al.,* 2002). The ^{15}N-urea solution (10.4 atom%, 5.0 g.L^{-1}) was sprayed in a fine mist, which limited the formation of drops and ensured a homogeneous labeling of the leaves. After the labeling, the branch bag was kept on all night, then very carefully removed the next morning to avoid any contamination among trees. The timing of the foliar labeling is summarized in **Figure VI.1**.

6.2.5. Sampling protocol

Leaf and twig samples were collected from 12 of the selected trees per treatment before labeling to determine their natural ^{15}N baseline abundance. After ^{15}N labeling, leaf material from each labeled branch was sampled over a chase period of 14 days. At three sampling dates (day 0.5, 1, 2), five small leaf disks (38 mm²/disk, 1 disk per leaf and per date) were removed with a

hole-puncher from five randomly selected leaves along the labeled branch. For the three dates, this sampling procedure was repeated on the same set of five leaves. At day 4, the remaining part of the five punched out leaves was taken. One additional leaf was also collected from six trees per treatment to assess total protein quantification and ^{15}N assimilation into proteins. We chose day 4 as a sampling date because the turnover of foliar proteins, especially Rubisco, is rapid enough to allow an incorporation of ^{15}N into foliar proteins within that time, as shown for ^{15}N labeled rice leaves by Suzuki *et al.*, (2001). At day 7, a second set of five leaves per branch was hole-punched as described above. At day 14, the whole branch (both wood and remaining leaves) plus several leaves from the terminal apical twig of the selected trees were harvested (**Figure VI.1**).

Each sampled branch was split by annual growth unit [current year (Y), 1-year-old (Y-1) and older (<Y-1)]. All samples were gently and carefully washed in distilled water, frozen in liquid nitrogen and stored at -80°C. The samples were then freeze-dried [Dura-Top (r), Dura-Dry (r), FTS Systems (r), Stone Ridge, NY, USA], weighed and ground into a fine powder in a ball mill (CEPI SODEMI CB2200, Cergy, France).

6.2.6. Foliar protein analysis

Soluble proteins were extracted from each leaf powder sample: 20 mg of leaf powder mixed with a ball mill (Retsch MM 301, Gmbh & Co, Germany) in a micro-tube with 500 µL of extraction buffer [62.5 mM Tris HCl pH 6.8, 2 % (v/v) SDS, 10 % Glycerol and 28 mM DTT] twice for 45 seconds. The samples were centrifuged twice at 32,000 g for 20 min (17°C). Protein concentration from the solubilized pellet was determined by RC-DC Protein assay (Biorad RC DC Protein Assay 500-0121). Soluble proteins were precipitated with four volumes of cold acetone and 28 mM of DTT and stored at -20°C overnight. The following day, proteins were collected by centrifuging at 16,000g, 4°C for 10 min, then washed four times with 1.5 ml of cold acetone and 28 mM of DTT. After each washing, the mixture was stored 30 min at -20°C, then centrifuged at 16,000 g, 4°C for 15 min. The final pellet was air-dried at room temperature. Aliquots (1 mg) from the protein pellets were weighed into tin capsules for isotopic analyses.

6.2.7. Elementary and isotopic analyses

Total N concentrations (% of dry matter, DM) and ^{15}N isotopic abundance (atom%) of the wood and leaf samples were measured with an elemental analyzer (NA 1500 NCS, Carlo Erba, Milan, Italy) coupled to a Delta-S isotopic ratio mass spectrometer (Finnigan-Mat, Thermoquest Corp.,

San Jose, CA, USA). Analyses were carried out at the SilvaTec platform (UMR Silva, INRA Grand Est-Nancy, France).

6.2.8. Isotopic calculations

The isotopic abundance for N in atom% (A_N %) is defined as

$$AN\% = \frac{15N}{14N+15N} * 100 \quad (13)$$

The enrichment of ^{15}N (atom %) in each compartment (wood, leaves) is defined as

$$15Nexcess = AN\%(\text{labeled compartment}) - AN\% (\text{unlabeled compartment}) \quad (14)$$

where $A_N\%_{\text{ labeled compartment}}$ is the ^{15}N abundance of the labeled compartment, $A_N\%_{\text{ unlabeled compartment}}$ is the natural ^{15}N abundance of the unlabeled compartment, with a $A_N\%_{\text{ unlabeled compartment}}$ of about 0.368306888 ± 0.00306 atom% for leaves and 0.370893333 ± 0.00127829 atom% for wood. The same calculations were carried out for ^{15}N atom% in leaf proteins. The natural ^{15}N abundance [atom% of the unlabeled proteins] was 0.36606 ± 0.00011.

The concentrations of ^{15}N (mg.100g^{-1} DM) incorporated by labeling in the Dry Matter (DM) of the leaf or in the leaf protein pool was calculated as:

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

where [N] is the N concentration (mg.100mg^{-1} DM) of the leaf or of the leaf protein extract.

The ^{15}N amount (g) incorporated by labeling in each compartment was calculated as

$$^{15}N \text{ amount} = \frac{^{15}N concentration}{1000} * \frac{DM}{100} \quad (16)$$

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

6.2.9. Total N and ^{15}N allocation in branches and ^{15}N partitioning in leaf proteins

Allocation of total N or ^{15}N is related to the distribution of N or ^{15}N within the different parts of the labeled branches (Dickson 1989). Allocation of N and ^{15}N represented the ratio (%) of the amount of N or ^{15}N incorporated into a given branch compartment (wood, leaf) relative to the total amount of N or ^{15}N incorporated in the corresponding whole branch.

$$Nor^{15}Nallocation(\%) = \frac{Nor^{15}Namountofthebranchcompartment}{Nor^{15}N\text{amount of the whole branch}} * 100 \quad (17)$$

Partitioning of N or ^{15}N represented the ratio (%) of the amount of N or ^{15}N incorporated into the leaf proteins relative to the total N or ^{15}N amounts incorporated into the leaf DM.

$$Nor^{15}Npartitioning(\%) = \frac{Nor^{15}Namount \in leafproteins}{Nor^{15}N\text{amount of the whole leaf}} * 100 \quad (18)$$

6.2.10. Statistics

We used general linear mixed-effect models to compare the seasonal dynamics of predawn water potential, tree growth, N and ^{15}N concentrations and N allocation between dates and treatments. Normality and homoscedasticity of the standardized residuals were graphically checked using quantile-to-quantile and residual-vs-predicted plots. When the distribution was not normal, a logarithmic or an arcsin (root square/100) transformation was used. Individual tree was a random factor while treatment and date of sampling were explanatory fixed factors in the models. A Tukey test was performed as a post-hoc analysis. Data were analyzed with the R software package (http://www.r-project.org, version 3.2.2, 2016-10-31).

6.3. Results

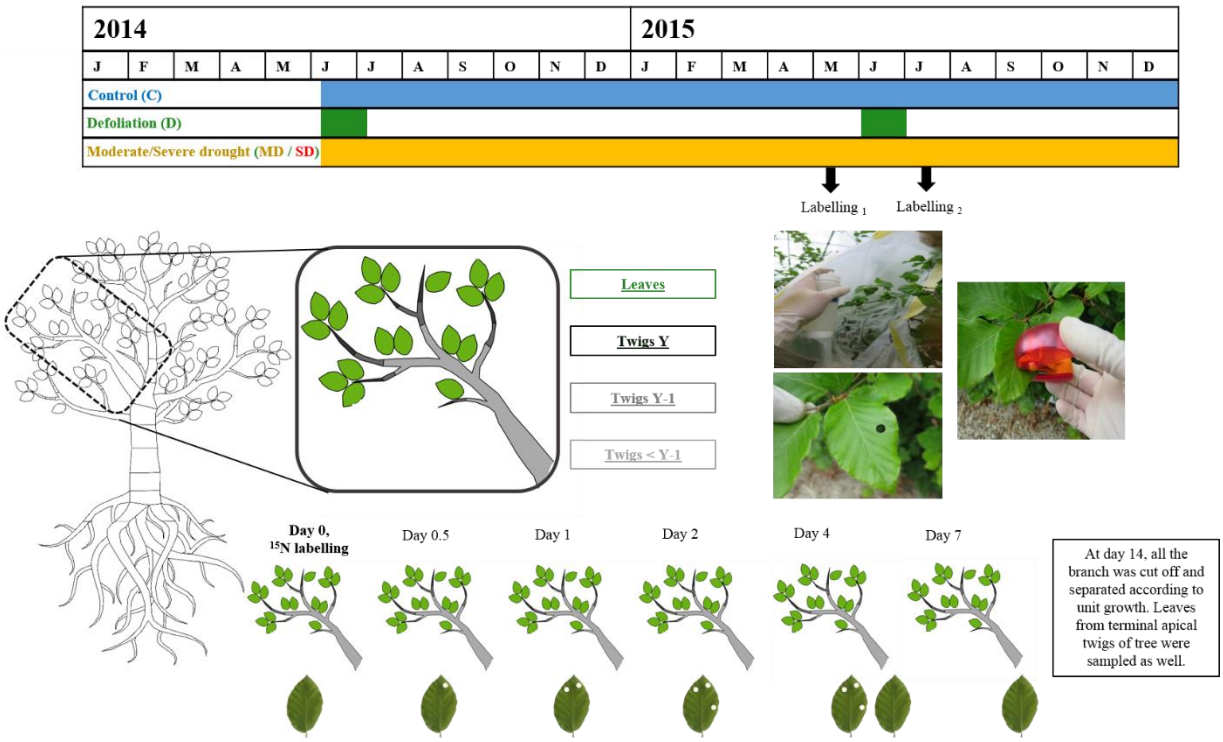

Figure VI.1. Schematic representation of the ^{15}N labeling experiment. Experimental schedule (top): Four treatments were applied over two years (2014 and 2015) with control (C), moderate (MD) and severe soil water deficit (SD) and defoliation (D) treatments (a 75% removal of the foliage, grey box). One branch per tree was labeled with enriched ^{15}N–urea on one set of trees in spring (LAB 1) and on a second set of trees in summer (LAB 2). Sampling procedure (bottom): At day 0, unlabeled leaves were taken to determine N% and baseline ^{15}N natural abundance. At day 0.5, a set of five leaves was hole-punched, then punched again at day 1, 2 and 4. At day 4, one intact leaf per tree was also sampled to estimate ^{15}N assimilation into foliar proteins. At day 7, another set of five leaves was hole-punched. At day 14, all the leaves and twigs from the labeled branch were harvested. Twigs were analyzed by annual growth unit (Y, Y-1 and <Y-1, where Y is year). In addition, leaves from the apical twigs were sampled to assess the long-distance transport of ^{15}N from the labeled leaves.

6.3.1. Changes in soil and twig water content

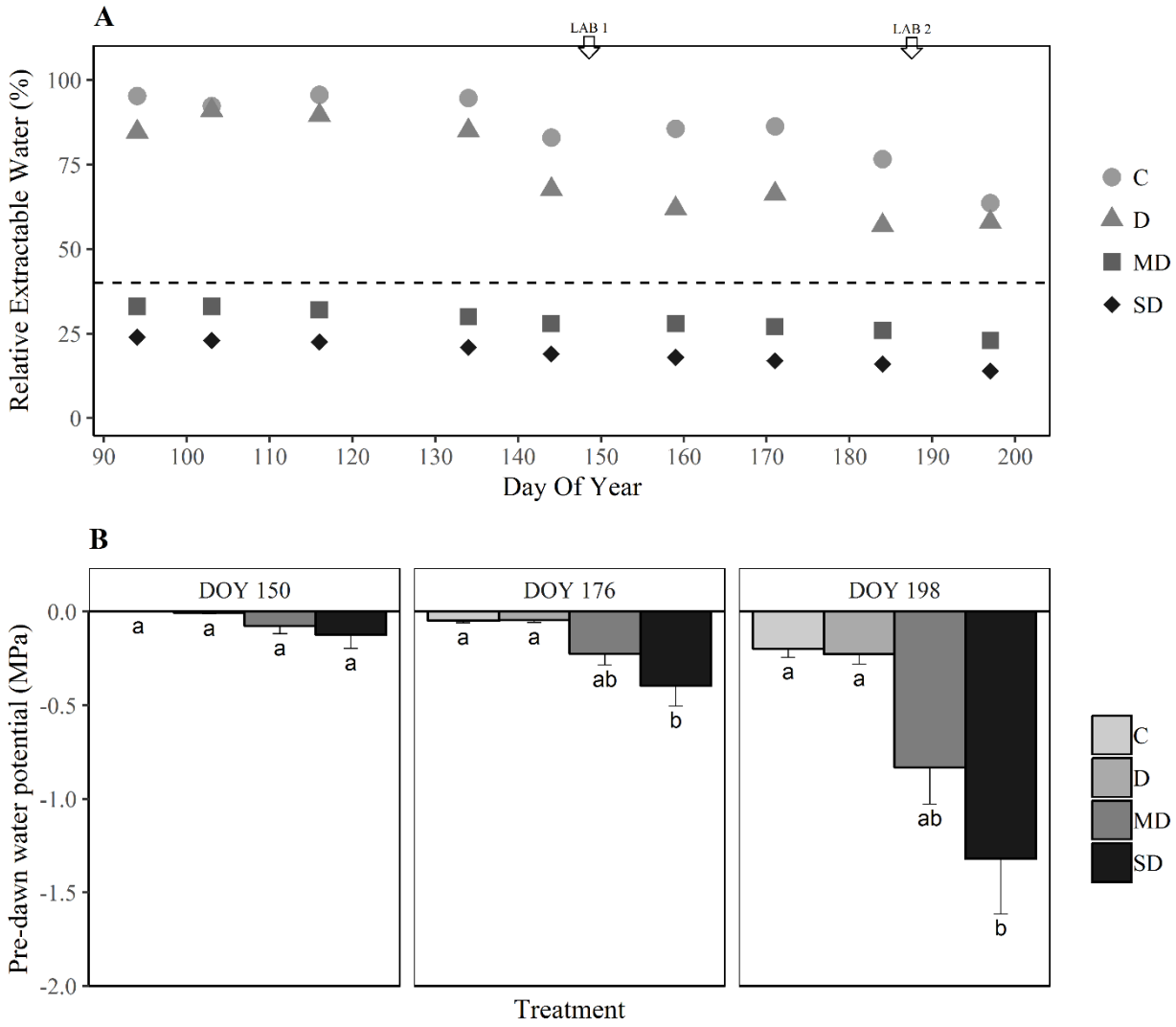

Figure VI.2. Seasonal dynamics for soil Relative Extractable Water (REW) during the growing season of 2015 (A) in 9-year-old beech trees, and average pre-dawn water potential for twigs at the time of the two labeling experiments (B) at days of year (DOY) 150, 176 and 198 in control (C), defoliation (D) and moderate (MD) and severe soil water deficit (SD) treatments. In A, grey arrows indicate the dates of the two labeling experiments (LAB1 and LAB2) and the dashed line indicates the threshold value of REW below which stomatal conductance is impacted, according to Granier et al., (1999). In B, different letters indicate a significant difference ($p<0.05$) in pre-dawn twig water potential between treatments for a given date; mean, ± SE; n=24.

The seasonal monitoring of the relative extractable water (REW) in the soil showed a progressive increase in the soil water deficit parallel to a continuous decrease of REW during the growing season in both drought treatments (MD and SD, **Figure VI.2.A**). In both MD and SD treatments, REW was below 0.4 for the duration of the experiment, whereas REW for the irrigated control (C) and defoliated (D) treatments remained above 0.4. As a result of the progressive seasonal soil water depletion, pre-dawn water potential (ψ_{pd}) in twigs decreased from -0.2 MPa (DOY 150) to -0.9 MPa for MD, and to -1.2 MPa for SD (DOY 198); ψ_{pd} always remained above -0.2MPa in C and D trees (**Figure VI.2.B**). Average pre-dawn twig water potential for the severe drought treatment (SD) was significantly lower ($p<0.05$) than the values for the C and D treatments at DOY 176, and pre-dawn twig water potential in both the MD and SD treatments were lower than the C and D trees at DOY 198.

6.3.2. Leaf N concentrations and ^{15}N partitioning in leaf proteins after a 4-day chase period

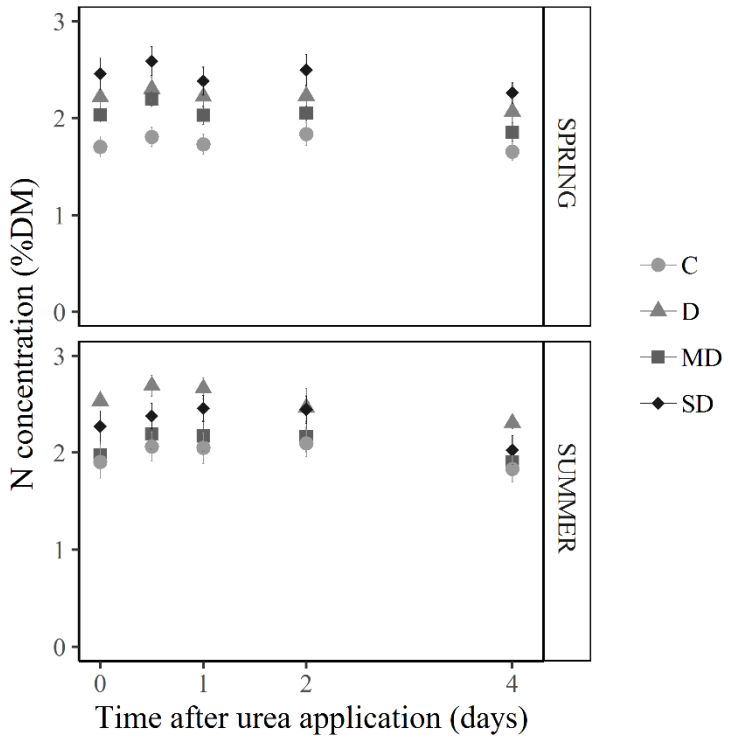

Figure VI.3. Nitrogen (N) concentrations (% DM) in leaves collected on the labeled branches of 9-year-old beech trees in spring (top) and summer (bottom) at days 0.5, 1, 2 and 4 after labeling in the four treatments: control (C), defoliation (D), moderate (MD) and severe soil

water deficit (SD). In each season, a set of 12 trees per treatment was used and one branch per tree was labeled. Values are mean ± SE, n=12.

Leaf N concentrations did not vary significantly before and after ^{15}N-urea labeling (**Figure VI.3**), with values comprised between 1.8 and 2.5 % DM. Thus, the application of ^{15}N-urea did not have any fertilizing effect. Regardless of treatment and season, ^{15}N concentrations in the leaves increased significantly with time until day 4, then stabilized (**Figure VI.4**). For a given treatment, ^{15}N concentrations in the leaves were not significantly influenced by season, with the exception of MD where ^{15}N concentrations during the chase period were significantly higher in summer than in spring.

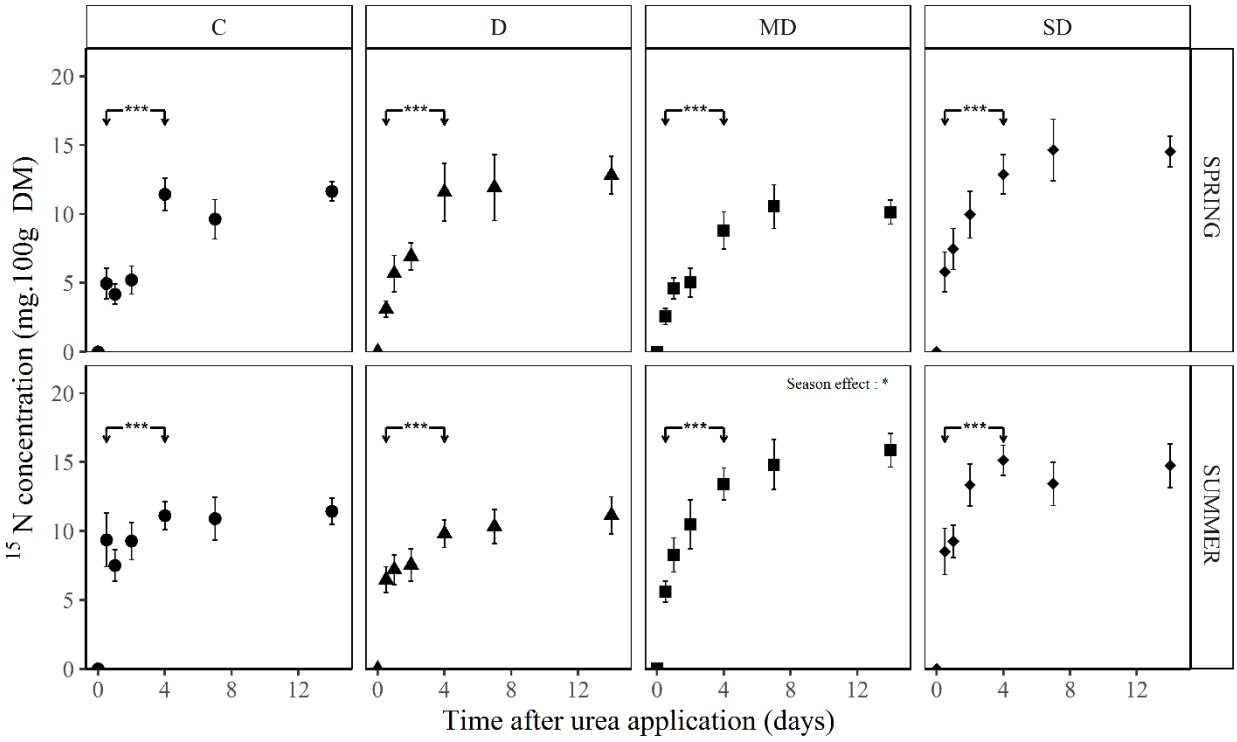

Figure VI.4. Dynamics of ^{15}N concentrations (mg.100g-1 DM) in leaves of 9-year-old beech trees in spring (top) and summer (bottom) for 14 days after labeling in the control (C, disc), defoliation (D, triangle), moderate (MD, square) and severe soil water deficit (SD, diamond) treatments. The same five leaves from each labeled branch were hole-punched at days 0.5, 1 and 2. Then, these leaves were harvested at day 4. Five new leaves were then chosen along the labeled branch and were hole-punched at day 7. At day 14, all the foliage remaining on the branch was harvested. The same protocol was applied for both seasons (spring and summer). The effect of time after urea application was calculated only when the same leaves

were used from day 0.5 to day 4 (ns: p > 0.05, *: p < 0.05, **: p< 0.01, ***: p< 0.001). We tested the effect of season between spring and summer and significant differences were noted "season effect". Values are mean ± SE; n=12.

Total N and ^{15}N in leaf proteins were assessed 4 days after labeling (**Table VI.1**). There was a global effect of treatment on total leaf N (*p<0.001*) and on total N in leaf proteins (*p<0.001*) but no effect on the incorporation of ^{15}N into leaf proteins (**Table VI.1**). No seasonal effect was observed on total leaf N or total N in leaf proteins, whereas a significant seasonal effect (*p<0.01*) was noted on ^{15}N in proteins and on ^{15}N partitioning (*p<0.01*). In spring and summer, both total N concentrations and N concentrations in leaf proteins were higher in D and SD leaves than in C and MD leaves. N partitioning values indicated that most of the leaf N was invested in soluble proteins (60 to 68 % of total leaf N) without any significant treatment effect. After a 4-day chase period, the proteins were enriched in ^{15}N and there was no statistical difference among the ^{15}N concentrations in proteins in response to treatment or season, except for the MD trees where a slight seasonal effect was noted, with no season x treatment interaction (**Table VI.1**).

Table VI.1. Concentrations of total nitrogen (N) and ^{15}N in mature leaves, N and ^{15}N in leaf proteins and N or ^{15}N portioning (the part of protein concentration in total N or ^{15}N concentration) in 9-year-old beech trees 4 days after ^{15}N labeling. Labeling experiments were conducted in spring and summer for the four treatments: control (C), defoliation (D), moderate soil water deficit (MD) and severe soil water deficit (SD). Different letters indicate a significant difference between treatments for a given date (p<0.05). "Season effect" is indicated by an asterisk if a significant difference was found between spring and summer. Values are mean ± SE, n=6 except for C and D trees in summer where 1 value is missing, then n=5. Statistical values (represented as F and P values) of the season and treatment effect or the interaction between season and treatment are given for each variable.

		N total (g.100g^{-1} DM)		N in proteins (g.100g^{-1} DM)		N partitioning (%)		^{15}N total (mg.100g^{-1} DM)		^{15}N in proteins (mg.100g^{-1} DM)		^{15}N partitioning (%)	
Season	Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Spring	C	1.65	0.10^{b}	1.02	0.11^{b}	60.1	3.1	8.38	2.46^{ab}	2.74	1.06	37.5	5.5
	D	2.32	0.02^{a}	1.50	0.07^{a}	64.8	2.6	15.28	3.06^{a}	5.62	1.12	37.8	5.9
	MD	1.86	0.15^{ab}	1.10	0.06^{b}	59.7	2.6	7.79	1.55^{b}	2.24	0.45	30.1	3.1
	SD	2.30	0.15^{a}	1.48	0.05^{a}	65.7	3.6	10.68	2.09^{ab}	5.29	1.37	47.4	8.10
Summer	C	1.91	0.23	1.15	0.15^{b}	64.9	2.3	12.71	1.57	5.10	0.49	44.7	2.6^{b}
	D	2.39	0.05	1.55	0.07^{a}	63.3	2.2	10.08	2.22	6.85	2.01	73.1	10.9^{a*}
	MD	1.99	0.09	1.19	0.06^{b}	60.2	3.3	14.08	2.23	5.71	0.73*	43.2	6.0^{b}
	SD	1.97	0.17	1.31	0.08^{ab}	68.1	5.1	14.63	1.47	5.48	0.97	41.1	10.0^{b}

Effect	F $_{value}$	P $_{value}$	F $_{value}$	P $_{value}$	F $_{value}$	P $_{value}$	F $_{value}$	P $_{value}$	F $_{value}$	P $_{value}$	F $_{value}$	P $_{value}$
Season	0.011	0.916	0.455	0.504	1.321	0.258	1.852	0.181	9.850	0.003	8.211	0.010
Treatment	6.674	0.001	16.15	<0.001	1.703	0.183	0.853	0.473	3.091	0.039	1.747	0.111
Season * Treatment	2.017	0.128	2.095	0.117	0.210	0.888	3.210	0.033	1.148	0.348	3.239	0.057

In spring, 30% to 47% of the total leaf ^{15}N was incorporated in leaf proteins, without any significant treatment effect. A global seasonal effect ($p<0.01$) was noted, resulting in a general increase of ^{15}N partitioning in leaf proteins in summer, without any season x treatment interaction. In summer, interestingly, ^{15}N partitioning in the proteins of D leaves was significantly higher (73% of the total ^{15}N) than ^{15}N partitioning in the proteins of MD and SD leaves (43 and 41% of the total ^{15}N respectively).

6.3.3. Leaf and proximal branch N concentrations at the end of the chase period

Table VI.2. Concentrations of nitrogen (N, % DM) in leaves and twigs divided by annual growth unit (Y, Y-1 and <Y-1, where Y is year) in 9-year-old beech trees 14 days after ^{15}N labeling. Labeling experiments were conducted in spring and in summer for the four treatments: control (C), defoliation (D), moderate soil water deficit (MD) and severe soil water deficit (SD). Different letters indicate a significant difference between treatments for a given date ($p<0.05$). Asterisks in summer indicate a significant difference between seasons for a given treatment. Values are mean ± SE; n=12. Statistical values (represented as F and P values) for season and treatment effect and their interactions are given for each compartment.

		Leaves		Twigs Y		Twigs Y-1		Twigs < Y-1	
Season	Treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Spring	C	1.66	0.10^{c}	0.86	0.03	0.41	0.03	0.46	0.03
	D	1.97	0.10^{ab}	0.85	0.05	0.51	0.02	0.45	0.06
	MD	1.81	0.09^{bc}	0.85	0.04	0.5	0.02	0.38	0.03
	SD	2.13	0.09^{a}	1.02	0.06	0.68	0.07	0.45	0.03
Summer	C	1.83	0.10^{b}	0.61	0.04^{a*}	0.44	0.06	0.37	0.05
	D	2.28	0.12^{a}	0.79	0.03^{ab}	0.56	0.03	0.4	0.03
	MD	1.86	0.10^{b}	0.81	0.05^{ab}	0.59	0.03	0.39	0.02
	SD	2.08	0.11^{ab}	0.98	0.08^{b}	0.67	0.06	0.44	0.04

Effect	F_{value}	P_{value}	F_{value}	P_{value}	F_{value}	P_{value}	F_{value}	P_{value}
Season	2.942	0.090	8.077	0.007	0.604	0.439	1.538	0.219
Treatment	8.721	<0.001	11.011	<0.001	9.987	<0.001	1.368	0.261
Season*Treatment	1.435	0.238	2.266	0.087	0.819	0.487	0.649	0.586

N concentrations in the leaves and twig samples 14 days after labeling are shown in **Table VI.2**. A strong treatment effect was noted in both leaves and Y twigs; there was no treatment effect in <Y-1 twigs. A seasonal effect was noted in Y twigs only. No season x treatment interaction was detected at $p<0.05$. In spring, leaf N concentrations were influenced by the treatments with more N in SD and in D than in C. In summer, leaf N concentrations were higher in D than in C and MD. In both spring and summer, a significant SD treatment effect on N concentrations was found on both Y and Y-1 twigs compared to C, whereas an MD treatment effect was found only in summer on Y twigs. Overall, N concentrations in Y twigs in the C treatment were higher in spring than in summer. Both a seasonal and a treatment effect were found for Y twigs, whereas only a treatment effect was found on Y-1 twigs.

6.3.4. Biomass, N and ^{15}N allocation between leaves and proximal branches after a 14-day chase period

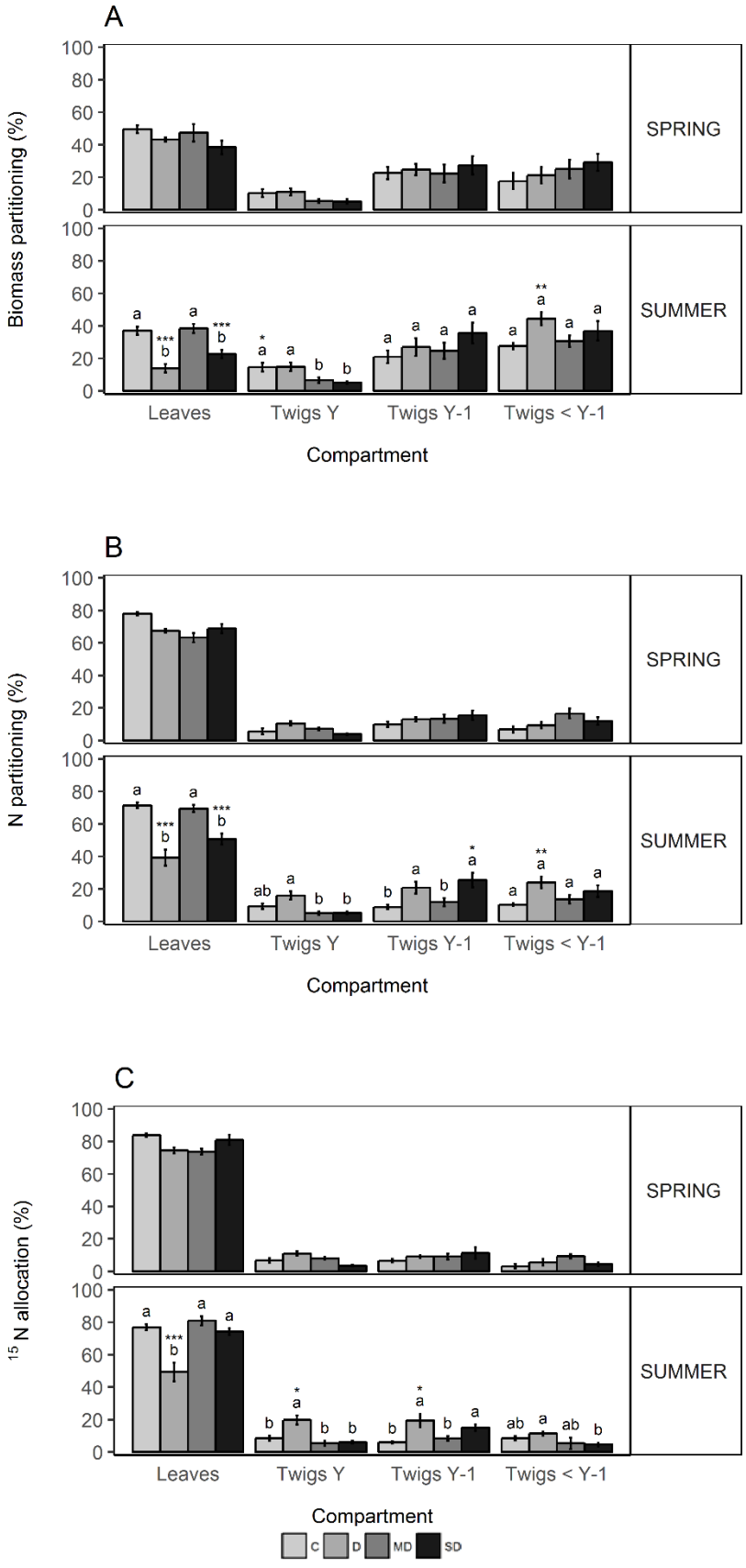

Figure VI.5. Biomass (A) nitrogen (B) and ^{15}N (C) allocation (%) between leaves and twigs according to annual growth unit (Y. Y-1 and <Y-1, where Y is year) on 9-year-old beech trees

14 days after ^{15}N labeling in spring and summer for the four treatments: Control (C), defoliation (D), moderate (MD) and severe soil water deficit (SD). Values are means ± SE; n=12. Treatment difference for a given compartment is shown with different letters ($p < 0.05$). Seasonal differences are shown with stars in the summer section (ns: $p > 0.05$, *: $p < 0.05$, **: $p< 0.01$, ***: $p< 0.001$).

Table VI.3. Statisticals values (F and P) for each variable given in Figure VI.5; biomass partitioning, nitrogen partitioning and ^{15}N allocation for the effect of treatment, compartment (Cmpt) or the interactions between them (Treatment*Cmpt) in spring and summer.

		Biomass partitioning		Nitrogen partitioning		^{15}N allocation	
Season	Effect	F_{value}	P_{value}	F_{value}	P_{value}	F_{value}	P_{value}
Spring	Treatment	1.0963	0.3610	1.2320	0.3097	1.3742	0.2638
	Cmpt	88.8318	<0.001	500.4165	<0.001	649.6055	<0.001
	Treatment*Cmpt	1.7464	0.0865	2.1432	0.0316	1.8090	0.0746
Summer	Treatment	2.0092	0.1269	2.573	0.0664	5.286	0.0035
	Cmpt	46.8348	<0.0001	232.394	<0.001	489.262	<0.001
	Treatment*Cmpt	8.0710	<0.0001	13.298	<0.001	14.711	<0.001

There was no treatment effect on biomass partitioning, but a compartment effect was observed, with a treatment interaction in summer (**Figure VI.3**). In spring, there was no significant effect of treatment on biomass partitioning and leaves accounted for the highest biomass (more than 40%, **Figure VI.5.A**). In summer, C and D trees had higher Y twig biomass partitioning than MD and SD whereas leaf partitioning was higher in MD and C than in D and SD. A seasonal effect was found on SD tree leaves with higher partitioning in spring than in summer; D trees also displayed a significant seasonal effect on leaves and <Y-1 twigs.

There was no treatment effect on N partitioning, but a significant treatment x compartment interaction was noted, especially in summer (**Figure VI.5.B**). A markedly significant compartment effect was noted on ^{15}N partitioning (**Figure VI.5.C**). Treatment significantly impacted ^{15}N partitioning in summer with both a compartment effect and a compartment x treatment interaction. No significant treatment difference was found in spring on N (Figure VI.5.B) or ^{15}N (**Figure VI.5.C**) partitioning. As a consequence of defoliation, D trees showed a significant reduction in N and ^{15}N partitioning in leaves and Y twigs in summer compared to other treatments, though there was also a seasonal reduction compared to spring. SD trees had less N and ^{15}N partitioning in Y-1 twigs in summer compared to C and MD.

6.3.5. ^{15N} long-distance transport

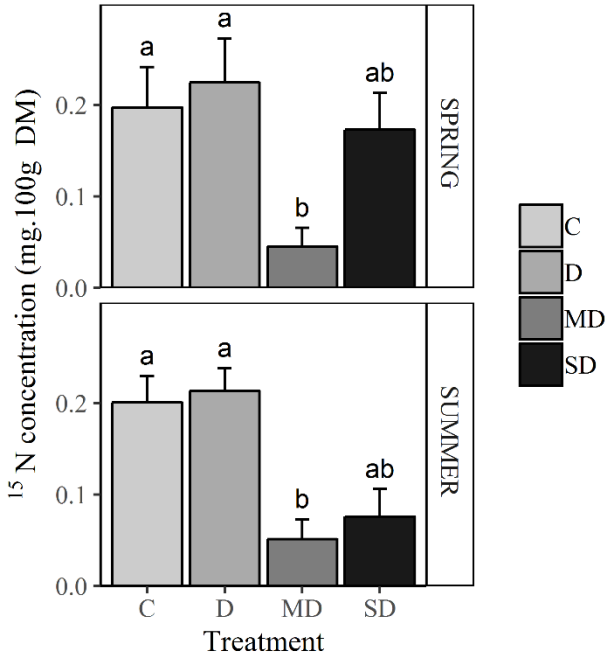

Figure VI.6. Long-distance transport of ^{15}N (mg.100g^{-1} DM) from the leaves of labeled branches to the leaves of the apical terminal twigs on 9-year-old beech trees 14 days after

labeling in spring (top) and summer (bottom) for the four treatments: control (C), defoliation (D) and moderate (MD) and severe soil water deficit (SD). Different letters indicate significant differences ($p < 0.05$) among treatments for a given date; values are mean ± SE; n=12.

At the end of the chase period, the leaves sampled from the apical terminal twig showed a significant ^{15}N enrichment in all treatments regardless of season (**Figure VI.6**). However, this ^{15}N enrichment was lower in the MD leaves than in leaves from the other treatments ($p<0.05$). A decreased rate of ^{15}N enrichment was observed from spring to summer for SD leaves but was not significant at $p<0.05$.

6.4. Discussion

6.4.1. Use of a ^{15}N urea pulse to trace the N pool in leaves

Our experiment was conducted at two dates during the growing season: in spring (DOY: 148) and in summer (DOY: 187) and in both cases, we applied ^{15}N urea to mature leaves of beech trees to label the whole leaf N pool of a given branch. ^{15}N urea was historically used on fruit trees (Klein and Weinbaum, 1984; Rosecrance *et al.,* 1998) to study the impact of foliar N fertilization on fruit production. Recently, this approach has been successfully used to study the effect of foliar N fertilization on forest tree nutrition at young stages (Uscola *et al.,* 2014). In older forest trees, ^{15}N urea labeling, without any fertilizing effect, has also been used to study N translocation between below- and above-ground components in 9-year-old beech trees (Zeller *et al.,* 1998), though the method is rarely used in large trees for obvious practical reasons. In our ^{15}N experiment, our first concern was to avoid any significant increase in leaf N content due to the foliar labeling. We therefore used a small amount of highly ^{15}N-enriched urea. For each treatment and each date, our results show that the N concentration in the leaves was similar before and after labeling, and was typical of N concentrations found by El Zein *et al.,* (2011) in adult leaves of beech trees located near our experimental site.

6.4.2. Leaf N metabolism

A well-established relationship exists between photosynthetic capacity and leaf nitrogen content in plants (Field and Mooney, 1986). High N partitioning to leaves is essential for leaf metabolism and provides N to the photosynthetic machinery during the growing season (Evans and Seemann, 1989), since a large proportion of the plant's N is present in thylakoid membranes and in soluble proteins of the Calvin cycle that represent most of the leaf's nitrogen. Leaf metabolism displays differences between spring and summer (Millard and Proe, 1991; Gomez and Faurobert, 2002). Under non-limiting environmental conditions, these metabolic differences may be due to local meteorological conditions which vary with the seasons, but also to seasonal variations in tree growth, which induce seasonal variations in the source-sink balance. In fact, in beech, the initiation of radial growth and the reactivation of the cambium are mainly dependent on climatic variations and leaf photosynthesis. In addition, a negative correlation of beech growth with maximal temperatures in summer has been found (Cufar *et al.*, 2008, Michelot *et al.,* 2012). Consequently, both seasonal climatic changes and growth

changes in sink organs will affect the N metabolism of leaves, the source of carbohydrates fueling several metabolisms e.g. primary growth, transport, etc. (Peñuelas *et al.*, 2013). However, in our study we did not detect any significant effect of season on N concentrations in leaves or on ^{15}N incorporation in leaf proteins in non-stressed trees. This result led us to hypothesize that seasonal adjustments in beech tree photosynthesis in spring and summer, with leaf protein synthesis remaining quite stable, could be due to changes in activity more than to changes in the synthesis *per se* of soluble proteins of Calvin cycle.

6.4.3. Leaf N functioning under harsh stress

Defoliation causes an immediate loss of resources (C, N) and a reduction in photosynthetic surface area for the tree (Lovett *et al.*, 2002); this may affect leaf biochemistry and N assimilation in the remaining leaves, especially in June when up to 30% of total beech N is found in the leaves (El Zein *et al.*, 2011b). First, a higher leaf N content than in controls was effectively observed in spring 2015 in the leaves of our beech trees which had been submitted to severe defoliation the previous year (2014). Despite the loss of N and carbon created by the previous summer's defoliation, and the likely detrimental consequences for storage during the winter, more N was allocated to the new foliage produced the following spring in the defoliated trees. Then, in 2015, the beech trees defoliated in 2014 were submitted to a second defoliation. At the branch level, 75% of the leaves were removed in spring 2016, resulting in a loss of 0.07g ± 0.01 of N for the branch (results not shown), i.e. a loss of about 75% of the N initially present in the branch foliage. This should have caused a huge and immediate N constraint for the tree. However, one month after this summer defoliation, leaf N was still higher than in the controls. The ^{15}N tracer showed that more N was allocated to protein synthesis in the leaves of the defoliated trees. Such results suggest that compensatory processes exist in beech trees, enabling them to mitigate the negative effects of a past or current defoliation by allocating the needed N resources to the remaining leaves from other tree compartments to maintain photosynthetic capacity. In fact, changes in allocation of internal resources among organs are often observed in plants responding to defoliation. For example, an increased allocation of resources to leaves at the expense of root and stem growth have been found in grasses and in aspen trees (Macaduff *et al.*, 1989, Stevens *et al.*, 2008). In our study, secondary growth also decreased by 15 to 20% in the defoliated beech trees (unpublished data). The higher leaf N content we found in the defoliated trees could be due to extra N uptake by the roots. However, more probably, this additional N was remobilized from the N reserves located in perennial compartments with

woody tissue (i.e. root and trunk sapwood), which are known to be the main pools for stored N in deciduous tree species (Neilsen *et al.*, 1997, Millard and Grelet, 2010). Because defoliation changes the within-tree microclimate, notably providing better access to sunlight for the remaining foliage, a capacity to compensate for the loss of leaves with higher photosynthetic rates has been observed in both deciduous and evergreen species (Quentin *et al.*, 2011, Puri *et al.*, 2015). This increase in light availability has also been reported to cause an increase in leaf N and changes in leaf protein composition in sugar maple (Ellsworth and Reich, 1995). Our study also showed that leaves of D trees contained more proteins than control leaves. This increase in the protein pool in beech leaves could be due to an extra accumulation of soluble proteins, which could enhance photosynthetic capacity and also be a temporary N storage strategy. For instance, the Rubisco protein is a carboxylase known to be stored when in excess and to function as a vegetative storage protein (Millard, 1988, Dickson, 1989, Warren *et al.*, 2001). In response to the 2nd defoliation, the proportion of ^{15}N allocated to foliar proteins increased from 38% in spring to 73% in summer whereas this seasonal increase was less important in the C trees (+28%); this suggests rapid changes in leaf biochemistry for the D trees. Consequently, despite the N loss created by this new defoliation, the leaves of the defoliated trees accumulated more N than the controls and exhibited an important *de novo* protein synthesis. Such metabolic changes probably involve tree stores that play a buffer role in adjusting tree N requirements and may enhance tolerance for partial, repeated foliage loss; this has been also suggested in other studies on deciduous and evergreen species (Palacio *et al.*, 2012, Piper and Fajardo, 2014).

Prolonged drought stress in forest trees may induce a series of changes in the biochemical processes of photosynthesis and cause a decrease in photosynthetic capacity and a decline in leaf nitrogen content (Grassi *et al.*, 2005). In our study, both a moderate and a severe water stress were applied to beech trees for two successive growing seasons. Under severe water-stress, we observed that for a given leaf area, C assimilation in 2015 decreased from 10.5 $\mu mol.m^{-2}.s^{-1}$ in the leaves of the control treatment to 2.8 $\mu mol.m^{2}.s^{-1}$ in the SD treatment (Daniel Epron, personal communication). Beech is known for its sensitivity to drought, and in beech saplings, when a severe soil water deficit occurred, a decrease in photosynthetic performance was observed, notably through a loss of chlorophyll (Gallé and Feller, 2007). Stomatal and mesophyll limitations to CO_2 diffusion under moderate and severe drought affect the carboxylase activity of Rubisco (Grassi and Magnani, 2005) in reason of a low chloroplastic CO_2 concentration. A decline in both the activity and amount of Rubisco and an increase in amino acid pools known to play a role in tissue osmoprotection may also occur (Bode *et al.*,

1985; Fotelli *et al.,* 2002). In our study, we did not see any decline in leaf N concentrations in beech trees in response to a prolonged severe water stress, and ^{15}N was still incorporated into the leaf proteins. Such a result suggests that a high metabolic activity was maintained with both rapid catabolism of the absorbed urea and rapid anabolism into amino acids and proteins. Our results also show that the ^{15}N incorporation into the soluble protein pool in summer represented only 40% of the ^{15}N present in the leaves of the SD trees, meaning that about 60% of the ^{15}N was incorporated into N compounds other than proteins (possibly pigments or amino acids) at this period of intense metabolism for leaves. Glutamine or allantoine, two important amino acids for transport (Sauter and Van Cleve, 1992), could be involved, as could proline and asparagine, osmoprotectants which maintain cell integrity under limiting soil water conditions (Bode *et al.,* 1985, Fotelli *et al.,* 2002).

6.4.4. Impact of stress on short- and long-distance transport of leaf N

We studied how much leaf N metabolism and its transport out of the leaves would be disrupted by repeated abiotic stress (recurrent defoliation or prolonged soil water deficit) at two key growth stages for both primary and secondary growth. Through nutrient storage and remobilization processes, trees ensure their survival by limiting their dependency on mineral uptake during adverse environmental events like drought (Rennenberg *et al.,* 2006). N is one of the nutrients that can be transported in the phloem or xylem and continuously recycled within the tree (Grassi *et al.,* 2003), or be released in tiny amounts *via* soil rhyzo-deposition, as Sommer *et al.,* (2016) shown in beech. We applied ^{15}N-urea on mature leaves in spring and summer and estimated whether part of the ^{15}N pool was transported out of the leaves towards the bearing branch or beyond, or whether it stayed in the leaves and was recycled there. In the well-watered trees, the maximum level of ^{15}N incorporated inside the N leaf pool was obtained after a 4-day chase period and stabilized for the next ten days. It is likely that this stabilization was due to an important local turnover in the N pool within the leaves rather than to an intensive ^{15}N export to and unlabeled N import from other tree compartments. Indeed, 14 days after labeling, about 80% of the ^{15}N content found in the whole branch had remained within the leaves, and only 20% had been distributed along the twigs on the same branch. For the duration of our experiment and regardless of treatment or season, this ^{15}N distribution pattern between leaves and branch twigs remained unchanged. However, in summer, the ^{15}N allocation inside the branch was modified due to the drastic defoliation the tree had undergone one month before.

This defoliation caused a biomass imbalance between the remaining leaves and the bearing branch, and a higher ^{15}N allocation in twigs Y and Y-1 occurred at the expense of the leaves. We also sampled the leaves of the apical terminal twig on each tree and found that some of the ^{15}N incorporated in the leaf N pool had been transported over a long distance to the terminal twig. It is very probable that the N exported from the leaves was evenly distributed within the crown, though this was beyond the scope of our investigation. Our study showed that, even under severe drought, beech maintained long-distance transport of N from labeled leaves. The persistence of long-distance N transport from the leaves after two years of no irrigation was truly remarkable. However, the increase in drought intensity between spring and summer induced a decrease in the ^{15}N flux towards the leaves of the terminal twigs of trees. This indicates a decrease in N transport capacity from the labeled branch in the SD trees. Even though N metabolism at the leaf and branch scales was apparently unaltered by two years of drought, the lack of water and N uptake did also cause a change in leaf N transport inside the tree. Two possible complementary causes could be involved in severe water-stressed beech trees: (1) the necessity for the leafy branches to keep more of their N and to favor local recycling to the detriment of its export due to the unavailability of N from soil absorption, or/and (2) the appearance of a partial dysfunction of the hydraulic system, particularly in the phloem, as proposed by Dannoura *et al.,* (2018) which could alter long-distance N transport.

6.5. Conclusion

Even under extreme constraints, leaf N metabolism was maintained, possibly thanks to internal N recycling and transport within the tree. Such strategies would help trees to avoid nitrogen starvation and to cope with the recurrent drought stress predicted in the context of future climate change.