SEASONAL TRANSLOCATION OF FOLIAR NITROGEN WITHIN BEECH TREES AND ITS TOLERANCE TO STRESS: A 15N LABELING APPROACH (in preparation)

7. SEASONAL TRANSLOCATION OF FOLIAR NITROGEN WITHIN BEECH TREES AND ITS TOLERANCE TO STRESS: A ^{15}N LABELING APPROACH

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Running title: Seasonal distribution of leaf nitrogen under stress in beech trees

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ABSTRACT

According to current scenarios for climate change in France, extreme drought events are expected to occur more frequently. Such events will not only limit supply of water but also the availability of soil N for trees. Nitrogen is one of the main nutrient driving growth and productivity in many forests. One question that need to be addressed is how severe drought events affect the internal N cycle and the growth of a widespread deciduous forest tree species as beech (*Fagus Sylvatica* L.). We designed an experiment with young beech trees (*Fagus Sylvatica* L.) submitted to an artificial N shortage through a rainfall exclusion or partial defoliation. Both treatments were expected to drastically affect the N balance of the trees by decreasing access to water and nutrients in the soil or through a loss of leaf N. To follow the changes of within tree N fluxes and N uses in response to the treatments, we pulverized a low quantity of urea with a high ^{15}N enrichment on the whole foliage of these trees thus tracing the flux of leaf N into other organs of the trees during automn and winter and their remobilization for new growth in Spring. We aimed to test the following hypotheses: (1) drought and defoliation stress will alter N (^{15}N) dynamics (2) which tree compartment will be preferentially used to store N (^{15}N) during the winter (3) how the treatments will alter the use of N (^{15}N) for the new growth in Spring? The first results show that nitrogen cycling have already started when we cut trees in October, 2 weeks after the labelling. Less ^{15}N was found in leaves compared to woody organs (e.g : stem and branches). We also found that nitrogen recycling was not modified by severe constraints where branches served as major target organs. In following spring, leaves were still a major sink for N remobilisation despite less stored N found in perennial parts. A result like this could mean that leaf metabolism is maintened even under severe constraints which can be advantageous in case of better soil water conditions.

Keywords: Nitrogen, labelling, defoliation, soil water deficit, *F. Sylvatica* L.

7.1. Introduction

From fifty years, drought periods and heat waves have increased in worldwide as a consequence of global climate change and this trend is expected to increase in the coming decades (Coumou *et al.,* 2013, Wagner *et al.,* 2013, IPCC, 2013). These climate hazards can induce strong reductions of primary productivity (Piovesan *et al.,* 2008) and sometimes forest decline and tree mortality. Understanding of the physiological processes involved in tree dysfunctions and forest tree decline is a major concern (Bréda *et al.,* 2006, McDowell *et al.*, 2008, Sala *et al.,* 2010) Two main but non-exclusive physiological mechanisms have been proposed: hydraulic failure and carbon starvation (McDowell *et al.,* 2008, Sala *et al.*, 2012, Hartmann *et al.*, 2013). On one side, hydraulic failure may lead to mortality when partial or total loss of xylem function occurs in response to drought. On the other side, a carbon starvation is also possible when drought lasts long enough to create a carbon imbalance between sink demand and source supplies (photosynthesized or remobilized carbon). Besides these two hypothesis, some authors have recently suggested that a reduced nutrient availability in response to drought, especially nitrogen (N) which could play also an important role in tree dysfunction (Gessler *et al.,* 2016). However experimental evidence of how N cycling of adult forest trees could be modified by harsh conditions is still missing today. Available studies focused only on seedlings but these results are difficult to generalize if ontology have an impact on tree N balance, as noticed on C balance (Cavender and Bazzaz, 2000, Gilson *et al.,* 2014). Forest ecosystems have often developed on poor soils which were not convenient for optimal growth due to the low N availability (Raven and Andrews, 2010). To cope with this N deficiency, trees adopted dedicated strategy by optimizing N use efficiency and internal recycling (Vitousek, 1982). The internal N cycling consists in N storage in perennial structures (wood and roots) when N requirements are low and remobilization of these N reserves towards sinks (mainly the foliage) in case of N shortage when current demand exceeds root N absorption capacity due to the seasonality of plant growth (Chapin *et al.,* 1990). In fact, in early spring, N uptake by roots is not sufficient to sustain spring growth and trees rely on stored N as largely demonstrated on fruit trees (O'Kenney, 1975, Millard and Nielsen, 1989, Neilsen *et al.,* 1997, Tagliavini *et al.,* 1998, Cheng *et al.,* 2002, Dong *et al.,* 2002) and more recently on mature forest trees (El Zein *et al.,* a & b 2011, Valenzuela Nunes *et al.,* 2011, Bazot *et al.,* 2013). This net decrease of stored N observed in spring cannot be recovered fastly and so, during the vegetative season, trees rely mainly on N soil uptake by roots (Bazot *et al.,* 2013, Villar-Salvador *et al.,* 2015). In autumn, a mechanism called N resorption occurs and consists in the degradation of foliar proteins into

amino acids and the transport via the phloem towards bark and wood parenchyma where they are stored during winter under amino acids and vegetative storage proteins forms (Sauter *et al.*, 1989, Wetzel *et al.*, 1989, Stepien *et al.*, 1994, Millard, 1996). Mobilization and refilling of N stores lead to seasonal fluctuations in these storage pools. As N soil is often a growth-limiting factor in natural forest ecosystems (Rennenberg *et al.*, 1998) and the ability of trees to store and redistribute N resources internally is a fundamental process conditioning their survival. A limited soil N availability caused by a stress like drought will end to an inability of roots to explore the soil and to a decrease of microbial activity (Kreuzwieser and Gessler, 2010, Creeger *et al.*, 2014), increasing the risk that N uptake will not be sufficient enough to refill tree internal reserves. Consequently, Gessler *et al.*, (2016) hypothesized that the internal N cycling, could become critical for tree survival when drought occurs in early spring and summer especially in deciduous tree species.

In relation to the Gessler's hypothesis previously presented (2016), we explore in our study how the tree internal N cycle is modified by a prolonged drought or a yearly manual defoliation in eight-year-old beech trees submitted to two years of constraints. As mentioned above, a soil water deficit is expected to decrease both N soil availability and uptake by trees. In another way, any drastic defoliation may cause a harsh loss of N in trees because thirty-height percent of N are located in leaves in June on beech trees (El Zein, 2011). So by these two severe constraints, we expect to decrease the tree N availability leading to adjustments on internal tree N metabolism to maintain fundamental tree functions (growth, maintenance and storage). To follow these adjustments, we labeled the whole foliage of control, defoliated and water stressed beech trees with ^{15}N-urea before leaf senescence in autumn. Then, we followed the fate of ^{15}N from senescing leaves toward perennial organs in the whole tree during the winter storage, and then its remobilization for spring growth.

We chose to work on European beech trees (*Fagus sylvatica* L.) trees, one of the most widespread and abundant species in Europe, because it is known to be more drought sensitive than other European broad-leaves species (Zang *et al.*, 2014; Zimmermann *et al.*, 2015) but paradoxaly it is also one of the most resistant to mortality. Given its survival capacity, we hypothesized that its resources management is particular efficient to face to constrains. Various authors also emphasize the remarkable potential for recovery after drought stress of *Fagus sylvatica* (Elling *et al.*, 2007). In the present study, we made the following hypotheses: (1) drought and defoliation applied repeatedly for two years will create a significant decrease of the tree N pool; (2) due to this N reduction, the stressed tree will intensify its leaf N recycling

and export more N in autumn toward perennial organs compared to a control tree in an attempt to counterbalance a marked decrease in total N winter storage level. (3) The expected N storage decrease in the stressed trees will impact the level of remobilized N available for growth increment and canopy establishment in spring. (4) Finally, if the N remobilization is source driven (Millard and Grelet, 2010), a reduced growth without any changes in N mobilization intensity and its partitioning between perennial organs and new formed organs (twigs, leaves) will be expected in stressed trees compared to controls.

7.2. Materials and methods

7.2.1. 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. 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 (**Figure VII.1.A**); 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 soil in the drought treatments was isolated by a rigid waterproof plastic sheet 1.80 meters depth buried vertically around the area. 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 tree dysfunction and 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 hydraulic status of the chosen trees for the experiment in each treatment (8 trees in C and D, 5 in MD and SD in September 2015 and 6 trees in C and D, 3 in MD and SD in June 2016) was checked by measuring pre-dawn water potential in twigs (ψ_{pd}) in September 2015 and in June 2016. We sampled the twigs (one per tree) before sunrise and performed the ψ_{pd} measurement with a pressure chamber (PMS Instruments, Albany, OR, USA).

7.2.2. Soil characteristics and soil water content 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:

$$\mathrm{REW}=100^{*}\ \frac{\mathrm{TAWC\text{-}R}}{\mathrm{TAWC}} \qquad (19)$$

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).

7.2.3. Foliar ^{15}N labeling procedure

The labeling experiment was performed at end of September 2015 (DOY: 271), before leaf fall. The timing of labeling is summarized in **Figure VII.1**. Forty-four trees were randomly chosen for labeling. On each tree, a crown bag made of polyethylene was placed over the total foliage of the tree 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).

The 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 plastic bag was kept on all night, then very carefully removed the next morning to avoid any contamination among trees. A net was put all around the tree to collect all the litter from the labeling through the winter fall.

7.2.4. Sampling protocol

Green leaves were sampled in July 2015 2 months prior the labeling to measure the leaf N concentration in the control, defoliated and water stressed trees (n=12 in each treatment). In October 2015, one month after the labeling, we harvested 8 trees (2 trees per treatment) in view to assess the incorporation of ^{15}N in the internal N cycle and its presence in perennial storage organs. Then, trees were harvested at two key phenological dates (El Zein, 2011) after the labeling: 1) in February 2016, 5 months after labeling at the theoretical highest storage level of N in perennial organs; and 2) in June 2016, 9 months after labeling at the theoretical end of N remobilization, once leaf expansion was done. We harvested 18 trees in February and June (6 C; 6 D; 3 SD; 3 MD), i.e. a total of 44 trees were labeled and harvested during this experiment. Ten unlabeled trees (3 C; 3 D; 2 MD; 2 SD) were also harvested in October 2015 to assess the natural abundance of ^{15}N in each tree compartment. Each tree was separated in its compartments (leaves, branches, trunk and roots). Roots were separated according to their diameter: fine roots ($d<1mm$), lateral roots ($1<d<3mm$) and main roots ($d>3mm$). We collected the litter in February 2016 with use of litter net. Each compartment was weighted to get the fresh mass, immediately frozen in liquid nitrogen, and then stored at -80°C. Then compartments were freeze-dried (Dura-Top (r), Dura-Dry (r), FTS Systems (r), Stone Ridge, NY, USA), weighed to determine the dry matter (DM) and ground into a fine powder with a ball mill (CEPI SODEMI CB2200, Cergy, France). The timing of labeling and harvest is displayed in **Figure VII.1**.

7.2.5. Growth measurements

For each treatment, height and diameter of labelled trees were measured at the end of the vegetative season in 2015 with an electronic caliper and a beam, respectively. In June 2016, the spring primary growth (trunk and shoots) was estimated on the sampled trees by measuring the length of the shoot 2016 on both on the trunk (primary axis) and of three branches per tree (secondary axis) randomly chosen in the canopy of each tree.

7.2.6. Foliar variable measurements

In October 2015, several foliar characteristics were assessed by sampling randomly 100 leaves per tree on the 8 labelled and 10 additionnal unlabeled trees (6 C; 6 D; 3 MD; 3 SD). The individual leaf area of these 100 leaves was measured using a portable area meter (LI 3000 A, LI-COR, Lincoln Nebraska, USA) then dried 48h at 100°C and weighed. The mean individual leaf area, the leaf mass per area (LMA), the total number of leaves and the total leaf area of trees were calculated based on the relationship between the leaf biomass and the leaf area. These measurements were also repeated on the 18 labeled trees harvested in June 2016 once leaf expansion was achieved. In order to have the effect of the defoliation made the year before on the current vegetative season leaf expansion, we calculated the initial values (number of leaves, LMA and total leaf area) for defoliated trees in 2015 by multiplying found values of harvested trees in October 2015 by 75% (*e.g* the intensity of defoliation made in 2015).

7.2.7. Nutrient resorption efficiency

Nutrient resorption efficiency was calculated as described by Killingbeck (1996) and more recently by Hai-Yang *et al.,* (2018)

$$\mathrm{NuR} = \frac{(\mathrm{Xgr} - \mathrm{Xsen}) * \mathrm{MLCF}}{\mathrm{Xgr}} * 100\% \quad (20)$$

Where Xgr and Xsen are the N concentrations of green (taken in July) and senescent leaves (taken in February) respectively. MLCF correspond to the mass loss correction factor corresponding to the percentage of leaf mass remaining in senesced leaves comparing to the green leaves (Vergutz *et al.,* 2012). As our specie is a deciduous temperate species, we used an MLCF value of 0.784 as preconized in Vergutz *et al.,* (2012).

7.2.8. Elementary and isotopic analyses

Total N concentration (% of dry matter) and ^{15}N isotopic abundance (atom%) of the different tree compartments (leaves, branches, trunk, roots, litter) were measured using an elemental analyzer (Eurovector, Redavalle, Italy) coupled to an Isoprime (Elementar UK). Analyses were carried out at the isotopic platform of B&PMP (INRA, Montpellier, France).

7.2.9. Isotopic calculations

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

$$A_N\% = \frac{^{15}N}{^{14}N + ^{15}N}\ 100 \quad (21)$$

The enrichment of ^{15}N in each compartment in each compartment is defined as

$$^{15}N_{excess} = A_N\%_{(labeled\ compartment)} - A_N\%_{(unlabeled\ compartment)} \quad (22)$$

where $A_N\%_{labeled\ compartment}$ is the ^{15}N abundance of the labeled compartment, $A_N\%_{unlabeled\ compartment}$ is the natural ^{15}N abundance of the unlabeled compartment, with a $A_N\%_{unlabeled\ compartment}$ of about 0.368306888 ± 0.00306 atom% to 0.370893333 ± 0.00127829 atom% according to the compartment. The unlabeled compartment came from the 10 unlabeled trees harvest in October 2015.

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

$$^{15}N\ concentration = {}^{15}N_{excess} \times \frac{[N]}{100} \times 1000 \quad (23)$$

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

$$^{15}N\ amount = \frac{^{15}N\ concentration}{1000} \times \frac{DM}{100} \quad (24)$$

where DM is the dry matter (g) of the compartment. For practical reasons related to the impossibility of excavating fully the whole root compartment especially under drought treatment, we will not present the ^{15}N amount in the root system.

7.2.10. Total N and ^{15}N allocation in the aerial tree compartments

The total N or ^{15}N allocation is related to the distribution of N or ^{15}N within the different compartments (leaves, branches, trunk) of the whole aerial system (Dickson, 1989). Allocation of N and ^{15}N represented the ratio (%) of the amount of N or ^{15}N incorporated in a given compartment relative to the total amount of N or ^{15}N incorporated in the whole aerial system.

$$N\ partitioning\ or\ ^{15}N\ allocation = \frac{N\ or\ ^{15}N\ amount\ of\ aerial\ compartment}{N\ or\ ^{15}N\ amount\ of\ the\ whole\ aerial\ system} * 100 \quad (25)$$

7.2.11. Statistics

We applied a General Linear Model to our data to test the effect of treatment and date on each variable. To fit the assumption of normality, we carried out a log transformation on ^{15}N concentrations and an arcsin (root-square/100) transformation on partitioning (%). 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). Values are presented as average ± SE.

7.3. Results

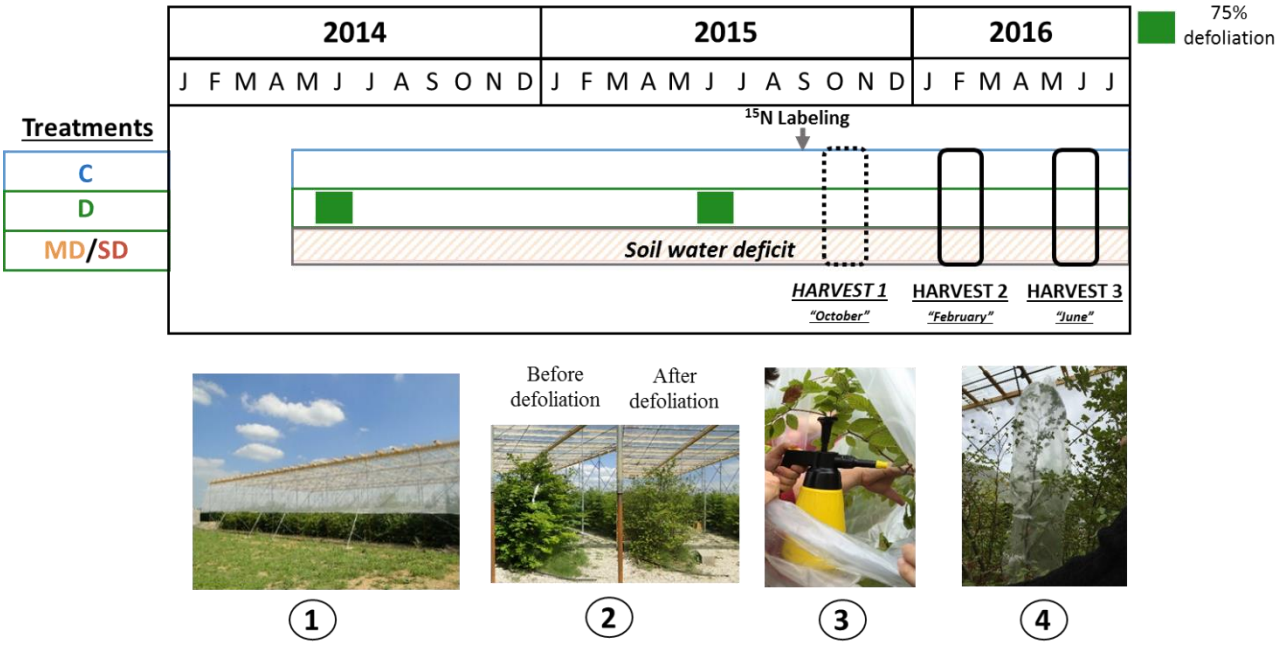

Figure VII.1. Schedule of the experiment since the onset of treatments in 2014 (photography 1 and 2). The foliar labeling was made in September 2015 with urea sprayer in a fine mist (photography 3), tree bag was installed before the labeling, remained during the night after labelling then removed the morning after (photography 4). First, we made a harvest one month after the labeling to confirm that the tracer was incorporated in perennial organs via leaf N resorption. Then, harvesting was made at two key phenological dates in February and June 2016. C is for Control, D for Defoliation, MD and SD for Moderate Drought and Severe Drought respectively.

7.3.1. Monitoring of water changes in soil and tree

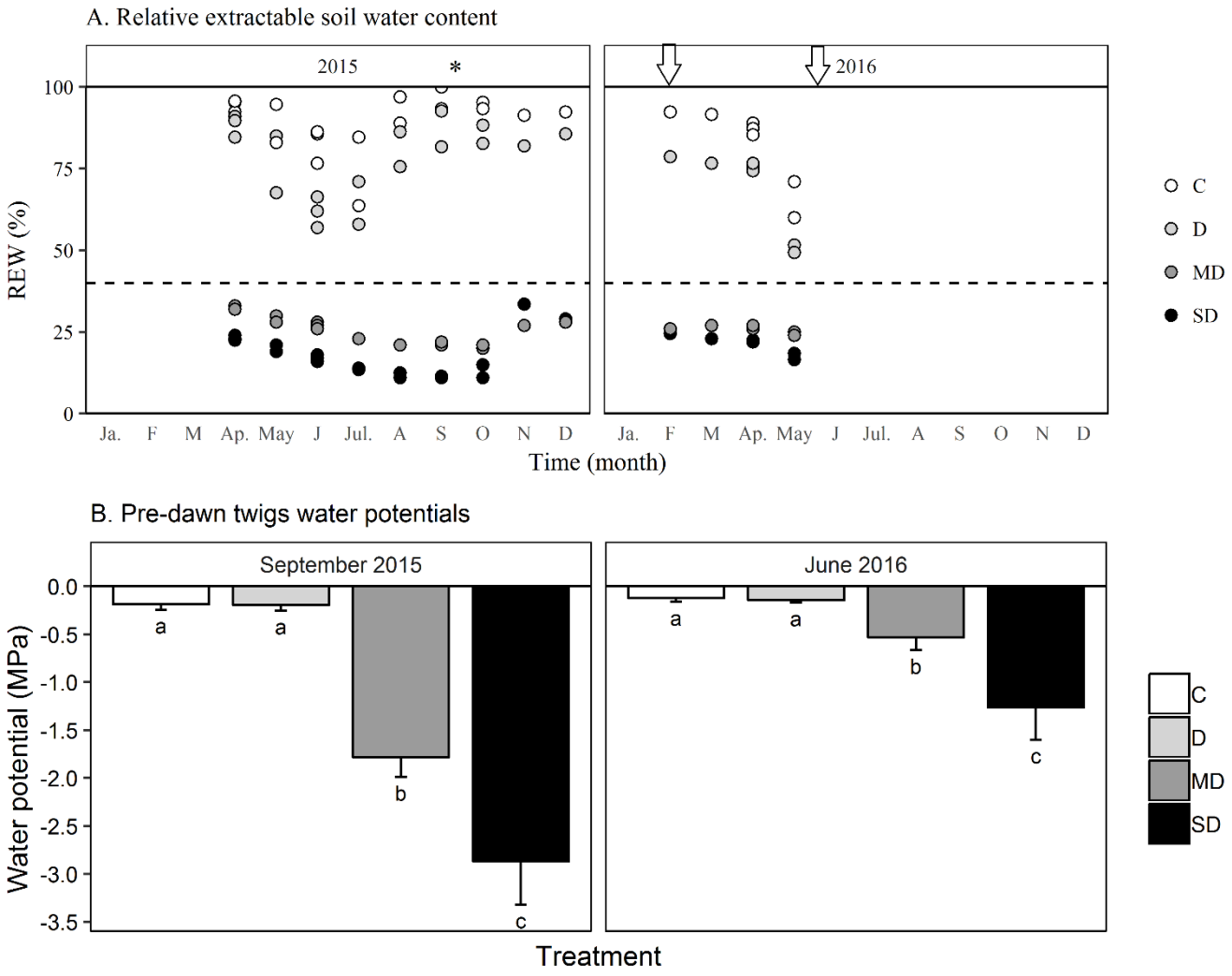

Figure VII.2. Seasonal dynamics of the relative extractable soil water content (REW, A) and the average pre-dawn water potential of twigs (B) in young beech trees during the year 2015 and 2016 in four treatments: moderate soil water deficit (MD), severe soil water deficit (SD), defoliation (D) and control (C). The dashed line (A) indicates the threshold value of REW from which the stomatal conductance is impacted according to Granier et al., (1999). The star indicates the labeling time and the two arrows indicate the harvesting times. In B, different letters means a significant difference ($p<0.05$) between treatments for a given date. Mean ± SE, n=8 trees in C and D, 5 in MD and SD in September 2015 and 6 trees in C and D, 3 in MD and SD in June 2016 for pre-dawn water potential of twigs.

The seasonal monitoring of the relative extractable water (REW) showed the progressive increase of soil water deficit with a continuous decrease of REW during the vegetative season in 2015 in the two treatments MD and SD (**Figure VII.2.A**). After the leaf fall, we observed a small increase of REW due to a small irrigation (40 mm). During all the experiment, REW stayed below the threshold of 0.4 in the MD and SD treatments. REW in C and D treatment showed a slight decrease after leaf expansion in spring 2015 and 2016, before the beginning of the irrigation, but it was above the threshold of 0.4 during all vegetative seasons. As a result of the progressive soil water depletion, the pre-dawn water potential (ψ_{pd}) of twigs (**Figure VII.2.B**) was lower in both drought-treatment (MD and SD), ranged between -1.5 and 3 MPa respectively, and were lower ($p<0.001$) than C and D trees. SD trees displayed significant lower water potential values than MD trees ($p<0.05$). In 2016, the pre-dawn water potential (ψ_{pd}) of twigs of C and D trees was significantly lower ($p<0.05$) than the one of twigs of MD and SD trees.

7.3.2. Impact of two years of stress on development of the aerial system and on spring growth of the third year

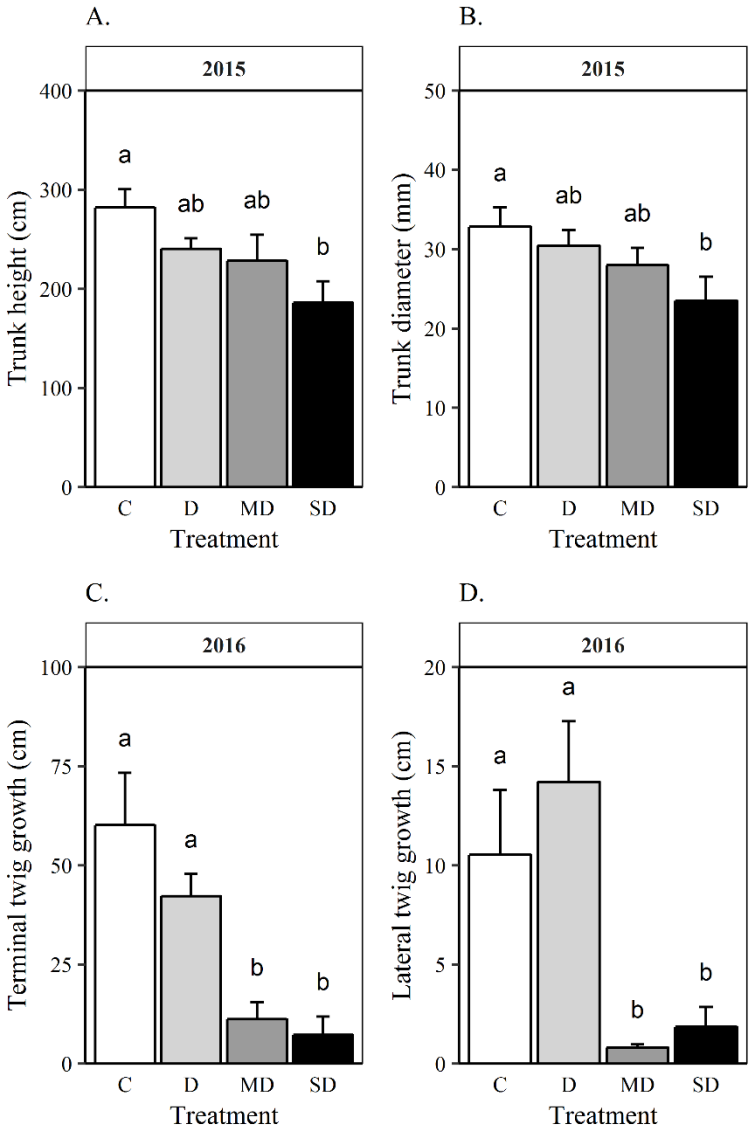

Figure VII.3. Mean height (A) and diameter (B) of the trunk at the end of the vegetative season 2015 and the mean terminal twigs (C) and lateral twigs (D) growth after the spring growth in 2016 in young beech trees in four treatments: moderate soil water deficit (MD), severe soil water deficit (SD), defoliation (D) and control (C). Mean ± SE, n=6 for C and D and n=3 for MD and SD. Different letters mean a significant difference between treatments.

At the end of the vegetative season in 2015, i.e. after 18 months of constraints, we measured the diameter and the height of the trunk of trees (**Figure VII.3.A and VII.3.B**). The height and diameter of SD trees was smaller than C trees (-34% in height and -28% in diameter on average) whilst moderate drought (MD) and defoliation (D) did not affect significantly the height and diameter in 2015. In June 2016, we were not able to access to the diameter increment because

secondary growth was not finished when trees were harvested. We measured the primary growth of the terminal and one lateral twigs (**Figure VII.3.C and VII.3.D**). MD and SD trees exhibited significant reduction in growth (*p<0.001*, -80% on trunk growth and -85% on lateral twigs growth on average*)* while defoliation did not impact the growth of terminal or lateral twigs.

7.3.3. Changes with time of leaf characteristics in response to treatments

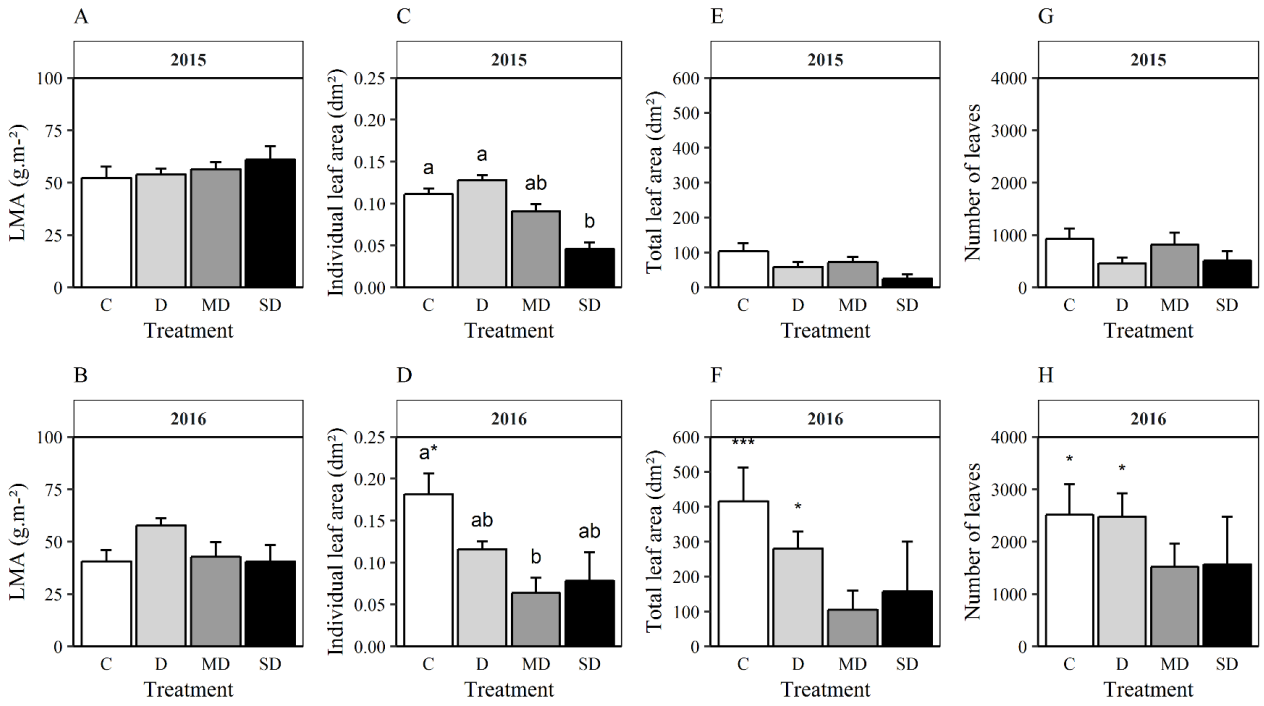

Figure VII.4. Change with time of leaf characteristics with leaf mass area (LMA; A,B), individual leaf area (C,D), total leaf area (E,F) and number of leaves per tree (G,H) at the end of vegetative season 2015 (top) and after the spring growth 2016 (bottom) in young beech trees in four treatments : moderate soil water deficit (MD), severe soil water deficit (SD), defoliation (D) and control (C). Mean ± SE. n=3 trees for MD and SD and n=6 trees for D and C. Different letters indicate significant difference (p<0.05) between treatments, stars indicate significate difference between years.

No effect of treatments were found on LMA (**Figure VII.4.A and B**) even if D trees seems to have a slight but not significant increase in LMA compared to others treatment in 2016. The individual leaf area was impacted by the most severe soil water deficit (SD) in 2015 (0.05 dm² in SD trees compared to 0.11 dm² for C trees) (**Figure VII.4.C**) but in 2016, it was MD trees

which had lower individual leaf area (0.06 dm²) than C trees (0.17 dm²) (**Figure VII.4.D**) An effect of the time between 2016 and 2015 was found on total leaf area on C (*p<0.001)* and D (*p<0.01)* trees (**Figure VII.4 E and F**). No significant effect of treatment was noted on the number of leaves in both vegetative seasons but an effect of time was noticed on C and D trees (*p<0.01).*

7.3.4. Evaluating of the autumnal ^{15}N distribution among the tree compartments one month after the labeling

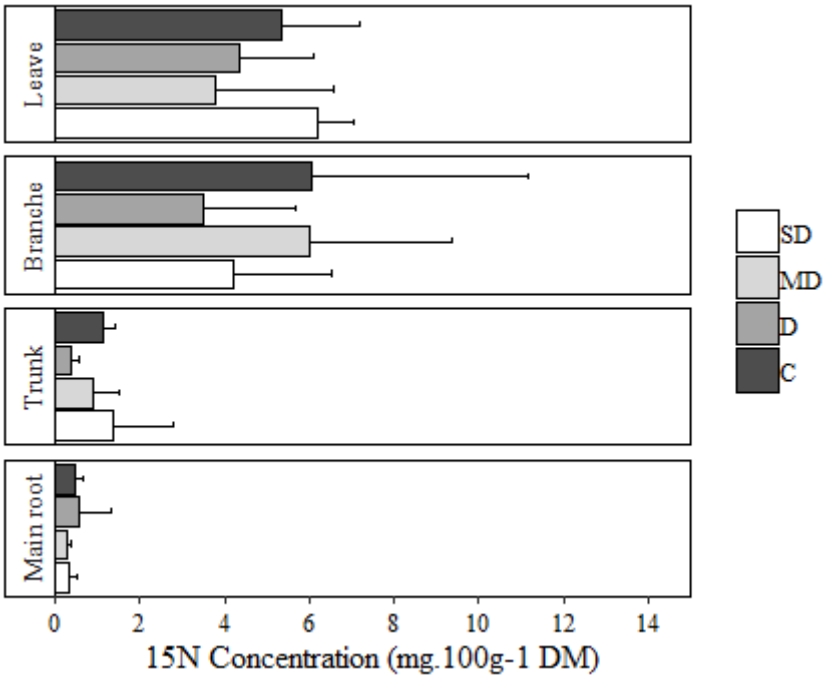

Figure VII.5. ^{15}Nitrogen concentration (mean ± SE, %DM) in above (leaves, branches, trunk) and belowground (main roots) compartments of young beech trees in October 2015 in the four treatments: moderate soil water deficit (MD), severe soil water deficit (SD), defoliation (D) and control (C). Mean ± SE, n=2.

We made the ^{15}N labelling directly on leaves to follow the leaf N resorption to perennial organs. Thanks to the first harvest, we wanted to verify the incorporation of ^{15}N and quantify if N resorption already occurred in mid-October. We found labelled nitrogen into all organs from leaves to roots. (**Figure VII.5**).

7.3.5. Nitrogen efficiency resorption at the end of 2015-leaf fall

Table VII.1. N concentration of green leaves (N green, %DM) in July 2015 and of marcescent leaves (N sen, %DM) in winter 2016 and the nitrogen resorption efficiency (NuR, %) in the four treatments: moderate soil water deficit (MD), severe soil water deficit (SD), defoliation (D) and control (C). n=12 for N green for all treatments in July; n=6 for N sen for C and D treatments; n=3 for MD and SD treatments. Different letters indicate significate difference between treatment while stars indicate a effect of season between summer (Ngreen) and autumn (Nsen).

	N green	N sen	NuR
	%DM	%DM	%
Treatment			
C	1.83 ± 0.10^{b}	$0.47 \pm 0.07^{ab***}$	58
D	2.28 ± 0.08^{a}	$0.66 \pm 0.10^{a***}$	56
MD	1.86 ± 0.10^{b}	$0.36 \pm 0.12^{b***}$	63
SD	2.08 ± 0.11^{ab}	$0.51 \pm 0.06^{ab***}$	59

The N concentration of green leaves on mid-July 2015 ranged between 1.83% DM to 2.28 % DM for C and D trees, respectively (**Table VII.1**), leaf N concentration of D leaves were higher than MD and C leaves in July. In winter the N concentration in the marsescent leaves varied from 0.36% (C) to 0.66% (D) and leaf N of D leaves were higher than leaf N of MD leaves. A strong ($p<0.001$) significant effect of the time between July and February has been found in all treatment As a consequence, the NuR calculated ranged between 56% for D to 63% in MD treatment.

7.3.6. Changes in N and ^{15}N concentration in tree compartments between winter and spring

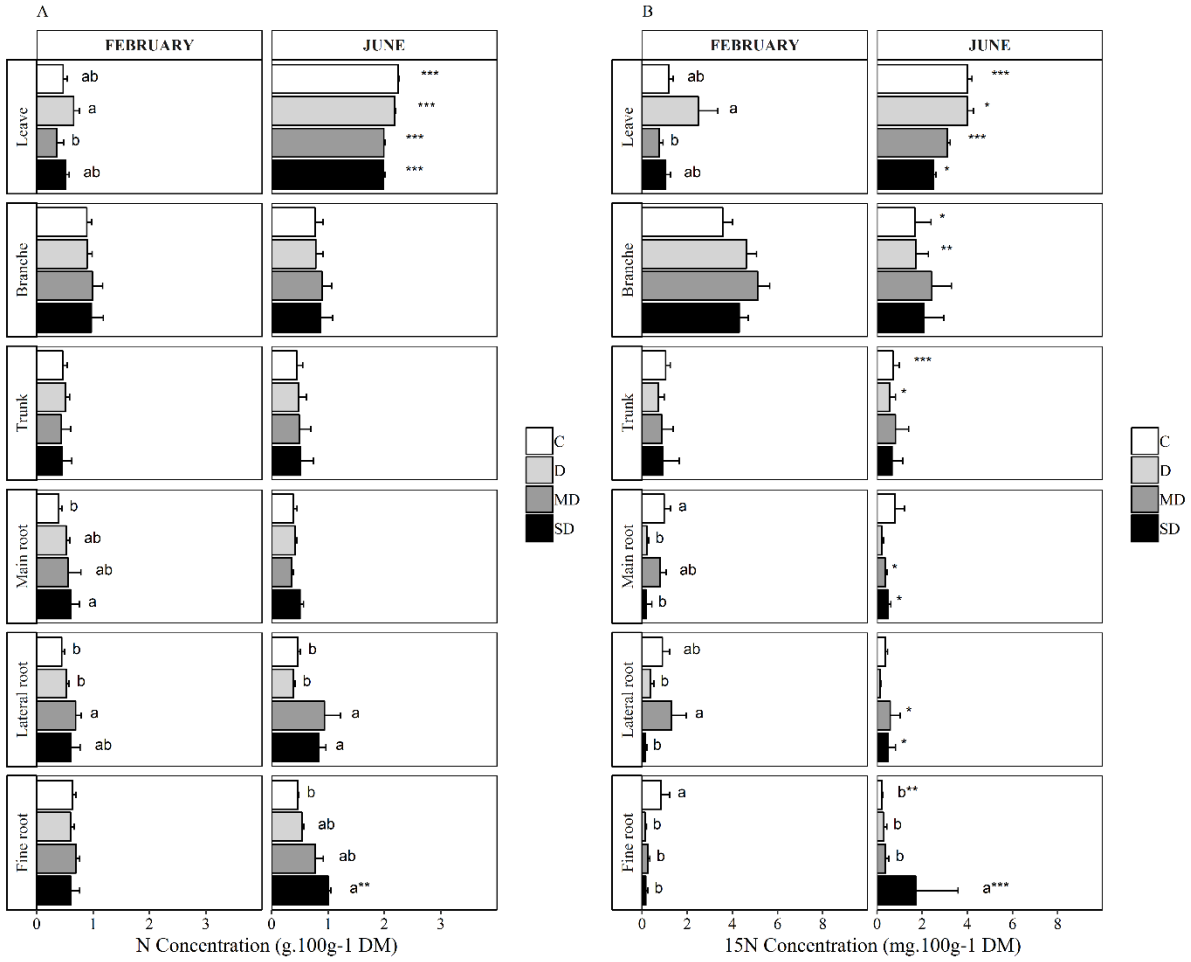

Figure VII.6. Changes with time of N (A) and ^{15}N (B) concentrations (mean ±SE, %DM) in aboveground (leave, branche, trunk) and belowground (main roots, lateral roots, fine roots) compartments of 10 year-old beech trees sampled in February and June 2016 in the four treatments : moderate soil water deficit (MD), severe soil water deficit (SD), defoliation (D) and control (C). Mean ± SE. n=3 trees for MD and SD and n=6 trees for D and C. A stars means a significant season effect for a given treatment and a given compartment. * p<0.05 ; ** p<0.01 ; *** p<0.001. Please note that leaves correspond to the marcescent leaves in February and to new leaves from new spring growth in June.

The **figure VII.6** presents the N (**Figure VII.6.A**) and ^{15}N (**Figure VII.6.B**) concentrations of each tree compartment in February and June. In February, N values of marcescent leaves ranged from 0.35 to 0.65 $g.100^{-1}$ DM according the treatments. D trees displayed higher leaf N

concentrations than MD trees. N concentration in main roots of SD trees were higher (0.60 g.100g^{-1} DM) than C trees (0.39 g.100g^{-1} DM) while N concentrations of lateral roots of MD trees (0.69 g.100g^{-1} DM) were higher than in C and D trees (0.44 and 0.52 g.100g^{-1} DM). In June, an effect of soil water deficit were notified on N concentration on lateral roots with higher N concentration on MD and SD (0.83 and 0.93 g.100g^{-1} DM) trees than C and D trees (0.47 and 0.39 g.100g^{-1} DM) and in fine roots where SD trees had higher N concentrations than C trees (1.01 g.100g^{-1} DM for SD and 0.46 g.100g^{-1} DM for C). An effect of season, meaning a significant difference between June and February were measured on leaves for all treatment with more N concentrations in spring than in winter (*p<0.001).* Another effect of season with higher N concentration in June than in February were noticed on fine roots from SD trees (*p<0.01).*

When regarding the ^{15}N concentration in February, *e.g* N which come from leaf N resorption in previous autumn, an effect of defoliation were notified with higher ^{15}N concentrations in leaves of D trees (2.48 mg.100g^{-1} DM) compared to MD trees (0.75 mg.100g^{-1} DM). In June, we found an effect of treatment only on SD trees for fine roots (1.73 in SD versus 0.21 mg.100g^{-1} DM in C trees). Time has an effect on leaves concentrations from trees in all treatment while ^{15}N concentrations of branches displayed lower values in June than in February on D (*p<0.01)* and C trees (*p<0.05).* Lower values were also found for trunk concentrations in C (*p<0.001)* and D (*p<0.05)* trees. Finally, a significant season effect were also found on MD and SD trees for main and lateral roots and on C trees for fine roots. In summary, higher N and ^{15}N concentrations were found in July on leaves while ^{15}N concentrations in branche were lower in June than in February.

7.3.7. Changes of N and ^{15}N quantity in the compartments of the aerial system between winter and spring

Table VII.2. Seasonal changes of biomass, nitrogen and ^{15}N amounts in aerial compartments of young beech trees (leaves, branches and trunk) in four treatments: moderate soil water deficit (MD), severe soil water deficit (SD), defoliation (D) and control (C) in February and June 2016. Different superscript letters indicate significant difference between treatments for a given date. A significant seasonal effect is indicated with stars (*; p<0.05, **; p<0.01; ***; p<0.001). Note that leaves correspond to marcescent leaves in February and to new leaves from spring growth in June.

		Biomass (g)			N quantity (g)			^{15}N quantity (mg)		
Organs	Treatment	February	June	Seasonal effect	February	June	Seasonal effect	February	June	Seasonal effect
LEAVE	**C**	48.5 ± 15.6	146.6 ± 36.8		0.25^{a} ± 0.09	3.29^{a} ± 0.84	***	0.63^{a} ± 0.22	6.08^{a} ± 1.71	***
	D	20.2 ± 5.3	122.1 ± 30.2	***	0.12^{ab} ± 0.03	2.67^{ab} ± 0.66	***	0.41^{a} ± 0.1	4.93^{a} ± 1.23	***
	MD	28.1 ± 13	38.9 ± 13.2		0.12^{ab} ± 0.07	0.78^{b} ± 0.27	*	0.24^{ab} ± 0.13	1.2^{ab} ± 0.39	*
	SD	5.5 ± 2	43.1 ± 32.6	*	0.03^{b} ± 0.01	0.87^{b} ± 0.66	***	0.05^{b} ± 0.01	1.11^{b} ± 0.88	**
BRANCHE	**C**	168.8 ± 97.2	255^{a} ± 84.4		1.51 ± 0.93	1.76 ± 0.54		3.93 ± 1.72	2.8 ± 0.67	
	D	147.6 ± 49	184^{ab} ± 37		1.32 ± 0.45	1.4 ± 0.26		6.33 ± 2.59	2.62 ± 0.55	
	MD	115.2 ± 50.8	106.7^{ab} ± 18		1.05 ± 0.44	0.94 ± 0.19		4.16 ± 1.77	2.35 ± 0.59	
	SD	47.3 ± 25.1	58.9^{b} ± 5.1		0.46 ± 0.27	0.46 ± 0.03		1.68 ± 1.03	0.96 ± 0.12	
TRUNK	**C**	395.1 ± 93.5	674.8 ± 180.6		1.67 ± 0.44	2.06 ± 0.73		3.69^{a} ± 0.8	2.33 ± 0.63	
	D	349.5 ± 106.9	394.2 ± 52.5		1.56 ± 0.48	1.25 ± 0.2		1.94^{ab} ± 0.63	1.03 ± 0.13	
	MD	276.6 ± 99.7	340.8 ± 137.4		0.81 ± 0.23	1.02 ± 0.48		1.38^{ab} ± 0.24	1.18 ± 0.56	
	SD	129.3 ± 80.6	296.5 ± 95.4		0.41 ± 0.23	1.02 ± 0.34		0.53^{b} ± 0.21	1.15 ± 0.53	

A significant seasonal effect were noticed on D (*p<0.001)* and SD (*p<0.05)* trees on leaves biomass but not on MD and C trees (**Table VII.2)**. Higher biomass of twigs were found on C trees compared to SD trees (255g versus 58.9g, *p<0.05*). No effect of soil water deficit or defoliation were found on the biomass of trunk and no effect of defoliation were found in any compartment compared to C trees. Higher leaf N quantity were measured on C trees (0.25g) compared to SD trees (0.03g) in February and in June (3.73g for C and 0.87g for SD) and C was also different from MD in June (0.78g). Significant difference of season were found in all treatment for leaves N quantity. When regarding N quantity on branches and trunk in February and June, no effect of treatment were found. Finally, the soil water deficit reduced drastically the ^{15}N quantity measured in leaves (0.05 mg in SD and 0.63 mg in C trees) but also in trunk (0.53 mg in SD and 3.69 mg in C trees) at the end of the resorption period. In June, the soil water deficit reduced also drastically the ^{15}N quantity found in the leaves (1.11 for SD trees) compared to C and D trees (6.08 and 4.93 mg, respectively). The same tendency was observed in the trunk and the branches but these differences were not significant. The ^{15}N quantity did not change significantly in the branches and the trunk among the seasons in the four treatments whereas it increased in the leaves between February and June. This increase were more important in the C and D treatments (+6.27mg for C and +5.92mg for D) than in the drought treatments (+1.26mg for MD and +1.22mg for SD).

7.3.8. Biomass, nitrogen and ^{15}N partitioning and allocation among tree compartments

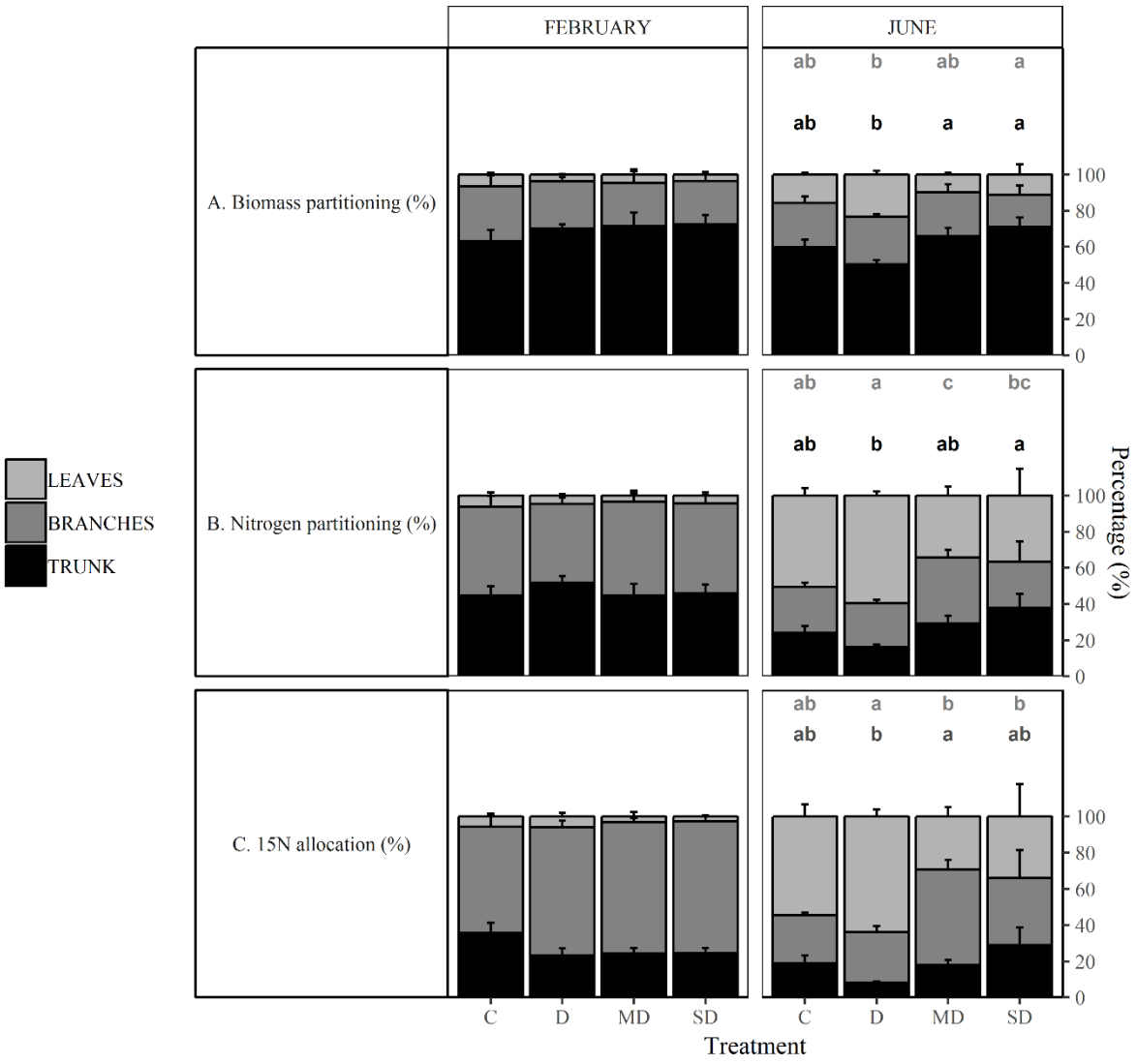

Figure VII.7. Biomass (A), nitrogen (B) partitioning and ^{15}N (C) allocation between aboveground organs (leaves, branches, trunk) of young beech trees in February and June 2016. Each line of letters indicates significant differences between treatment for a given organ: lower black letters for trunk, middle grey letters for branches and upper letters for leaves. n=6 for C and D and n=3 for MD and SD. Note that leaves correspond to marsescent leaves in February and to new spring leaves in June.

In February, the biomass partitioning (**Figure VII.7.A**) were mainly on trunk which represented up to 60% in all treatment followed by branches biomass. Litter biomass were minor with less than 10%. No difference on biomass partitioning was found in February among treatments. In June 2016, at the theoretical end of spring remobilization, the biomass of trunk consists still as the main partitioning, but, contrary to February, the biomass partitioning was different between

D and MD and SD trees with higher trunk biomass and lower leaves biomass in SD than in D trees.

When regarding N partitioning (**Figure VII.7.B**) in February, in all treatments, the trunk and branches were the two highest contributors with more than 40 % for each and only less than 10% left in litter. In June, the main organs for N partitioning was leaves in all treatments but higher values were observed in D trees than in MD and SD trees while C trees had higher N partitioning on leaves than MD. The N partitioning in trunk was higher in SD trees compared to D trees.

The ^{15}N (**Figure VII.7.C**), *e.g* the mobile N which come from leaf N resorption, in February were mainly located on branches (up to 70%) followed by trunk and very few in leaves. In June, the ^{15}N which is remobilized for spring growth were located mainly on leaves for C and D trees. In MD and SD trees, the ^{15}N stay mainly into branches (40%) where it was also mainly located in February. The ^{15}N partitioning in leaves was strongly lower in drought treatments than in D. The ^{15}N partitioning in trunk was the lowest in all treatments.

7.4. Discussion

In the present study, we submitted trees to prolonged drought or defoliation stress during two years and the first consequence was a reduction of the growth in the drought treatment whereas defoliation did not affect growth. Reducing growth following drought events is a common response of beech trees in various site (van der Werf *et al.,* 2007; Charru *et al.,* 2010). Is N limitation could explain the decrease of growth rate following drought or defoliation?

We found during the summer 2015 that trees under water deficit or defoliation still have high leaf N concentration, especially in D trees. Such high N concentration on leaves might be not entirely related directly to photosynthetic machinery but e.g on osmotic tolerance with amino acids, such as proline, which can act as an osmoprotectant or to compensatory photosynthesis after the defoliation. Such high partitioning to leaves and more particularly to foliar protein could be also a strategy for local nitrogen storage as hypothesized in others studies (Ourry *et al.,* 2001; Millard *et al.,* 2007). During 2015-vegetative season, high tree N quantity must had be found on leaves and, consequently, defoliation must had resulting of a major loss of N and through the decrease of REW observed since the beginning of our experimentation on drought treatment soil N availability must be decreased.

In autumn, prior to dormancy, deciduous species shed their leaves. Nutrient resorption is a fundamental process by which tree can withdraw nutrients from senescing tissues prior to abscission (Lu *et al.,* 2012). Our ^{15}N foliar labelling experiment was made at the end of September. At the time of labelling, leaves was still green and we can be confident that senescence was not occurring at this time. Tracking the storage process by applying enriched nitrogen was one goal of this study. This goal was reached as we found ^{15}N on leaves but also on woody parts one month after the labelling, in October. The color of leaves changed between the time of labeling and the time of the first harvest passing from pronounced green to lighter green or yellow indicating that change in pigments or chlorophyll shall occur. As pigments are made of N, it was a good way to determine if senescence occurs visually. Consequently, we can be confident that N resorption already occurred between labelling and the first harvest.

7.4.1. Branches as the main storage location of N from autumn resorption

Recycling N from leaves to perennial parts shall end in February (El Zein, 2011), at the time of our second harvest. Indeed, our results go on this way as N and ^{15}N concentrations found on

leaves in February was lower than in October ($N_{leaf\ in\ July} > N_{leaf\ in\ October} > N_{leaf\ in\ February}$). Such result indicate that in October, leaf N resorption was not finished. Previous studies have shown that nutrient resorption can account for ~31% of annual tree nitrogen (Cleveland *et al.,* 2013) and where temperate deciduous tree may exhibit the most N resorption (Hai-Yang *et al.*, 2018). Low leaf N concentration was found on litter indicating that N resorption was very efficient among treatment. Compared to vegetative season, about 60% of leaf N was recycled in all treatment and it was in accordance with those found in meta-analysis by Hai-Yang *et al.,* (2018). This result contradict our second hypothesis which was that under stress, trees will intensify its leaf N recycling. However, we can hypothesize that the rate of N that was able to recycle in autumn was not so far from 60%. Indeed, the remaining leaf N in the litter was probably a mix from structural N and non-structural N as we found small but still concentration of ^{15}N in the litter. Considering only the aerial parts of trees (excluding roots) leaf N containing in the litter represented less to 5 percent. However, total resorption were not possible because some N shall be fixed into structure and, so, cannot be recycled.

We found that closest organs from leaves, *e.g* branches, were the main sink organs for recycled N and less to distant organs such as roots and trunk as found on other studies on Mediterranean oak species (Mooney and Hays, 1973; Cherbuy *et al.*, 2001; Palacio *et al.*, 2018). It could be a strategy for trees to keep N near from leaves for the next spring to reduce the cost associated to the remobilization. Another reason is considering that youngest twigs was the most active perennial organs and could suffer more from freezing damage, as negative temperatures and cold episode occurred often in Lorraine's Region during winter and amino acids such as proline can act as a protection against freeze hardiness (Janska *et al.*, 2010).

7.4.2. Impacts of two years of soil water deficit or consecutive defoliation on internal N quantity

If the aim of the defoliation was to significantly decrease internal N pool as up to 30% of total N in June (El Zein *et al.*, 2011), our results tend to say that it was not the case. Indeed, same N quantity has been found stored in trunk in D and C trees. In contrast to defoliation, trees which presented the lowest values of pre-dawn water potentials (SD) exhibited lower N quantity than control trees on aerial perennial organs. Under mild drought, soil exploration could be increased as observed by Gruber *et al.,* (2013) by increasing the total absorptive surface of root systems towards high nutrient soil patches (Kiba and Krapp, 2016). In our study, considering the dryness of the soil of the drought treatment, no root growth was possible (J.Levillain, personal

communication). Despite an efficient leaf N resorption of MD and SD trees, the limitation or even suppression of the root N uptake during consecutives vegetative seasons induced N reserves very scarce compared to irrigated trees. Consequently, under continuous severe N limitation due to drought, trees might be not able after a certain duration of soil water deficit to meet their N demand.

7.4.3. Spring remobilization could be impacted by recurrent drought treatment but not through defoliation.

In spring, stored N are remobilized and used for initial growth of beech trees (El Zein *et al.,* 2011a; Bazot *et al.,* 2013). As in 2015, 2016-growth was repressed in MD and SD trees in both trunk and branches. A decrease of trunk growth has been also found on D trees. Others studies found also a decrease of growh following defoliation on seedlings of *Quercus velutina* (Wiley *et al.,* 2013), saplings (Maguire *et al.,* 2015, Schmid and Palacio, 2017) or on mature trees of *Northofagus pumilio* following defoliation (Piper, 2015). After two consecutive yearly defoliation, D trees exhibited the same number of leaves than C trees. Following defoliation, higher LMA was generally found in others studies (Millard *et al.,* 2001; Nabeshima *et al.,* 2001). When computing multidinuous data on experimentation on tree mortality worldwide, Greenwood *et al.,* (2017) showed that tree species with lower SLA (which is 1/LMA) showed lower mortality responses. Whereas LMA was not significantly different among treatments in October 2015, after the new leaf flush in 2016, LMA in D trees was still not significantly but slightly higher than control. Adaptation of deciduous trees to defoliation has been suggested by authors (Krause *et al.,* 1993; Piper, 2015) and making smaller leaves to reduce the cost in case of defoliation could be an adaptation to defoliation. However, in our study, smaller individual leaves has been noticed only after the second defoliation making this assumption only true when recurrent defoliation occurs.

Contrary to carbon remobilization that is sink driven, N remobilization in spring is thinking to be source driven and may not be affected by current N availability in the soil during the growing season (Millard, 1996; Millard and Grelet, 2010). Bud removal experiments have demonstrated that N remobilization is driven by the size of the storage pool and not by the sink strength (Millard *et al.,* 2001; Millett *et al.,* 2005). To sustain leaf flush and spring growth, trees mainly used the recycling N in perennial organs from previous season (*e.g* ^{15}N) in C and D treatment but not at the same rate than in MD and SD treatment. The main sink for N remobilization in spring was leaves as 50-60% of recycled N in previous winter was remobilized in C trees

whereas only 35% and 30% in MD and SD trees. Sustain leaf metabolism is still a key priority for trees under disturbance but disruption of transport system could also explain why only one third of the remobilized N was found on leaves. Both disruption of transport system and the source-driven hypothesis may explain why less previous N recycled was allocated to new leaves flush.

7.4.4. Growth limitation as a consequence of N shortage?

Trees did not seem to be under nitrogen limitation following two harsh yearly defoliation. Consequently, the small decrease on tree growth after defoliation might be not a consequence of solely a N limitation so, others hypothesis could be made on what factors can limit growth. Although, we can hypothesize also that tree growth repression could come not from a single source but from a combination of a lack of carbon and nitrogen induced by defoliation, or by others nutrients such as calcium or potassium. Another explanation could be a tradeoff between storage and growth (Palacio *et al.,* 2014; Piper, 2015) such as a preventative allocation shift. Such tradeoff is thought to be an adaptation of deciduous species to defoliation, as it can be re-mobilized to meet demands for re-foliation and growth when root nutrient uptake fails (Millard *et al.,* 2001; Millard and Grelet, 2010). Finally, finding an explanation on why tree under defoliation did not present evidence of N limitation could have a response on belowground systems. D trees were under irrigation systems in the same way that C trees and tree roots system responses to defoliation should be central to regulating the long-term effects of defoliation. Study have found that following defoliation, root N uptake can decrease quickly after defoliation (Parsons *et al.,* 1983; Jarvis and Macduff, 1989; Kosola *et al.,* 2001) or not (Fotelli *et al.,* 2004; Peuke and Rennenberg, 2004). As compensatory mechanisms of photosynthesis could occur after defoliation as found on the same experiment in 2015 (**Chapitre V**), defoliated trees can invest more NSC to belowground organs and export to root, where the carbon is rapidly assimilated into amino acids at the site of N uptake (Roche *et al.,* 2017) leading to re-enhance of root N uptake. Such results could be also explain by our soil composition which come from an agricultural soil which is richer than natural forest soils regarding available nutrients.

Perennial tree lifecycle regarding N ressources consists to a strong remobilization of stored N to sustain growth and leaf expansion at the beginning of vegetative before rely on current N uptake and, finally, by a strong N resorption from leaves to perennial organs. Consecutive to the onset of soil water deficit, N uptake must had depressed leading to less new incomings to

sustain N metabolism. As we found strong N resorption, it could be a strategy for tress under severe soil water deficit to work as a "closed system". Our finding that less previous resorbed N has been reallocated to new leaf flush could argue as the view of "closed system". Indeed, even if allocate N to leaves is essential to have a production of new photosynthetates through photosynthesis, keeping more N to perennial parts could be a strategy to prevent risks from defoliation, which is a common drought response (Bréda *et al.*, 2006; Galvez *et al.*, 2011; Ryan, 2011). Furthermore, we also found that little but still N was "lost" in the litter (up to 30% of initial leaf N) and during a period of strong reduction of N uptake, such a loss could be hazardous for survival. As we fond strong growth reduction, our results could argue in favor of an allocation shift by decreasing growth in favor of storage. However, we must be careful with such interpretation as we not access to the quantity of N left on belowground organs. Finally, in the same way than for defoliation, we can also make the hypothesis that others coumpounds might be missed to explain this growth reductions such as carbon, potassium, calcium …

7.5. Conclusion

Currently, a growing debate on whether extreme disturbance associated with climate change (e.g. defoliation or drought) provoke N limitation in trees (Millard and Grelet, 2010). Our study provides a valuable knowledge about how internal N cycling and recycling could have been impacted by disturbance, by using a labelling tool. We were able to show a significant reduction of N stored in trees under severe soil water deficit which is really difficult to show under natural drought conditions. To succeed to prove that trees can be under N privation, we made 24 month of drought treatment, which underline also the resistance of this deciduous species to extreme constraints.