DYNAMICS OF NON STRUCTURAL CARBOHYDRATES (NSC) IN 10 YEARS-OLD FAGUS SYLVATICA L. YOUNGEST TWIGS FOLLOWING EXPERIMENTAL DEFOLIATION OR DROUGHT WHICH INDUCED MORTALITY (in preparation)

V. DYNAMICS OF NON STRUCTURAL CARBOHYDRATES (NSC) IN 10 YEARS-OLD FAGUS SYVATICA L. YOUNGEST TWIGS FOLLOWING EXPERIMENTAL DEFOLIATION OR DROUGHT WHICH INDUCED MORTALITY

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ABSTRACT

Plants acquire carbon via photosynthesis and use most of it as substrate to sustain their metabolisms (e.g respiration) and to build structural biomass. A smaller fraction is retained in the form of nonstructural carbohydrates (NSC). NSC pools are broadly referred as storage because they support metabolism at night and when carbon assimilation is insufficient to meet demand. In addition, NSC show a seasonal dynamic in deciduous trees. Mobilization and refilling of the NSC stores lead to fluctuations in these storage pools. As future climate scenarios under ongoing climate change predict that drought frequencies and intensities will increase in several regions worldwide, the ability to understand future tree reponse to and survival of water deficit has become increasingly important. Due to the critical role of storage for survival under stress, these predicted changes have led to a recent renewed interest in NSC storage, which is increasingly acknowledged as a key component in tree and ecosystem models. Despite their critical role in the plant carbon balance, our understanding of the dynamics of NSC in perennial plants remains limited. Seasonal dynamics of NSC have been well assessed in several temperate forest key species (*Quercus, Fagus*). However, the contribution of stored NSC to sustain internal plant metabolisms against abiotic stress or the existence of NSC thresholds in these species are poorly understood. Our main goal is to explore seasonal NSC dynamics and their potential biological significance in the context of extreme constraints. To do that, we used a dedicated experimental design with 10-year-old *Fagus sylvatica* L. which have been submitted for three years to repeated artificial defoliation and intense soil water deficit. NSC concentrations (soluble NSCs and starch) in twigs of control and stressed trees were assessed at key physiological dates during each season from 2014 to 2016. We found that following three years of treatments, NSC concentrations were altered in response to extreme constraints, starch and others soluble sugars had different patterns meaning a different biological significance. Our results seems to indicate that slight but crucial change of sugars composition have led to increase survival of trees under severe water deficit treatment.

Keywords: soil water deficit, defoliation, soluble sugars, *Fagus Sylvatica L.,* Non-structural carbohydrates, branches, osmoregulation

5.1. Introduction

Climate change might result in more intense and more frequent drought events particularly in temperate regions (IPCC, 2013). Even if forest ecosystems are known to be resilient and have faced to disturbance for century, this climate-mediated increases in disturbance could be exceed the capacity of forest to cope with such rapid changes (Reyer *et al.,* 2015; Seidl *et al.,* 2016). Indeed, recent evidence of drought-induced mortality has been reported in many regions of the world resulting of a renewed interest in studying forest mortality in the scientific community (McDowell *et al.,* 2008; Sala *et al.,* 2010; Allen *et al.,* 2010; Anderegg *et al.,* 2015; Johnson *et al.,* 2016). Relevant key physiological mechanisms are needed for models to reliably predict forest responses to environmental changes especially when mortality occurs (McDowell *et al.,* 2013) and carbon metabolism is one of these (Martinez-Vilalta *et al.,* 2002; McDowell *et al.,* 2008).

Trees fix CO_2 from atmosphere via photosynthesis that allows providing critical source of C for various sinks such as growth, respiration, turgor maintenance, supply solutes for water movement or into mechanisms involved in defense (Chapin *et al.,* 1990; Korner, 2003; Sevanto *et al.,* 2014; O'Brien *et al.,* 2015). According to the time in the season and the environmental conditions the carbon source-sink balance may lead to periods of carbon surplus or deficit. For example, when soil water content becomes limiting, trees close their stomata to limit their water loss by transpiration but this impacts also negatively the photosynthesis (Bréda *et al.,* 2006; Palacio *et al.,* 2008; Hartmann *et al.,* 2013; Piper and Fajardo, 2014; Dickman *et al.,* 2015). Consequently, the carbon balance may become negative (McDowell *et al.,* 2008; Rowland *et al.,* 2015). To face to temporary negative carbon balance trees can use carbon stored in their perennials organs under form of starch and soluble sugars which constitute the non-structural carbohydrates (NSC). NSC quantity are dynamic within the seasons especially on deciduous trees, with increase starch concentrations in woody tissues at the end of the growing season (Barbaroux *et al.,* 2003; Damesin and Lelarge, 2003; Dietze *et al.,* 2014; Sevanto *et al.,* 2014; Hartmann *et al.,* 2016). These NSC are then used to maintain the winter metabolism during the non-leafy period (cold tolerance, maintenance respiration) and are also fundamental for new leaf growth during the next spring (Penning de Vries, 1975; Sakai and Larcher, 1987; Wanner and Junttila, 1999; Ogren, 2000).

When photosynthesis is altered by drought or by a loss of photosynthetic tissue through defoliation or branch mortality it has been hypothesized that trees are forced into C storage dependency to meet continuous carbon demand for osmoregulation, respiration and defense against pathogens (McDowell *et al.,* 2008; Sala *et al.,* 2012). In the long term, trees might not survive if carbon supply by photosynthesis and stored NSC does not meet overall carbon demands (Martinez-Vivalta *et al.,* 2012). Several studies have focused of the role of carbon into mortality processes but this role need more investigations because contradictory results were observed about NSC dynamics under disturbances (Piper, 2011; Hartmann *et al.,* 2013; Zhao *et al.,* 2013) as summarized in a recent report by Alice Delaporte (2015, thesis).

In the cohort of organs involved in carbon metabolisms under drought, leaves play a crucial role in balancing the risks of carbon starvation and hydraulic failure through the stomata regulation, since they are the site of carbon assimilation and sugar production, as well as the main source of water loss for trees. Consequently, by bearing leaves and being the more proximal perennial organs of the new photoassimilates production, we hypothesized that twigs have a critical role in carbon acquisition strategy. Indeed, with the study of twigs, we search have a good proxy of the metabolic adjustments made under stressful conditions but little is known about the specific response of twigs which supporting leaves (Salmon *et al.,* 2015). Moreover, when disturbances such as drought or defoliation limit growth, change of NSC concentration should take place in tissues proximate to those where primary growth occurring, e.g twigs (Bazot *et al.,* 2013; Piper *et al.,* 2016) because trees usually cover their demand by first mobilizing stored resources from the closest possible storage sites (Landhausser and Lieffers 2003).

NSC are mainly formed by both starch and soluble sugars. Soluble sugars and starch have different functions: while starch act as a buffer, soluble sugars are involved as osmoticum into immediate metabolic functions such as defense, respiration, growth, osmoregulation … (Dietze *et al.,* 2014). Whereas depletion of soluble sugars is very rare, starch depletion can be found in some organs (Martinez-Vivalta *et al.,* 2012). One mechanism which has a crucial role to prevent mortality is the possible interconversion from long chain of carbon products to smaller one by the intermediate of an enzyme such as amyloglucosidase which can cut starch granules into fructose and glucose (Schimpf and Stosser, 1984; Lacointe *et al.,* 1993). Such interconversion are only possible under sufficient amount of water and under a certain range of temperature (Sauter, 1967; Sauter and Ambrosius, 1986). But interconversion from starch to lower C products are not the only possible mechanisms of interconversion because glucose and fructose can combine to form sucrose, the main form of sugar used for transport. By studying differents sugars through starch to smaller ones, we can have a better idea of metabolic adjustment operate under disturbance.

In this study, we propose to get further in the understanding on how tree cope with disturbance which can lead to mortality by investigating the effect of extreme drought stress or defoliation on NSC concentrations and more particularly on the composition of individual sugars in specific organs, proximal twigs, on *Fagus Sylvatica* L. trees, a deciduous broadleaf species, widespread in Europe. The current distribution of *F.Sylvatica* L. is limited by cold winters in the east, drought in the south and long winter and freezing events in the north. Beech trees grows typically on moderate dry to moderaly moist soil conditions (Ellenberg and Leuschner, 2010). Numerous dendrochronological and ecophysiological studies suggest that European beech growth is more sensitive to disturbance like drought than others major broad-leaves species in Europe (Dulamsuren *et al.,* 2016) but it has been also reported that seedlings recover quickly from severe drought episodes (Aranda *et al.*, 2005 ; Gallé and Feller, 2007) and might indicate that local internal physiological adjustments could occur. One key to partially understand this particularity of beech sensitivity is to study to response in term of sugars concentrations and compositions which might be reflect the physiological adjustments operate when beech is under severe disturbance.

In the present study we explored, at key phenological stages for NSC dynamics in beech (Barbaroux *et al.*, 2002) the influence of a defoliation or a drought on seasonal patterns of of twigs carbon reserves. We focused on starch and soluble sugars by detailing main specific sugars. We made the following hypotheses: (1) we made the hypothesis that working on twigs might be a good proxy of NSC dynamics and, consequently, we would be able to find the same dynamics that was found in previous study on beech (2) Then, under soil water deficit or defoliation, the concentration of low-weight sugars will be increase due to interconversion of starch into sucrose, glucose or fructose to cope with specific metabolic demand (*e.g* osmoprotection). (3) Finally, when mortality occurs, we expect more starch depletion than soluble sugars depletion.

5.2. Materials and methods

5.2.1. Site and experimental design

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 submitted to three different treatments for three years (2014, 2015, 2016): (1) control (C) in which the trees were regularly irrigated (n=336 trees); (2) defoliation (D) in which the trees were submitted to a yearly defoliation and regularly irrigated (n=336 trees). Manual defoliation of the trees in treatment D was done in June for the two consecutive years (2014 and 2015) and consisted in 75% of removal of the whole foliage, making sure the removal was homogeneously distributed throughout the tree crown. In 2016, a third manual defoliation was made earlier in the vegetative season (May) and at a higher severity (90% of removal) to constraint more C metabolism; (3) drought (Dr.) where the trees were submitted to soil water deficit (n=336 trees). The drought stress level 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. 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 (8 trees per treatment) was checked by measuring pre-dawn water potential in twigs (ψ_{pd}) montly during vegetative season in 2014, 2015 and 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). At the end of each vegetative season, height and diameter were measured using a caliper or a beam respectively.

5.2.2. 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:

$$\mathrm{REW}=100 * \frac{\mathrm{TAWC-R}}{\mathrm{TAWC}} \tag{9}$$

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).

5.2.3. Sampling protocol

Twenty-four trees (8 C; 8 D; 8 Dr.) were randomly chosen. Additional dead trees were chosen when mortality occurred (n=4 in October 2015, June 2016 and Octobre 2016). We have determined mortality on the base of sudden dryness of leaves and a change of color of the trunk (for dead trees taken in October) and trees which did not present budburst for dead trees taken in May. From October 2014 to October 2016, each year, twigs were sampled at two key phenological dates i.e.,i) at the theoretical minimum of NSC in May, 3 weeks after the end of leaf expansion, and ii) at the theoretical maximum of NSC in October, at the end of vegetative season. For each sampling date, three twigs were sampled on each trees. We took three twigs located at the crown at breast-height and made sure that twigs were representative of all twigs in the sampled trees and they were with enough material. Each sampled twigs was divided by annual growth unit between one-year-old twigs and current year-old twigs. One-year-old twigs were sampled at each date but current-year twigs were sampled only at end of each vegetative season because in May they were still undergoing lignification. We took off dormant buds. We were not able to make our samping during precise hours of the days, consequently, leaves samples were not assessed in this study. All samples were immediately frozen in liquid nitrogen, and then stored at -80°C. All samplings were then freeze-dried (Dura-Top (r), Dura-Dry (r), FTS Systems (r), Stone Ridge, NY, USA), weighed and ground into a fine powder with a ball mill (CEPI SODEMI CB2200, Cergy, France). The scheme of the experiment is presented in **Figure V.1**.

5.2.4. Non-structural carbohydrate analyses

Soluble sugars were extracted from 10 mg (±0.5) of each sample with 1 mL of 80% (v/v) ethanol/water at 80°C for 30 mn and then centrifugated at 10,000g for 10 min at 4°C. This step was repeated three times and the resulting supernatants were pooled together. The pellet was retained at -20°C waiting for starch analysis and the supernatants, containing soluble sugars, were dried overnight with a vacuum evaporator (Heto Maxi Dry Lyo Freeze-dryer) to eliminate ethanol. Dried extracts were rehydrated in 1.5 mL of ultrapure water, redissolved on ice by sonication and then filtered at 0.2 µm. Main simple sugars (sucrose, glucose, fructose, raffinose,) concentrations were determined by peak height measurements against standard calibrations on ionic chromatography (Dionex, Thermo Scientific, USA). Others sugars were also found but at lowest concentrations and they were summed as “others sugars”. The pellet was hydrolyzed with 0.02N NaOH for 30 min at 100°C, then cooled at ambient temperature, and hydrolyzed in glucose molecules with amyloglucosidase (from *Aspergillus Niger*, Sigma Aldrich, Saint Quentin Fallavier, France) for 30 mn at 50°C. Glucose was quantified using hexokinase and glucose-6-phosphate dehydrogenase followed by spectrophotometric determination of NADPH formation at 340 nm (Boehringer, 1984). Soluble sugars and starch concentrations were expressed in g.100g^{-1} DM.

5.2.5. Quantity and partitioning calculations

The quantity of differentiate sugars is defined as (2):

$$\text{Sugar quantity (g)} = \frac{\text{Sugars concentrations} * \text{Weight (DM)}}{100} \tag{10}$$

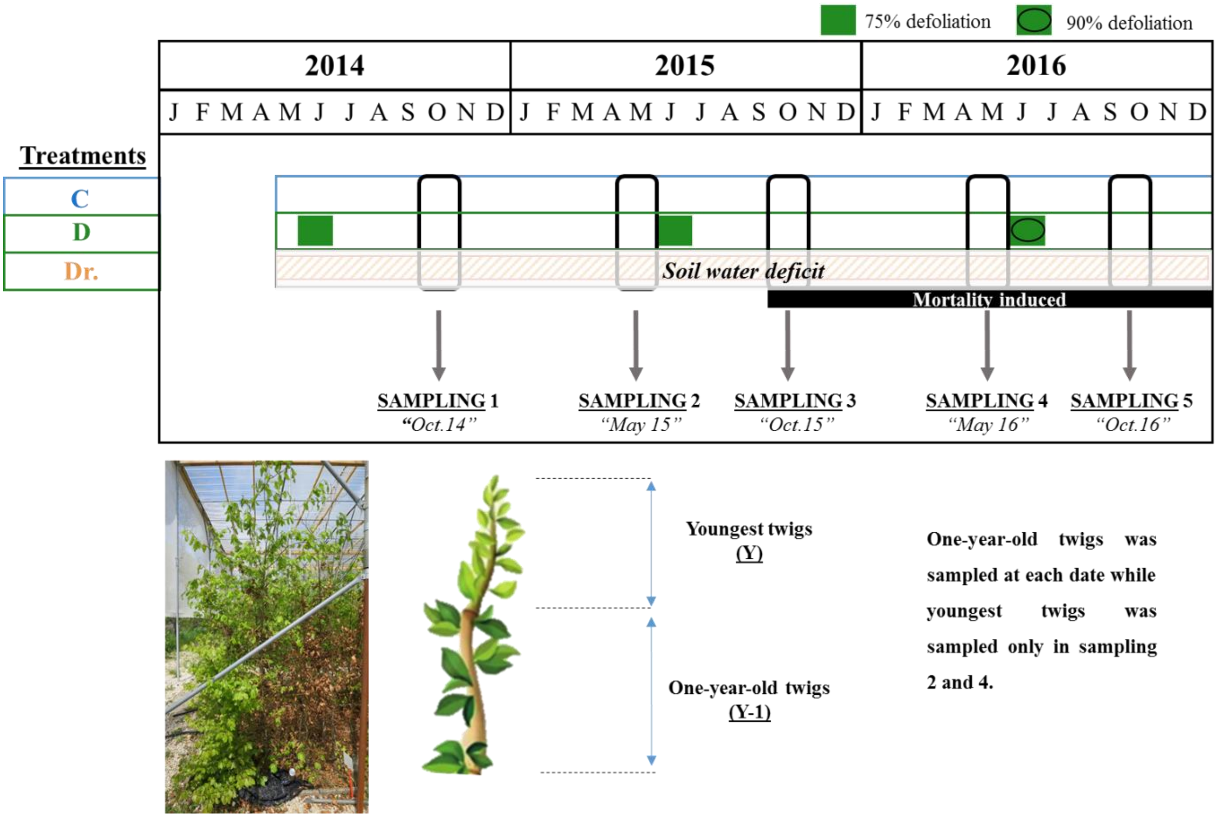
Partitioning of sugars is represented as the ratio (%) of the amount of a given sugar relative to the total amount of sugars (3).

$$\text{Given sugars ratio (\%)} = \frac{\text{Given sugar quantity}}{\text{Quantity of total sugars}} * 100 \tag{11}$$

5.2.6. Statistical analyses

We used general linear mixed-effect models to study seasonal pattern of predawn water potentials from 2014 to 2016. We included treatment and date of sampling as explanatory fixed factors and predawn leaf water potentials as a responses variable. Individual trees were modeled as a random factor. Similar general linear mixed-effect models were also used to study the growth of trees, NSC concentrations, total soluble sugars and differentiated soluble sugars under the three treatments and in the dead trees. If distribution was not normally distributed, a logarithmic transformation was used. Normality and homoscedasticity of standardized residuals were graphically checked using quantile-to-quantile and residual-vs-predicted plots. When a significant effect or interaction between effects were underlined, post-hoc tests were performed. 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. Differences were considered significant for $p<0.05$.

5.3. Results



One-year-old twigs was sampled at each date while youngest twigs was sampled only in sampling 2 and 4.

Figure V.1. Schematic representation of the NSC sampling experiment. Experimental schedule (A): Three treatments were applied over three years (2014, 2015 and 2016) with control (C), defoliation (D) and soil-water-deficit (Dr.). Two intensities of defoliation were applied with 75% of removal in 2014 and 2015 and with 90% of removal in 2016. Three branches per tree (24 trees, 8 in each treatment) were randomly choosen at each date and divided into annual growth. From 2015, twigs of dead trees were also sampled using the same procedure than living ones.

5.3.1. Monitoring of water changes in soil and twigs

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 drought treatments (**Figure V.2.A**). In drought treatment, 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 (ψpd) in twigs of drought trees were always statistically lower than those of C and D trees during the whole time of the experiment (**Figure V.2.B**).

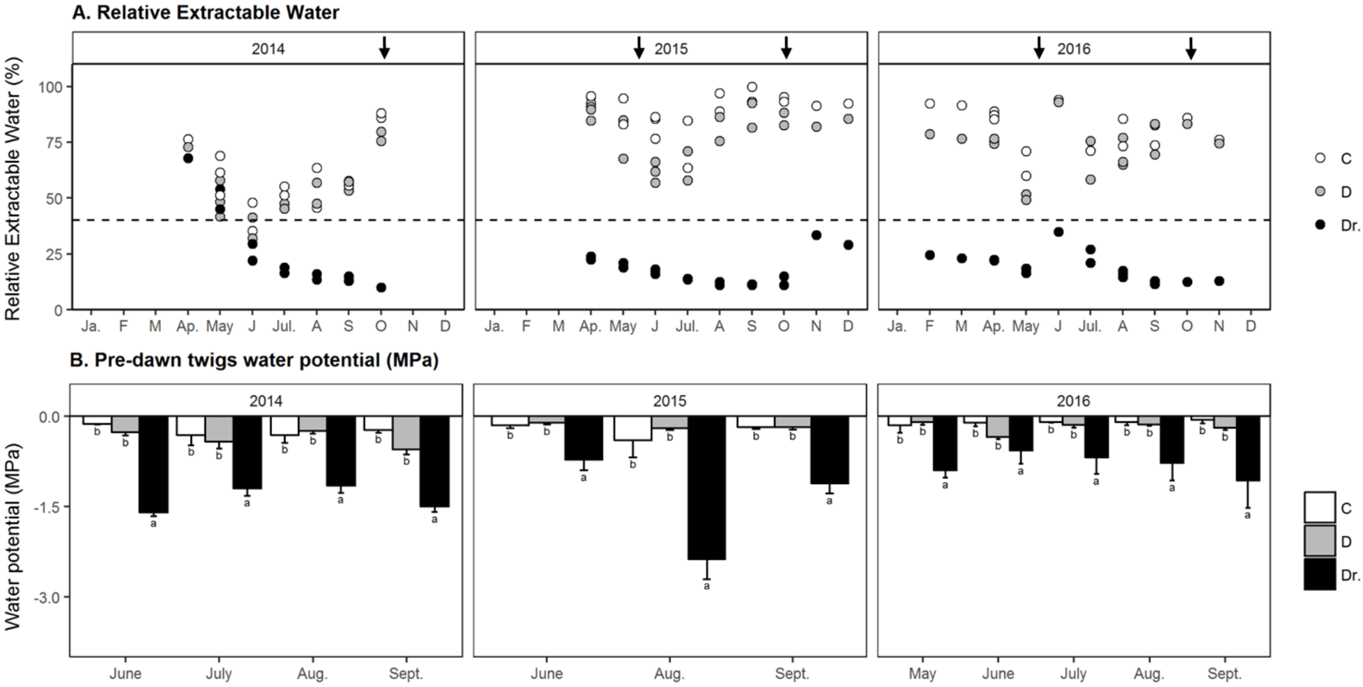


Figure V.2. Seasonal dynamics of the soil Relative Extractable Water during the growing season (REW, A) and the average of pre-dawn water potential of twigs (B) in 8-year-old beech trees since the start of the experiment in control (C), defoliated (D), soil water deficit (Dr.) treatments. Black arrows indicate the dates of the sampling. The dashed line indicate the threshold value of 40% of REW from which the stomatal conductance is impacted according to Granier et al (1999). Different letters means a significant difference (p<0.05) between treatments for a given date; mean ± SE, n=8.

5.3.2. Growth responses to drought and defoliation

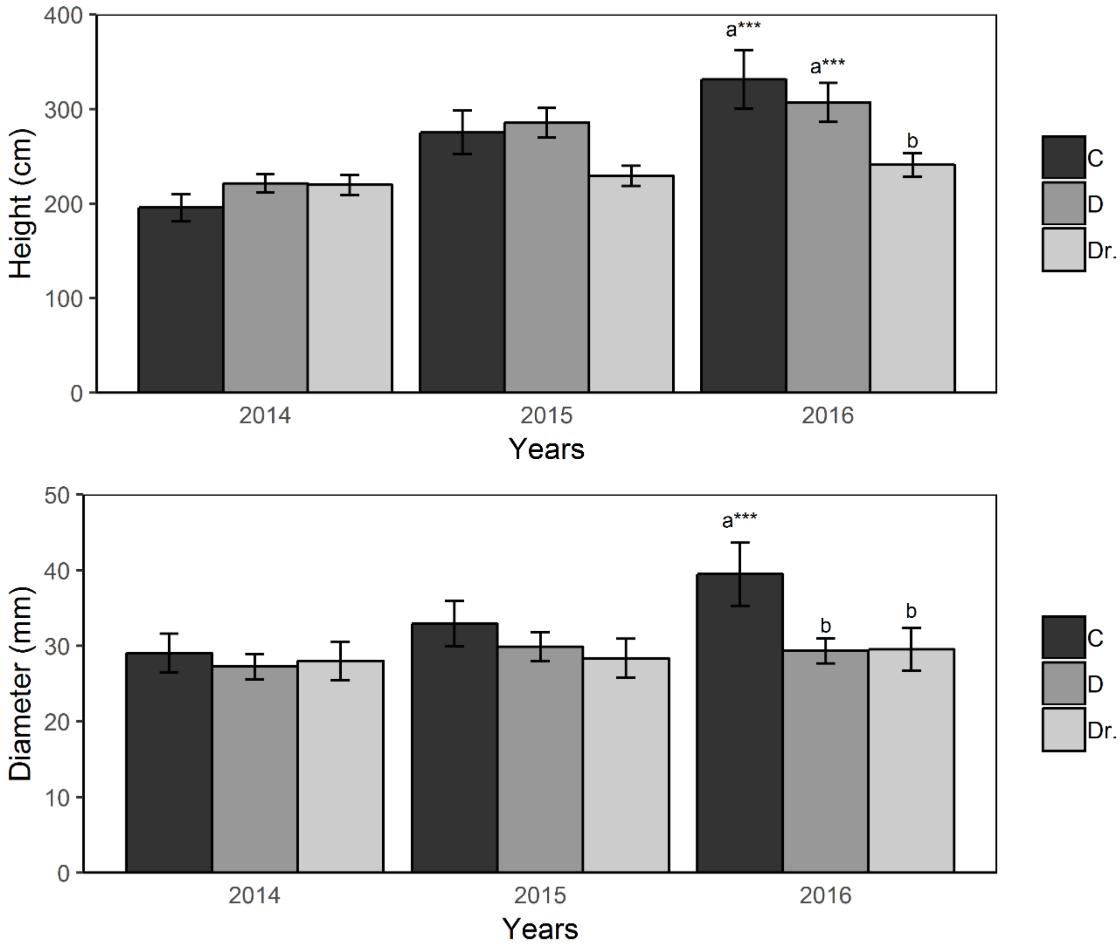


Figure V.3. Mean height (top, cm) and diameter (bottom, mm) soil water deficit (Dr.) or under defoliation (D), C is for control treatment, i.e well-watered trees and no defoliated in 2014,2015 and 2016. Mean ± SE. Different letters indicate significate difference between treatment for a given date while stars indicate a significate effect of time between 2014 and 2016, n=8.

At the consequence of soil water deficit, the height of drought trees was impacted quickly after the onset of treatment and showed lower height than control trees in 2016 ($p<0.001$) and also compared to defoliated trees ($p<0.05$) whereas defoliations had no effect on primary growth during three consecutive years (**Figure V.3**). In 2016, a significant effect ($p<0.05$) was found in D and Dr. trees on primary growth when comparing to the C trees. If we look at the effect of the time (*e.g* if height or diameter were different in 2016 compared to 2014), a significant effect was found on D (*$p<0.001$)* and C (*$p<0.001$)* trees but no significant difference was found on trees under soil water deficit. Considering the diameter, the only effect of the time was noticed on C trees (*$p<0.001$)* but not on D and Dr. trees.

5.3.3. Responses of starch and soluble sugars concentrations to drought and defoliation.

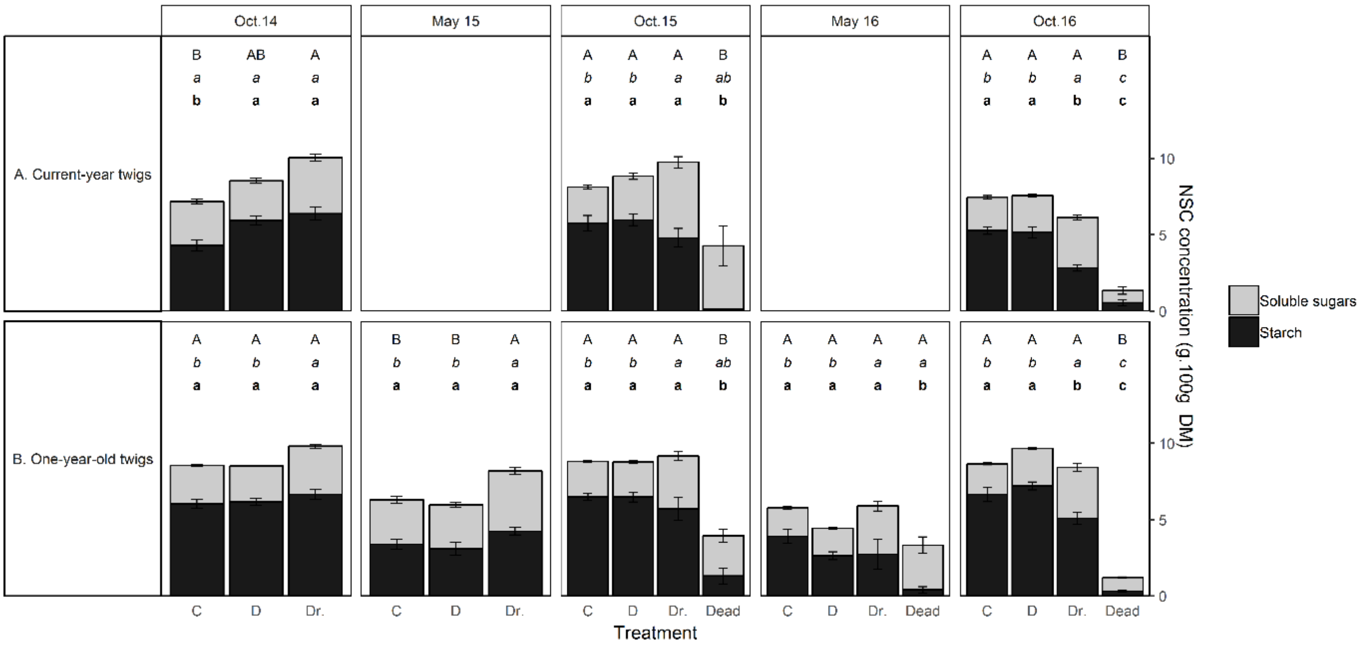


Figure V.4. Mean (±SE) concentration ($g.100g^{-1}DM$) of starch (black) and total soluble sugars (grey) on twigs from current year of sample (A) and one-year-old twigs (B) on trees from of soil water deficit (Dr.) or under defoliation (D) since the start of the experiment. Current-year branches were only sampled at the end of each vegetative season. C is for control treatment. We sampled dead trees in October 2015 and 2016 and in June 2016. Lower lowercase bold letters indicates differences between treatments for starch concentration, middle italic letters is for soluble sugars and uppercase letters is for total NSC difference. n=8.

First, we checked the effect of time between two consecutive sampling dates on one-year-old twigs meaning if total NSC concentration was higher or lower between two consecutive dates (**Figure V.4**). On trees which were irrigated on non-defoliated (control), strong significate differences was found always between two consecutives dates (*$p<0.001$).* Similar results was found on D trees (*$p<0.001$*). However, the only effect of time between two consecutive sampling dates on trees under soil water deficit was found between October 2015 and May 2016 (*$p<0.05$*).

Total NSC concentrations were not different between defoliated and control trees regardless the age of twigs but soil water deficit has an effect on total NSC concentrations in October 2014 on youngest twigs and in May 2015 on one-year-old twigs. When regarding only starch concentration, higher concentrations were found on D trees compared to C in October 2014 in youngest twigs and soil water deficit had an effect on starch concentrations only in October 2016 in youngest and one-year-old twigs. No difference were noticed on D trees regarding total soluble sugars concentration. Dr. trees exhibited higher soluble sugars concentration 6 month after the onset of drought treatment (October 2014), also in May and October 2015 and finally in May and October 2016.

Dead trees sampled in October 2015 displayed lower total NSC concentration and starch concentration but similar total soluble sugars concentrations. 24 month after the onset of drought treatment, dead trees which died from drought had lower starch concentration but similar NSC and soluble sugars concentrations. Finally, at the end of the experimentation and after 30 month of drought, lower NSC, starch and soluble sugars concentrations were found on dead trees compared to living ones.

5.3.4. Impact of drought and defoliation on concentrations of the main nonstructural carbohydrates.

Table V.1. Mean (±SE) concentration (g.100g^{-1} DM) of main sugars (starch, sucrose, glucose, fructose, raffinose) in one-year-old twigs of trees submitted to soil water deficit (Dr.) or defoliation (D) since the start of the experiment. C is for control treatment. We sampled dead trees in October 2015 and 2016 and in June 2016, n=8.

		Starch		**Sucrose**		**Glucose**		**Fructose**		**Raffinose**	
		(g.100g^{-1}DM)		(g.100g^{-1}DM)		(g.100g^{-1}DM)		(g.100g^{-1}DM)		(g.100g^{-1}DM)	
Date	**Treatment**										
		Moy	*ES*	*Moy*	*ES*	*Moy*	*ES*	*Moy*	*ES*	*Moy*	*ES*
October 2014	C	6.04	±0.29	2.28	±0.07^{ab}	0.04	±0.01^{b}	0.03	±0.00^{b}	0.16	±0.02^{b}
	D	6.17	±0.24	1.92	±0.04^{b}	0.07	±0.01^{ab}	0.06	±0.02^{b}	0.30	±0.02^{a}
	Dr.	6.68	±0.32	2.69	±0.06^{a}	0.18	±0.04^{a}	0.22	±0.06^{a}	0.06	±0.01^{c}
May 2015	C	3.39	±0.33	2.11	±0.14^{b}	0.32	±0.08	0.47	±0.07^{b}	0.02	±0.01
	D	3.08	±0.43	2.06	±0.08^{b}	0.31	±0.07	0.49	±0.06^{b}	0.02	±0.01
	Dr.	4.25	±0.23	2.68	±0.13^{a}	0.44	±0.06	0.79	±0.10^{a}	0.02	±0.01
October 2015	C	6.50	±0.24^{a}	2.08	±0.05^{b}	0.03	±0.01^{c}	0.02	±0.01^{c}	0.19	±0.02^{a}
	D	6.49	±0.33^{a}	1.96	±0.08^{b}	0.04	±0.01^{c}	0.04	±0.02^{c}	0.26	±0.03^{a}
	Dr.	5.71	±0.76^{a}	2.59	±0.30^{a}	0.37	±0.16^{b}	0.42	±0.14^{b}	0.06	±0.01^{b}
	Dead	1.32	±0.51^{b}	0.47	±0.18^{c}	1.01	±0.19^{a}	1.06	±0.20^{a}	0.01	±0.01^{c}
May 2016	C	3.91	±0.46^{a}	1.34	±0.07^{a}	0.21	±0.02^{b}	0.31	±0.04^{b}	0.01	±0.00
	D	2.65	±0.26^{a}	1.13	±0.05^{a}	0.27	±0.04^{b}	0.40	±0.04^{b}	0.00	±0.00
	Dr.	2.75	±0.97^{a}	1.01	±0.51^{a}	1.06	±0.33^{a}	1.06	±0.20^{a}	0.01	±0.01
	Dead	0.42	±0.20^{b}	0.72	±0.28^{b}	1.02	±0.18^{a}	1.13	±0.15^{a}	0.04	±0.02
October 2016	C	6.66	±0.48^{a}	1.66	±0.08^{b}	0.08	±0.04^{b}	0.07	±0.05^{c}	0.19	±0.02^{b}
	D	7.22	±0.26^{a}	2.05	±0.04^{b}	0.06	±0.01^{b}	0.04	±0.01^{c}	0.30	±0.01^{a}
	Dr.	5.10	±0.40^{b}	2.61	±0.18^{a}	0.17	±0.06^{b}	0.33	±0.09^{b}	0.21	±0.03^{b}
	Dead	0.27	±0.07^{c}	0.00	±0.00^{c}	0.85	±0.13^{a}	0.74	±0.12^{a}	0.00	±0.00^{c}
		F_{value}	P_{value}	F_{value}	P_{value}	F_{value}	P_{value}	F_{value}	P_{value}	F_{value}	P_{value}
Date		46.4	**<0.0001**	32.6	**<0.0001**	13.1	**<0.0001**	84.3	**<0.0001**	113.1	**<0.0001**
Treatment		1.17	0.3117	19.6	**<0.0001**	22.3	**<0.0001**	63.9	**<0.0001**	22.2	**<0.0001**
Interaction Date x Treatment		3.75	**0.0003**	3.27	**0.0014**	4.2	**<0.0001**	1.41	0.1885	10.05	**<0.0001**

A strong effect of treatment (except starch) and date was found for all sugars and a strong interaction between them (**Table V.1**). Tree under defoliation displays same concentration of each NSC than control except for Raffinose in October 2014 and October 2016. Drought has an effect on multiple sugars concentration in the short term after the onset of drought as represented by significant difference in sucrose, glucose and raffinose concentration in October 2014, sucrose and glucose concentration were still significantly different from control in May 2015. One year and a half after the beginning of soil water deficit, starch, glucose, fructose and raffinose concentrations were different from control whereas trees which died in the drought treatment displayed significant concentrations differences in all sugars from control. In May 2016, drought has an effect only on glucose and fructose concentrations and dead trees has lower starch and sucrose concentrations but higher fructose concentrations than control. Finally, in October 2016, drought has an effect on all sugars concentrations compared to control as same as dead trees.

5.3.5. NSC proportion

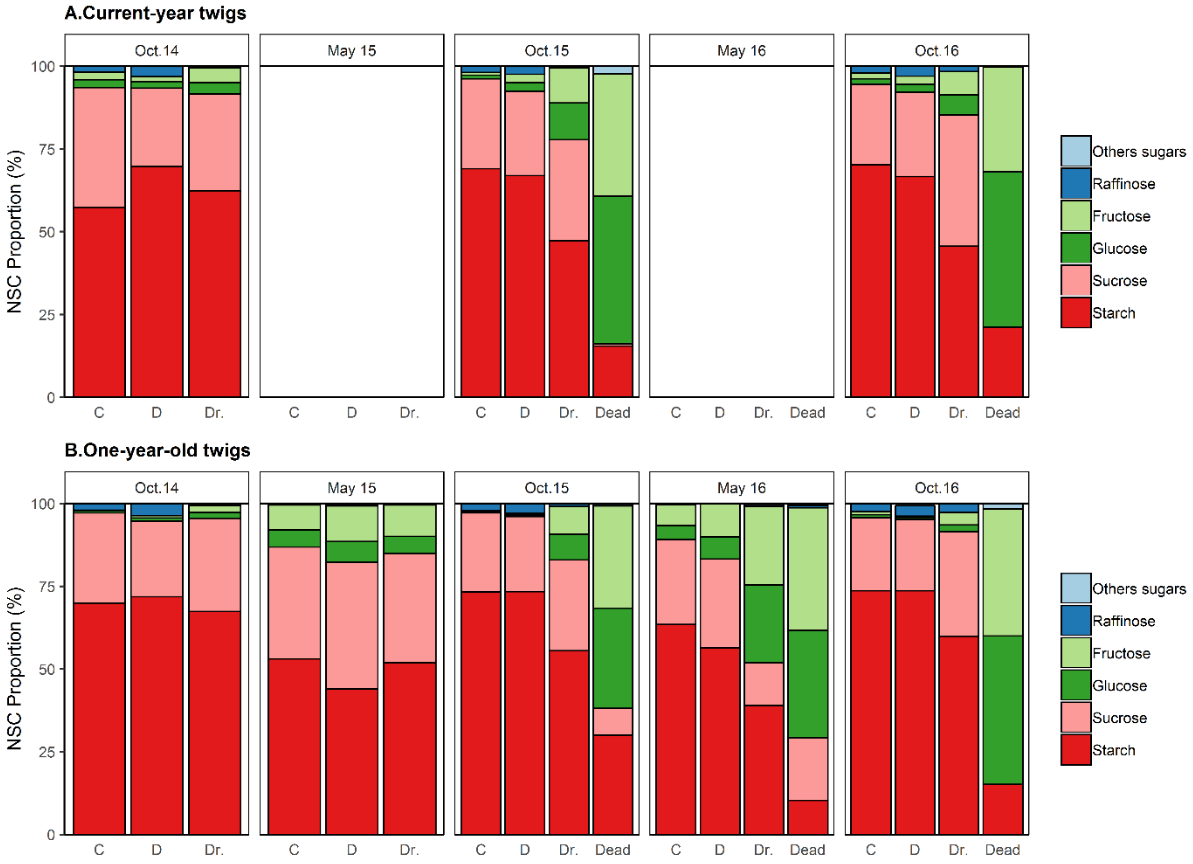


Figure V.5. Proportion (%) of differentiate sugars on current-year (A) and one-year-old (B) branches from tree under drought (Dr.) or under defoliation (D) or dead trees (Dead) since the start of the experiment, n=8.

For trees under control treatment, the highest proportion was starch the end of each vegetative season (average 70%) but C trees had a slight decrease of proportion of starch at the beginning of each vegetative season (average 55%) (**Figure V.5**). After starch proportion, the highest proportion of sugars were sucrose and fructose in May 2015 and 2016 but only sucrose in October 2014, 2015 and 2016. Similar ‘patterns’ were found on trees under defoliation. However, such results were not found on tree under soil water deficit. The proportion of starch decrease when drought time increase and the expanse of the increase of glucose and fructose proportion. Dead trees showed highest proportion for glucose and fructose while starch and sucrose were at minor proportion.

5.4. Discussion

Our first hypothesis was that twigs might be a good proxy to asses NSC dynamics. We found a strong significant effect of the time between two consecutive date of sampling on C trees with higher NSC concentrations at the end of vegetative season compared to the beginning of vegetative season. Our results was similar of those found on mature beech trees by Delaporte *et al.,* (2016) with higher NSC concentrations at the end compared to the beginning of vegetative season or by Bazot *et al.,* (2013) and Gilson *et al.,* (2014) on mature oak and a chronosequence of oak respectively.

Our study combine analysis of short and mid-term effect oftwo intensities of drought or a harsh defoliation on carbon storage dynamics. Under water stress, the behavior of young beech trees might be typical of an isohydric specie, with early stomatal closure that prevented desiccation while photosynthesis was shot down (McDowell *et al.,* 2008). Beech responses to drought might be determined mainly by the rate and degree to which water status is hydraulically regulated. Initially in our study, more than six hundred tree were either under defoliation or under drought treatment but, at the end of the study, 30 month later, no tree under defoliation died whereas 15% of trees under severe intensity of soil water deficit died during the experiment. Why some trees died while others survive is an important question which being debate in the scientific community (Manion 1981; Franklin *et al.,* 1987; Martinez-Vivalta *et al.,* 2002; McDowell *et al.,* 2008; Garcia-Forner *et al.,* 2015; Dai *et al.*, 2018) and the study of NSC dynamics could help for the understanding.

5.4.1. Twigs C metabolism was not impacted by photosynthetic tissue loss through defoliation

Despite three successive years of extreme defoliation (at 75% in 2014 and 2015 and at 90% in 2016), none of the defoliated trees died or showed evidence of die-back. A small effect was observed on both primary and secondary growth but only from 2016. Deciduous species, contrary to evergreen species (Harms and Mattson, 1992; Krause *et al.*, 1993), are known to be tolerant to herbivory (Piper and Fajardo, 2014; Piper, 2015). This is probably because deciduous species store their reserves in woody parts which are generally protect to herbivory (Millard *et al.,* 2001) whereas evergreen species stores also a significant part in the foliage. In our first initial hypothesis, we postulate that through loss of foliage due to defoliation, C metabolism would be impacted and result of difference on growth and NSC dynamics. However, following defoliation, beech trees did not present significant growth reduction compared to control. However, when an effect of time was found on the diameter of C trees, no effect was noticed on D trees. Such result highlight that despite no significant difference between C and D trees, defoliation started to have an impact on secondary growth. On the same experiment site when studying leaf N concentration following defoliation, leaf N was higher after defoliation suggesting that possibly photosynthetic compensatory mechanisms occurs after a defoliation (**Chapitre V)** which has been also hypothesized in other studies (Canadell and Lopez-Sorian 1998; Eyles *et al.,* 2009). Increasing of photosynthetic rates following defoliation increase the amount of C products. Despite the loss of major photosynthetic products, we found same NSC dynamics than in control whereas no difference in terms of starch proportion were found. We could hypothesis that storage might be enhanced at the expense of growth especially in 2016 when the defoliation intensity were at the highest rate. However, explaining why secondary growth were constraint at the end of the experiment after three consecutive defoliation could be related with limitation of others crucial elements such as potassium or nitrogen (Palacio *et al.*, 2018).

5.4.2. NSC dynamics under soil water deficit

Our second initial hypothesis made the assumption that both drought intensity and duration had an impact on sugars concentration. In a first approach, by following the total NSC concentrations, we did not find significant differences among treatments. But the story behind is not ended because studies have shown that the composition in NSC types could be modified by stress conditions. Indeed in a response of severe soil water deficit, soluble sugars proportion can increase at the expanse of starch, probably in a result of interconversion soluble NSC – Starch (Mencuccini, 2014). Indeed, our results showed an increase of fructose and glucose proportion in expanse of starch in the severe soil water deficit treatment. Starch, sucrose, glucose and fructose are thought to make up >80% of NSC in temperate trees (Hoch *et al.*, 2003) but in our study of young beech trees, this pool of 4 non soluble and soluble sugars make up more than 95% of NSC, whatever the treatment. Soluble sugars has multiple metabolic roles (Hartmann *et al.,* 2016): for example, growth or defense (Dietze *et al.,* 2014; Fatichi *et al.*, 2014). Glucose and fructose can occurs as free monosaccharides or can be transformed into sucrose for transport (Gibon *et al.,* 2009) or used for osmoprotection of tissues against desiccation (Patakas *et al*., 2002) by maintaining cell turgor by osmotic adjustment to improve drought adaptation (Upadhyaya *et al.,* 2013). However, less sucrose was found in the twigs of the Dr. trees. This could be due either to a possible interconversion from sucrose to glucose and fructose since we have also found more glucose and fructose or to a transport of sucrose towards other organs such as belowground. On a study on the same experimental site, ^{13}C labelling has been made to examine how drought alters carbon transport from leaves to perennial part through phloem and they concluded that intensive drought affected phloem transport capacity (Dannoura *et al.,* 2018). Consequently, this result suggest that the decrease of sucrose is likely due to interconversion to osmoprotectant sugars or to transport. Our beech trees seems to privilege carbohydrate requirements for osmoregulation which can explain our low mortality rates.

5.4.3. NSC related to drought-induced mortality.

The mortality of trees under sever soil water deficit began in 2015 at the end of vegetative season when some trees began to be drier and finally, shed their leaves. We were expected that those tree were dead, sampled them, and it was confirmed at the beginning of the next vegetative season in 2016 when 2015- supposed-died trees did not burst their buds. Then, in 2016, others trees in drought treatment died also leading to a total percentage of tree mortality of 15%.

Now, we can ask what's happened for implying mortality on certain while others survived? Low mortality was enhanced firstly at the end of the second vegetative season under severe soil water deficit which reflect the high resistance of beech trees against severe water stress. Some reports highlight also that beech trees can be resistant and resilient against drought (Tegel *et al.,* 2014; Hentschel *et al.,* 2016). In a recent review, O'Brien *et al.,* (2017) defined key trait associated with drought-induced mortality in temperate trees which were wood properties, tree size and growth at the individual levels. The first common response of our young beech trees following drought was to reduce their stem growth which has been also found to decrease in others studies when subjected to soil water deficit (Voltas *et al.,* 2013; Hentschel *et al.,* 2016) and European beech are also known for their strong morphological plasticity (Schroter *et al.,* 2012). Moreover, we can exclude any mortality induced by biotic attacks given the fact that we were aware to prevent any external disease to weakness our young beech trees. Consequently, we could hypothesize that mortality could be a consequence of a local failure of hydraulic properties but hydraulic failure cannot be explain by itself otherwise we were find mortality earlier than after 18 month and especially at a higher rate. In 2015, after one year and a half of severe constraints, mortality occurs in our study and none of consequences presented above can explain by themselves why some trees died and others not. A combination of processes might explain the low mortality in our study. Interactions between water and C balance are numerous, for example xylem and root growth, xylem embolism repair or osmotic regulation. Recovery of a functional xylem is the result of the replacement of embolized vessels with new functional ones (Brodribb *et al.,* 2010) and studies hypothesize that sugars play a crucial role in the creation of an osmotic driving force to refill embolized xylem vessels (Secchi and Zwieniecki, 2011; Bloemen *et al.*, 2016). We observed NSC concentration significantly lower in twigs of dead trees than living trees indicating that potential local NSC depletion could occur. More than local NSC depletion, our study highlight potential interconversion indicated by decrease of starch concentrations combined with decrease of soluble sugars concentrations in dead trees. Interestingly, sucrose proportion was lower in dead trees than living ones indicating also a possible sucrose conversion to glucose and fructose. We found lower starch concentration but no total starch depletion. Starch depletion was rarely observed in studies, for exemple, only in roots (Hartmann *et al.,* 2013). This result confirmed one of our initial hypothesis which is that more than depletion, availability might be more important (Sala *et al.*, 2012; Pantin *et al.*, 2013; Deslauriers *et al.*, 2014). In case of low water availability, the transport of NSC from source to sink could be disrupted and lead to mortality, which might be the case in our study and the research of minimum threshold of NSC concentrations required for survival could be like the Quest for the Grail.

5.5. Conclusion

Our results emphasize the tight C regulation which might occur on twigs under defoliation or drought. We found that under a long period of soil water deficit, local C storage seems to be previligiated but when drought became longer and still harsher, the need of C for osmoregulation lead to the breakdown of starch and might improve the chance of survive under severe soil water deficit. However, we also found that twigs from dead trees exhibited very low content of NSC which indicate that C content play a role when mortality occurs. In the other way, tree which loss a huge part of photosynthetic tissue maintain their C storage strategy all along the experiment. All these results argue for the high survival capacities of young beech trees to extreme constraint.