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THE EFFECT OF WARMING AND WATER DEFICIT ON THE NON-STRUCTURAL  
SUGARS VARIATIONS IN PICEA MARIANA

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## ABSTRACT

The aim of the study was to qualify and quantify non-structural carbohydrates (NSC) in stems of black spruce (*Picea mariana* (Mill.) B.S.P) seedlings. The effects of temperatures and water deficit on the seasonal NSC amount within the stem were also investigated. Weekly secondary growth observations of the stem was correlated to the sugars variations in order to define the most important sugars involved in wood formation in different environmental conditions (water deficit and warmer temperatures). The carbohydrates found in cambium and xylem were mainly composed of sucrose, pinitol, glucose, fructose, raffinose and starch. Water deficit decreased sucrose, while raffinose rose. Temperature had no effect on carbohydrates but amplified the effect of water deficit. Sucrose, pinitol and raffinose were correlated to cambium activity and xylem development, but changes in those carbohydrates quantities only had a little effect on cell development in the stem.

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## INTRODUCTION

In plants, carbohydrates are synthesized in leaves (the source), translocated to the growing tissues (the sinks) by the phloem in form of sucrose and stored as sucrose or starch (Roitsch and Gonzalez, 2004; Ainsworth and Bush, 2011). They play an essential role to sustain cell metabolism and growth. In the cambium zone, as well as in differentiating xylem (Uggla *et al.*, 2001; Antonova and Stasova, 2008) enzymatic activities converting sucrose into UDP-glucose, glucose and fructose (Uggla *et al.*, 2001) are observed in order to sustain wood formation. In poplars, the rates of cell division in cambium was positively linked with carbohydrates content (Deslauriers *et al.*, 2009). Metabolism regulation of growth hormones, like auxin (Sairanen *et al.*, 2013), and metabolic signaling pathway (O'Hara *et al.*, 2013; Bolouri Moghaddam and Van den Ende, 2013) also appeared to be drive by carbohydrates.

In stressing events, carbohydrates are one of the most utilised compounds in the plants. They are known to play a role in maintaining hydraulic function during severe drought (Sala *et al.*, 2012) and to increase freezing tolerance during cold season (Chen *et al.*, 2012) to cite only few examples. But carbohydrates can also be affected by biotic and abiotic stresses (see Smith and Stitt (2007) and Ericsson *et al.* (1996) reviews). For example, in general, a decrease of carbohydrates is observed when photosynthesis is less efficient or stopped due to temperature increase and/or water deficit. However, in case of higher night temperatures, amplification of respiration rate can bring an acceleration of starch consuming. Because of the lack of carbon energy reserve in the morning a signal is sent to

produce carbohydrates in high quantity when photosynthesis begin, leading to a rapid but not sustained sucrose rise (Turnbull *et al.*, 2002; Turnbull *et al.*, 2004). Also, in conifers, starch was found to increase with altitude (Hoch and Körner, 2012) but no effect of artificial warming was detected in the sugars and starch availability at high altitude (Streit *et al.*, 2013).

On the plant metabolism point of view, changes in temperature and water availability have been studied in many species from different age or growth (see review of Kozłowski and Parllardy (2002) and Kranensky and Jonak (2012) for more details). For black spruce (*Picea mariana* (Mill.) B.S.P.), the most important species in the boreal forest of North America, increased temperature and drought are both supposed to affect photosynthesis and growth : a rise of 8°C during day and night led to a growth reduction and a decrease of the root/shoot ratio on young seedlings (Way and Sage, 2008a). Moreover, such a temperature increase, negatively affected the needle morphology of black spruce by producing thinner and less dense needles with higher mesophyll cells per foliar surface, and the gas exchange with a decrease of CO<sub>2</sub> assimilation and photosynthesis inducing higher respiration rate (Way and Sage, 2008a, b). Therefore, these effects on the leaf morphology and on the gas exchange could decrease the carbon gain (both sugar and starch) leading to growth reduction. An increase of 5°C on ten-years-old black spruce had no effect on photosynthesis and respiration and allowed higher production of new shoots but a lower production of fine roots (Bronson and Gower, 2010). On a seasonal scale, a warming of 3°C was supposed to increase the period of cambium and xylem growth from 125 to 160 days (Rossi *et al.*, 2011), which means that more carbon will be needed to sustain growth for a longer period.

Warming also increases density of earlywood of Scots pine (*Pinus sylvestris* L.) (Kilpelainen *et al.*, 2007). Water deficit affects wood formation by shortening the duration of cell production (Gruber *et al.*, 2009) and decreasing the size of earlywood cells and cell production (Eilmann *et al.*, 2009; Gruber *et al.*, 2010).

Thus, both growth and other metabolic processes seem to need carbohydrates. During a stress event, it would thus be possible that those activities are in competition with carbon availability. It's indeed usually observed that growth is the first thing to suffer when stress event appear (McDowell, 2011). But it has also been observed that growth, respiration and non-structural carbohydrates could be independent, depending of the situation (Bansal and Germino, 2010).

Climatic models predict increases in temperature in the boreal forest of up to 3°C over the next 50 years, with the largest increases occurring in winter and spring, at resumption of plant growth (Plummer *et al.*, 2006). Changes in precipitation are also predicted with more extreme events, especially during winter (increase in precipitation) and summer (drought). Temperatures however are not expected to change in an equal way during the night and during the day: between 1950 and 1998, unlike the daily maximum temperature, the daily minimum temperature increased significantly, indicating that the nights were warmer (Bonsal *et al.*, 2001). These modifications could affect the gas exchanges of the plant and consequently the production of sugars as well as the production or utilisation of starch. As carbon is essential for plant growth, a change in carbon production could have repercussions for wood formation. Each carbohydrates are essential in different ways in the signal pathway and wood formation, therefore, it is essential to fully understand the growth

timings and dynamics of each of them in the black spruce and other species of the boreal forest under climate change to predict eventual changes in the productivity of biotopes that have obvious ecological and economic importance.

Thus, the aim of this study is the qualitative and quantitative assessment of carbohydrates and wood formation under different temperatures and irrigation regimes in two different tissues of the stem, cambium and xylem, of 4-years-old black spruce seedlings. The following hypotheses were tested: a) the warmer temperature and water deficit will negatively affect carbohydrate availability for xylogenesis and b) the key developmental phases of xylogenesis (cambial production, cell enlarging, cell-wall thickening, and mature cells) will be linked to carbohydrate availability. Two steps were necessary to verify these hypotheses: (1) to quantify the available carbohydrates in cambium and xylem and (2) to associate the available sugars with the forming tree ring.

## METHODS

### **Study area and experimental design**

The study took place in a greenhouse complex located at the Université du Québec à Chicoutimi, QC, Canada (48° 25' N, 71° 04' W, 150m above sea level). The mean annual temperature in 2010 and 2011 were 5.2°C and 2.2°C respectively. The higher mean temperature in 2010 was caused by a particularly hot winter and spring with a mean January-May temperature of -0.2°C compared with -4.5°C in 2011. The temperature in the summer of 2010 and 2011 were about the same with a mean of 18.1°C and 17.6°C respectively.

Two experiments were performed in a greenhouse divided in three independent sections and automatically controlled with a misting and windows opening systems for the cooling. About three hundreds black spruce seedlings were installed every year in each section for the two irrigation regime treatments (one hundred fifty plants per irrigation regime). Plants consisted of four years old seedlings transplanted in 4.5 L plastic pots filled with a peat moss, perlite and vermiculite mix, and left in an open field for the whole previous growing season and during winter. In April of each year, the seedlings were taken inside the greenhouse for the experiment and plant were fertilized with 1 g·l<sup>-1</sup> of NPK (20-20-20) fertilizer dissolved in 500 ml of water. Only the healthier trees were selected for the experiment, while the other ones were used in the buffer zone at the borders. Overall, the seedlings were 48.9 ± 4.7 cm in height, with a diameter at the collar of 8.0 ± 2.0 mm. Each

seedling was equipped with drip trickles to perform the irrigation. In each section, different irrigation and temperature regimes were applied (table 1). Control (named T0) corresponded with outside temperature, while the two other sections were subjected to a specific thermal condition in respect to control. In 2010, T2 and T5 experienced a temperature of 2 and 5 K higher than T0, respectively. In 2011, T6D and T6N were warmer of 6 K than T0 during the day or during the night, respectively (figure 1). For irrigation, control consisted in maintaining the soil water content over 80% of field capacity, while the other seedlings were submitted to a water deficit from mid-May to mid-June, when cambium was vigorously differentiating (Rossi *et al.*, 2009b; Rossi *et al.*, 2009a).

Table 1. Temperature regimes as control (T0), +2K (T2), +5K (T5), +6K during the day (T6D) and +6K during the night (T6N), and irrigation regimes in term of day of year (DOY) for 2010 and 2011

Year	Temperature regime	Irrigation regime (DOY)	
		Irrigated	Non Irrigated
2010	T0		
	T2	118-300	142-173
	T5		
2011	T0		
	T6D	118-300	158-182
	T6N		

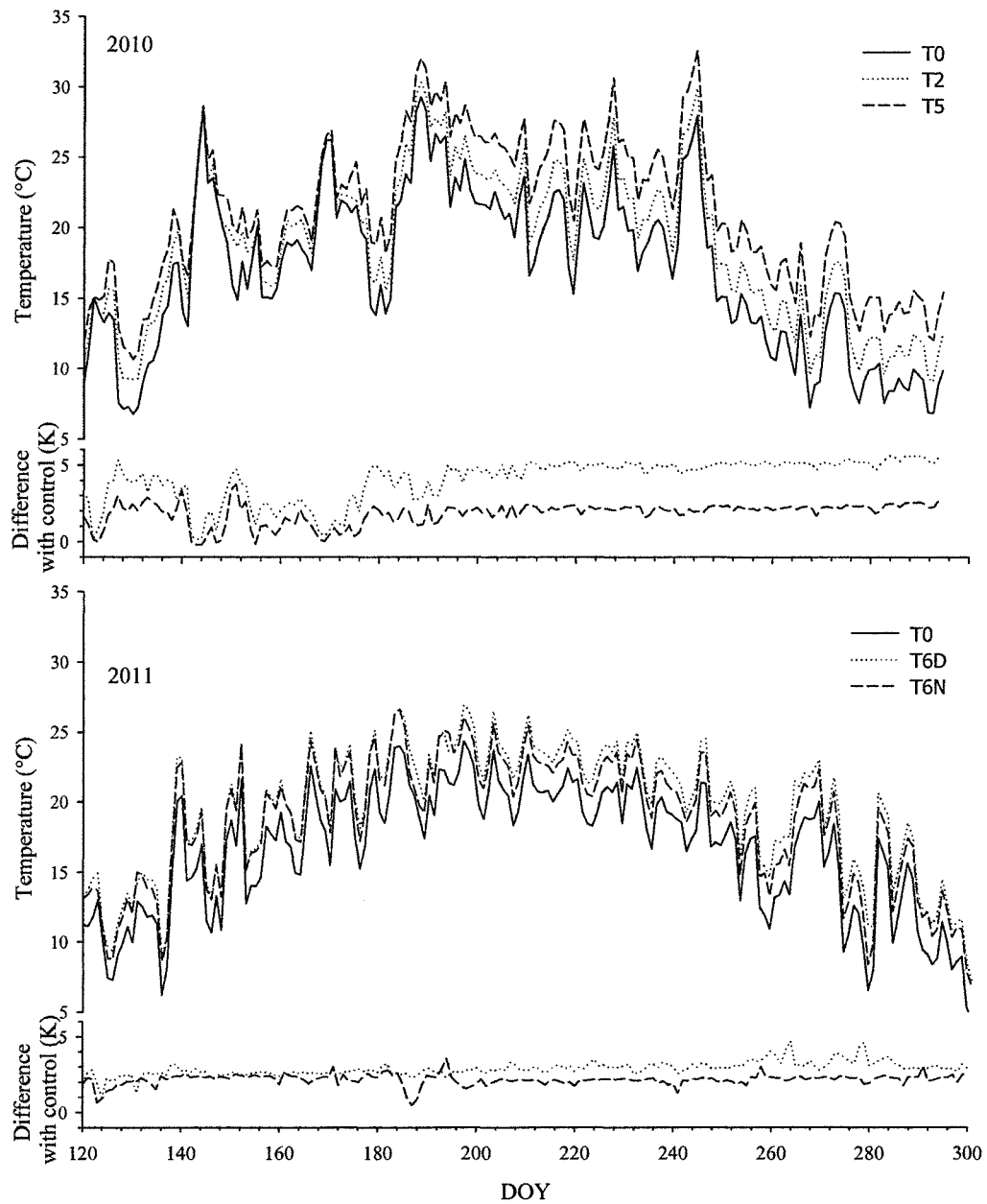


Figure 1. Daily temperature ( $^{\circ}\text{C}$ ) and delta of temperature in each thermal sector of the greenhouse for 2010 and 2011. Temperatures are presented as control (T0), +2K (T2), +5K (T5), +6K during the day (T6D) and +6K during the night (T6N). Delta temperature are presented as the elevated temperature minus the control with the corresponded line of the elevated temperature



## **Xylem growth**

From May to September, stem disks were weekly collected 2 cm above the root collar from 36 randomly-selected seedlings (6 seedlings  $\times$  3 thermal conditions  $\times$  2 irrigation regimes) from 8:00 AM to midday (Balducci *et al.*, 2013). The samples were dehydrated with successive immersions in ethanol and D-limonene, embedded in paraffin and transverse sections of 8-10  $\mu$ m thickness were cut with a rotary microtome (Rossi *et al.*, 2006a). In each sampled seedling, the length of apical shoot was measured.

The sections were stained with cresyl violet acetate (0.16% in water) and examined within 10-25 minutes with visible and polarized light at magnifications of 400-500 $\times$  to distinguish the developing xylem cells. For each section, the radial number of (i) cambial, (ii) enlarging, (iii) cell-wall thickening, and (iv) mature cells were counted along three radial files according to Rossi *et al.* (2006b). In cross section, cambial cells were characterized by thin cell walls and small radial diameters. During cell enlargement, the tracheids still showed thin primary walls but radial diameters were at least twice those of the cambial cells. Observations under polarized light discriminated between enlarging and cell wall thickening tracheids. Because of the arrangement of the cellulose microfibrils, the developing secondary walls glistened when observed under polarized light, whereas no glistening was observed in enlargement zones where the cells were still just composed of primary wall (Abe *et al.*, 1997). The progress of cell wall lignification was detected with cresyl violet acetate reacting with the lignin (Antonova and Shebeko, 1981). Lignification appeared as a colour change from violet to blue. A homogeneous blue colour over the

whole cell wall revealed the end of lignification and the reaching of tracheid maturity (Gričar *et al.*, 2005).

### **NSC extraction and assessment**

Each two weeks, 18 of the 36 seedlings used for xylem analysis were selected for sugar extraction. The branches were removed and the bark was separate from the wood to expose the cambial zone of the stem. The two parts (bark and wood) were plunged into liquid nitrogen and stored at -20°C. Dehydration was made by lyophilisation for a period of five days for both parts.

The cambium zone, including some cells in the enlargement, was manually separated by scraping with a chirurgical scalpel the inner part of the bark and the outset surface of the wood (Giovannelli *et al.*, 2011). After having removed the cambium, the wood was milled to obtain a fine powder.

The soluble carbohydrates were extracted following the protocol of Giovannelli *et al.* (2011). For the cambium, only 1-30 mg of powder was available and used for the sugar extraction, while 30-600 mg of powder was available for wood. Samples with less than 1 mg of cambium powder were not considered, this quantity being too low as, after extraction of this quantity of powder, the sugar in the extract did not reach the detection limit of the High Performance Liquid Chromatography (HPLC). Soluble carbohydrates were extracted three times at room temperature with 5 ml of ethanol 75% added to the powder. A 100 µl volume of sorbitol solution (0.01 g/ml) was also added at the first extraction as an internal standard. In each extraction, the homogenates were gently vortexed for 30 minutes and

centrifuged at 10,000 rpm for 8 minutes. The three resulting supernatant were evaporated and recuperated with 12 ml of nano-filtered water. This solution was then filtered by the solid phase extraction (SPE) method using a suction chamber with one column of *N+ quaternary amino* (200mg/3ml) and one of *CH* (200mg/3ml). The solution was evaporated until 1.5 ml and filtered through a 0.45  $\mu\text{m}$  syringe filter to a 2 ml amber vial.

An Agilent 1200 series HPLC with a refractive index detector (RID) and a Shodex SC 1011 column with guard column, equipped by an Agilent Chemstation for LC systems program, was used for soluble carbohydrates assessment. Calculations were made following “the internal standard method” described in Harris D.C. (1997). A calibration curve was created for each carbohydrate using pure sucrose, raffinose, glucose, fructose (Canadian Life Science) and D-pinitol (Sigma-Aldrich). All fitting curve had  $R^2$  values of 0.99 and a F value near one, indicating that each sugar had a ratio 1:1 with sorbitol.

The quantity of sugar loss during extraction was calculated by comparing the concentrations of sorbitol added at the beginning of the extraction, to those of un-manipulated sorbitol. The percentages of loss were then calculated and added to the final results.

Xylem powder was also used for starch extraction, performed according to Chow and Landhäusser (2004). The extraction consisted of adding 5 ml of 80% ethanol at 95°C to 50 mg of powder. The solution was vortexed for 30 minutes and centrifuged, and the supernatant was removed. This step was repeated twice. The starch was solubilised with NaOH 0.1M and acetic acid 0.1M and digested with an alpha-amylase solution at 2000 U/ml and amyloglucosidase 10 U/ml. PGO-color reagent and H<sub>2</sub>SO<sub>4</sub> 75% were added to

the solution 24 hours later. Starch was assessed using a spectrophotometer at 533 nm (Chow and Landhäusser, 2004).

### **Statistical analysis**

Pearson correlations were separately performed for cambium and xylem to identify the relationships among soluble carbohydrates. Samples in each tree part were pulled together without distinction of treatments. The mean sugar concentration were compared between irrigation regimes with Wilcoxon non-parametric analysis ( $P \leq 0.05$ ). Analyses were conducted using the NPAR1WAY procedure in SAS. However, because of very low number of sample in some DOY ( $N < 3$ ), some test could not be performed.

For determining the effects of temperature and water treatments on starch, samples were separated into treatment and compared for the whole growing season. Kruskal-Wallis and Smirnov tests ( $p \leq 0.05$ ) were made using transformed  $\log(x+1)$  values.

The relationship between soluble carbohydrates and xylem growth was assessed by performing canonical correlations using the CANCELL procedure in SAS 9.2 (SAS Institute Inc., Cary, NC). The two sets of variables consisted of soluble carbohydrates concentrations (raffinose, sucrose, glucose, pinitol and fructose) and number of cells in the cambial zone and differentiation. The procedure finds the linear combinations allowing the canonical variables to have the largest possible correlation. Each canonical variable was uncorrelated with all the other canonical variables of either set except for the corresponding canonical variable in the opposite set.

## RESULTS

### **Secondary growth**

In the cambial zone, similar annual trends and amounts of cambial cells were observed between the two treatments (figure 2). In May, on average 6 closely spaced cells were observed in the cambial zone. From DOY 125 to 251, the number of cambial cells fluctuated with values ranging between 5 and 10, with maximum observed on DOY 187-194 in 2010 and 139-147 in 2011. Once annual activity had ended and the cambium stopped dividing, the number of cells in the cambial zone gradually decreased to the minimum value, corresponding to quiescence conditions of the meristems. Between 4 and 6 cambial cells were observed in autumn, a lower number than at the beginning of the season (Figure 2). The only dissimilarities that could be observed were the 1 to 3 more cells in the irrigated plants than the non-irrigated ones just after the water deficit period in the control temperature and +2K in 2010.

Cell enlargement also showed similar trend between the treatments (figure 2). As for cambium, the maximum of cell enlargement (4 to 5 cells) occurred in the middle of the season in 2010 and at the beginning in 2011, with some difference: in 2010 the maximum occurred at DOY 215, 208 and 201 for the control temperature, +2K and +6K respectively. The difference of 1 or 2 cells along the growing season in 2010 didn't seem to be related to irrigation or temperature treatments. Despite that the cell enlargement began before the

experiment in 2010, the end of enlargement was the same in both year between DOY 251 and 258, irrespective of the treatments.

For cell wall formation, the trend was essentially similar to that of enlargement. Its maximum appeared one to four weeks after the culmination of cell enlargement with 6 to 10 cells. The difference of 2 to 5 cells observed after the stress period between the control and the water deficit treatment was not correlated to the treatment. The end of cell enlargement and wall formation always occurred on DOY 272, irrespective of the year and treatment.

Total carbohydrates in the cambium and the xylem had similar trends within the temperature and water treatments. In the cambium, sucrose was 2 to 30 times more abundant than the other sugars. Therefore, total available sugar in the cambium was represented mostly by the variation in sucrose (figure 2). In the xylem (figure 2), significant differences between the water deficit treatments were discernible and were mostly influenced by fructose in 2010 and by sucrose and pinitol in 2011. No trend was observed between the water deficit and the control treatment, before, during or after the water deficit period.

The sum of soluble carbohydrates in cambium and xylem (figure 2) showed similar variations with wood formation. When cambial and needle growth started, between DOY 120 and 130, the amount of carbohydrate was high in the cambium. A decline was observed between DOY 150 and 170, which was most pronounced in 2010 with amount near 0. A second decline was also observed in the middle of July (DOY 208 in 2010 and 196 in

2011). Xylem pattern was mostly symmetric of the cambium one. This aspect will be fully analysed in the last section of the results.

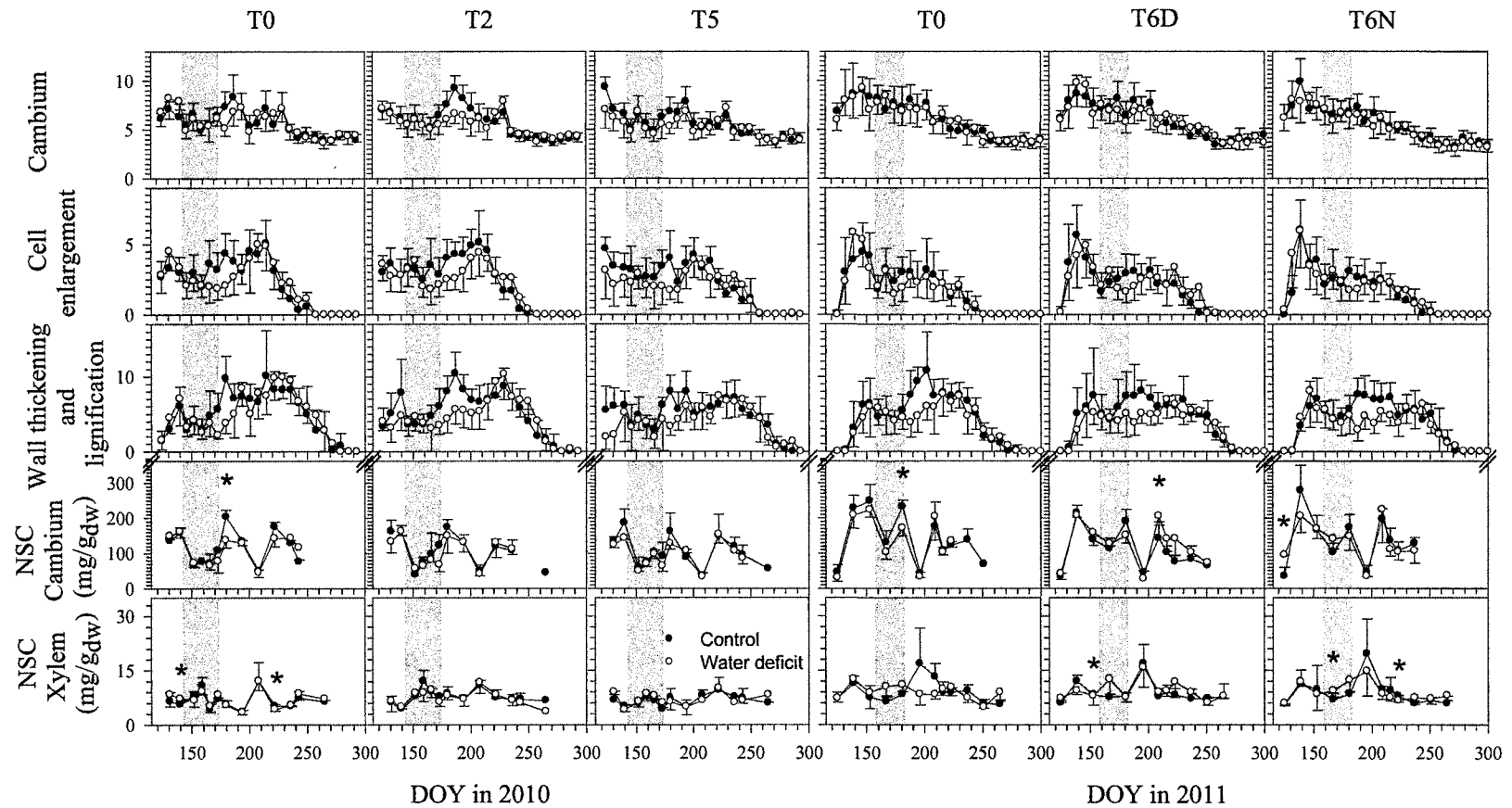


Figure 2. Wood formation in black spruce seedling illustrated by the number of cells in the cambial zone, in the phase of enlargement and wall formation in 2010-2011 for the three thermal conditions. Plants subjected to water deficit are represented with an open dot and irrigated plants have a close dot. Shade part represents the water deficit period. Asterisk (\*) represent significance with  $p \leq 0.05$



### Identification and quantification of carbohydrates in black spruce

Within the 438 trees collected in 2010 and 2011, a total amount of 438 xylem and 351 cambium samples that had more than 1 mg of material were analysed for soluble carbohydrates. They were distributed almost equally in every treatment but had a high variability of weight within a treatment (table 2). Figure 3 present a chromatogram obtained by the evaluation of the carbohydrates in the HPLC.

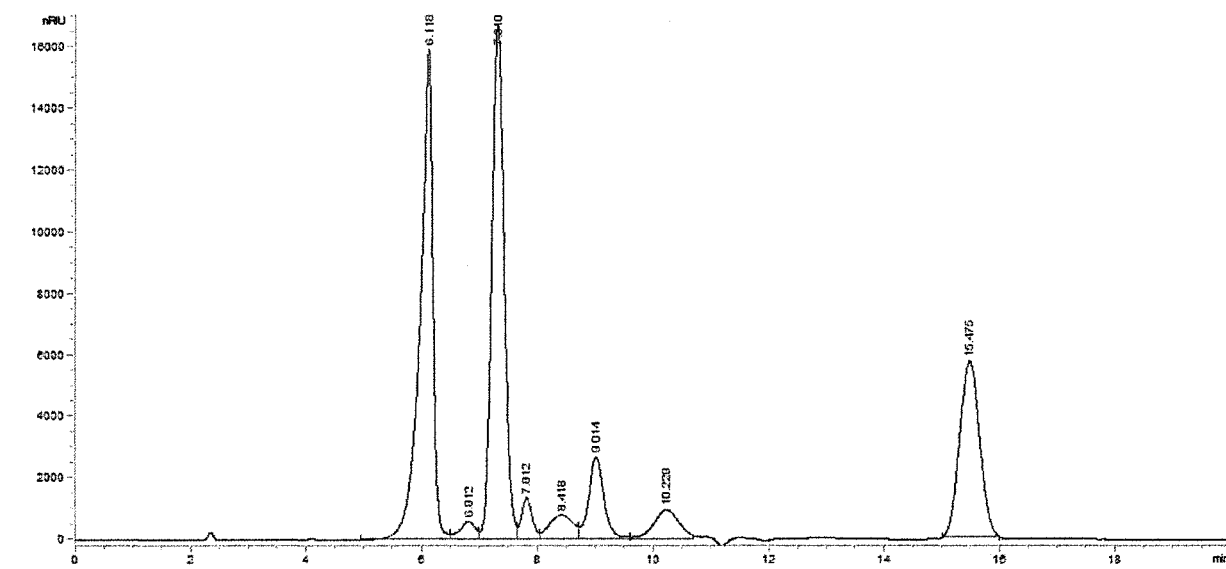


Figure 3. Chromatogram of carbohydrates present in the black spruce

Table 2. Weight of the collected powder ( $\geq 1$  mg) with standard deviation (S.D.) in cambium and xylem and number of analysed samples for water deficit (S), control (C) and temperature treatment in 2010 and 2011

Treatment	Cambium		Xylem		Number of sample analysed	
	Mean (mg)	S.D.	Mean (mg)	S.D.	Cambium	Xylem
<b>2010 Total</b>	<b>12.05</b>	<b>10.82</b>	<b>110.97</b>	<b>42.01</b>	<b>180</b>	<b>219</b>
2010 T0S	15.69	13.80	97.00	38.07	31	37
2010 T0C	17.62	11.75	100.83	40.84	32	38
2010 T2S	7.83	7.07	121.32	44.48	25	37
2010 T2C	10.59	9.20	121.39	45.96	29	37
2010 T5S	8.73	6.14	115.67	37.29	28	32
2010 T5C	10.59	10.92	110.54	40.50	35	38
<b>2011 Total</b>	<b>13.08</b>	<b>12.59</b>	<b>85.61</b>	<b>16.06</b>	<b>171</b>	<b>219</b>
2011 T0S	13.23	10.86	90.21	17.55	26	36
2011 T0C	14.21	14.44	86.68	12.02	32	36
2011 T6DS	13.57	11.96	85.54	12.74	26	37
2011 T6DC	12.91	13.79	83.65	18.91	29	37
2011 T6NS	11.42	11.77	85.53	13.41	27	36
2011 T6NC	12.95	12.78	82.21	19.64	31	37

In cambium and xylem, five carbohydrates were detected: sucrose (in a total of 743 samples), pinitol (789), fructose (785), glucose (782) and raffinose (491). The absence of raffinose in a large amount of sample was related with the small quantity of this sugar in tissue of black spruce during the growing season and with the detection capacity of the

HPLC which was near the limit for this specific sugar. That was also explaining the high standard deviation associated with the mean (table 3).

In the cambium, sucrose (mean of 61 and 70 mg/g<sub>dw</sub> for 2010 and 2011 respectively) was the most abundant soluble carbohydrate followed by pinitol (between 25 and 32 mg/g<sub>dw</sub>). Raffinose was the less abundant with quantity less than 5.6 mg/g<sub>dw</sub> (table 3). In xylem, three components had similar mean values, with fructose been the most abundant soluble carbohydrate (between 1.8 and 2.0 mg/g<sub>dw</sub>) followed by sucrose and pinitol. Raffinose still had the lower value with a mean of 0.18 mg/g<sub>dw</sub> for 2010 and 2011 (table 3). The quantity of soluble carbohydrates found in both cambium and xylem was higher in 2011 compared with 2010.

Starch was analysed in 235 samples of xylem and detected in 200 of them. It was present in all treatments and along the growing season in both years with a mean of 0.28 mg/g<sub>dw</sub>.

Table 3. Mean and standard deviation (S.D.) of carbohydrates for the cambium and xylem of 2010 and 2011, all treatments confounding

NSC (mg/g <sub>dw</sub> )	2010		2011	
	Cambium	Xylem	Cambium	Xylem
Sucrose	61.18±41.12	1.78±1.21	70.56±44.20	1.99±1.18
Pinitol	25.17±10.08	1.60±0.59	32.45±13.99	2.15±0.94
Fructose	13.25± 7.09	2.02±1.10	16.13± 6.06	2.50±1.40
Glucose	9.33± 6.99	1.65±0.96	10.58± 4.65	2.07±1.71
Raffinose	1.91± 2.78	0.12±0.21	5.62± 7.27	0.23±0.28

### Variation of carbohydrates in cambium and xylem along the growing season

#### *Cambium*

The variations of non-structural carbohydrates in the cambium were not uniform during the growing season but some association was found (table 4). Fructose was highly correlated ( $p < 0.001$ ) with glucose ( $r=0.88$ ) while sucrose was correlated with pinitol ( $r=0.53$ ). Raffinose had a different pattern of seasonal variation as this sugar was only slightly correlated with sucrose, pinitol and fructose ( $r=0.15-0.17$ , table 4).

In 2010, sucrose changed according to a W pattern with two drops at DOY 160-170 and 210 where concentration was near 0 mg/g<sub>dw</sub> for both water treatments (figure 3). In 2011, the drop of DOY 160-170 was less pronounced and the concentration reached zero at DOY 190. In both years, the amount of sucrose in the cambium was high at the beginning of cell

division and enlargement with value between 100 and 200 mg/g<sub>dw</sub>. In both years, pinitol concentration rather followed the seasonal variation of sucrose with the exception that concentration did not drastically drop between DOY 160-170 (figure 3). Although the concentrations of fructose and glucose were similar across water and temperature treatments, some differences occurred between years. In 2010, the variations of both sugars showed a messy pattern while in 2011, fructose and glucose increased at the beginning of tree-ring formation and gradually decreased toward the end of the growing season. With the exception of high value in water stressed plants (DOY 160-180) for both years, the concentration of raffinose was always very low in cambium during the growing season. Other high values were also observed in T6N treatment at the end of the tree-ring formation (figure 3).

### *Xylem*

In xylem, the seasonal variation of sucrose had a distinct pattern as this sugar had very low positive correlations with pinitol ( $r=0.18$ ) and raffinose ( $r=0.29$ ) and a negative correlation with fructose and glucose ( $r=-0.14$  and  $r=-0.21$ ) (table 4). The same situation was observed for raffinose with low correlation with all other sugars. However, the variation of fructose and glucose was highly similar with a correlation of  $r=0.98$ . The seasonal variation of pinitol in the xylem was also associated with fructose and glucose (table 4).

During the growing season the sucrose in the xylem (between treatments) was generally high at the beginning and at the end (figure 4). As for the cambium, a drop in sucrose concentration was observed in July at DOY 160-170 with concentration near zero. In

xylem, the variation of pinitol, fructose and glucose showed no specific seasonal trend. In all temperature treatments of both year, higher values were observed at DOY 210 in 2010 and 195 in 2011 (figure 4). The concentration of raffinose was always near zero mg/g<sub>dw</sub> throughout the growing season except, as for cambium, for high values mainly observed in water stressed plants during and after the water deficit treatment.

Starch did not follow the trend of the other sugars but had the same trend over the two years. Between DOY 118 to139, starch culminated and drop to almost zero at DOY 180. It stayed low until the end of summer where a small rise was detectable (figure 4).

Table 4. Pearson correlation matrix between carbohydrates in black spruce seedlings. All treatments and years were considered in the correlation. Xylem (n=438) is represented in the lower part of the matrix and cambium (n=351) in the higher part of the matrix. Asterisk represents significance with \*p ≤ 0.05, \*\*p ≤ 0.01, \*\*\*p ≤ 0.001

	Sucrose	Pinitol	Fructose	Glucose	Raffinose
Sucrose	–	0.53***	0.23***	0.04	0.17**
Pinitol	0.18***	–	0.30***	0.08	0.16**
Fructose	-0.14**	0.64***	–	0.88***	0.15**
Glucose	-0.21***	0.56***	0.98***	–	0.07
Raffinose	0.29***	0.11*	0.12*	0.12*	–

### **Effects of temperature and water deficit on soluble sugars**

Differences were observed between the irrigated and non-irrigated samples for each temperature treatments and year (figure 3-4) by using non-parametric two samples Wilcoxon test. In 2010, in the cambium, the control treatment had mostly significantly higher quantities of sucrose, pinitol and fructose for the T0 treatment, especially at DOY 180. For the T0 treatment, no data were available at DOY 159 (2010) for the water deficit treatment and for both treatment at DOY 265 due to the lack of available cambium powder. The same problem occurred at DOY 243 (T2, 2010) for both treatments, as well as DOY 265 for the stressed plants (T2 and T5, 2010). In the xylem of the same year, for the T0, plants subjected to water deficit had significantly higher quantity of NSC compared with the control ones, before or after the water deficit period. Both fructose and glucose had significant differences between the irrigation regimes just before the water deficit period at DOY 140 for the T0 and T5 treatments. The T2 and T5 treatments presented differences in sucrose and raffinose. Higher amount of sucrose in the control treatment was observed during or just after the water deficit period while the opposite happened in the same period for the raffinose. Nonetheless, significant higher amount of raffinose of the control treatment was observed at the end of the season at DOY 222 and 243.

In 2011, fewer differences were observed at T0 but sucrose had about the same behavior compared with 2010. Raffinose showed higher variations within the stressed trees and higher amount of that sugar during the water deficit period were observed. Missing data prevented to correctly perform comparison of sugar quantities for the same reason than for 2010 at DOY 237 and 251 for the water deficit treatment at T0 and at DOY 251 for both

treatments for T6N. The raffinose in the xylem followed the same pattern as for 2010 except that the stressed plants had higher values of this NSC at the end of the season for the T6N treatment. Sucrose trend was not the same along the temperature treatment and seemed to have a higher amount on stressed trees with temperature rise.

A simple general linear model (GLM) of least squares means was performed by using the seasonal mean amount of sugar for each treatment (figure 5-6). Results demonstrated that temperature had an effect on sucrose in the xylem of 2010, where the quantity of this sugar in T2 and T5 are significantly higher compared with T0. A water deficit effect was also found for the raffinose in the xylem of the same year (figure 5).

For cambium in 2011, an effect of temperature was found for fructose, where T2 was significantly different of T0, and for raffinose which all three temperature treatment had a significant difference between them. No difference was found in the xylem, except for the general water deficit effect in the raffinose, which was also found in the cambium for the same sugar (figure 5-6).



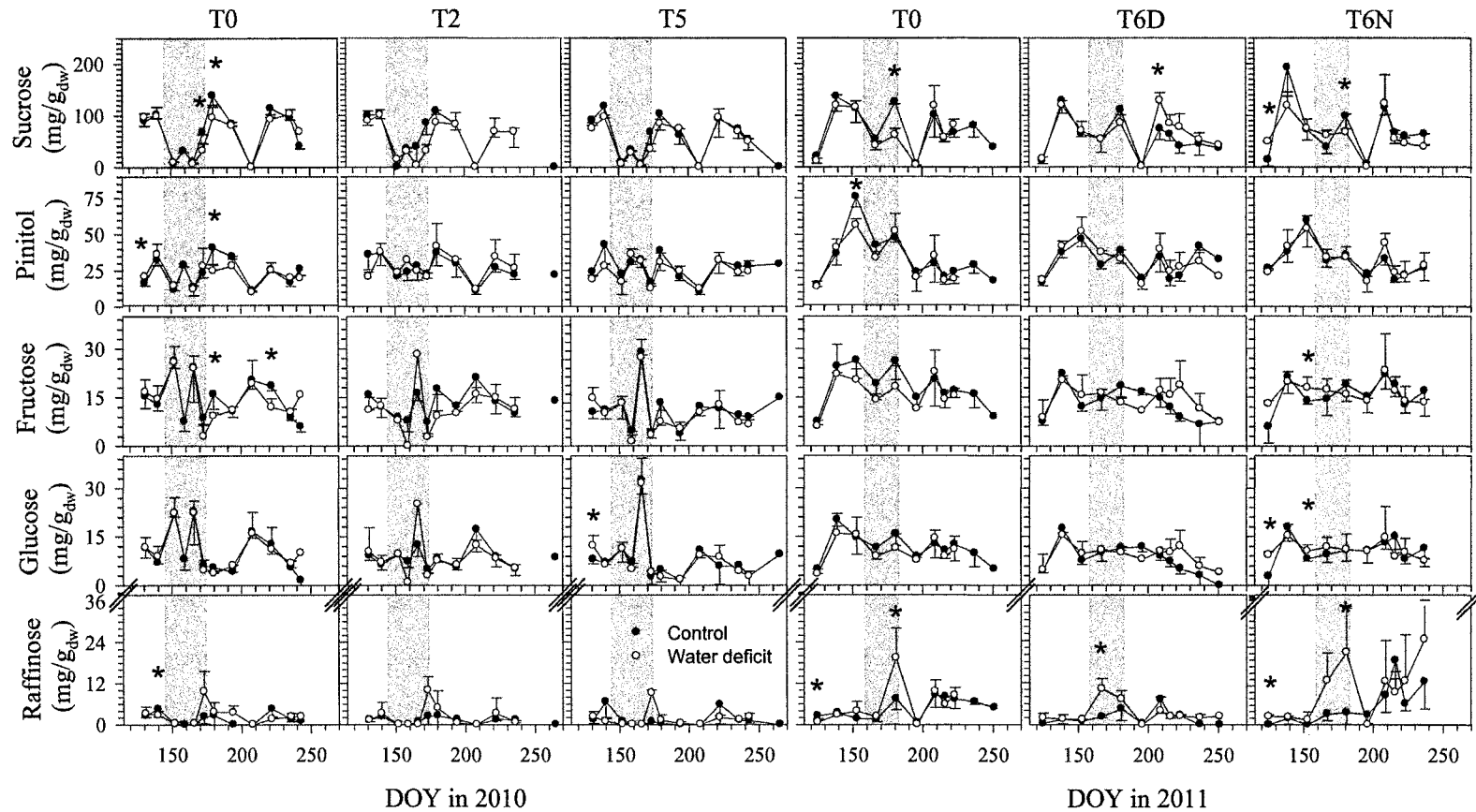


Figure 4. Soluble sugars in  $\text{mg/g}_{\text{dw}}$  in cambium for 2010 and 2011. Temperature treatments are represent as control (T0), +2K (T2), +5K (T5), +6K during the day (T6D) and +6K during the night (T6N). Plants subjected to a water deficit are represented with an open dot and irrigated plants have a close dot. Shade part represent the water deficit period. Asterisk (\*) represents significance with  $p \leq 0.05$

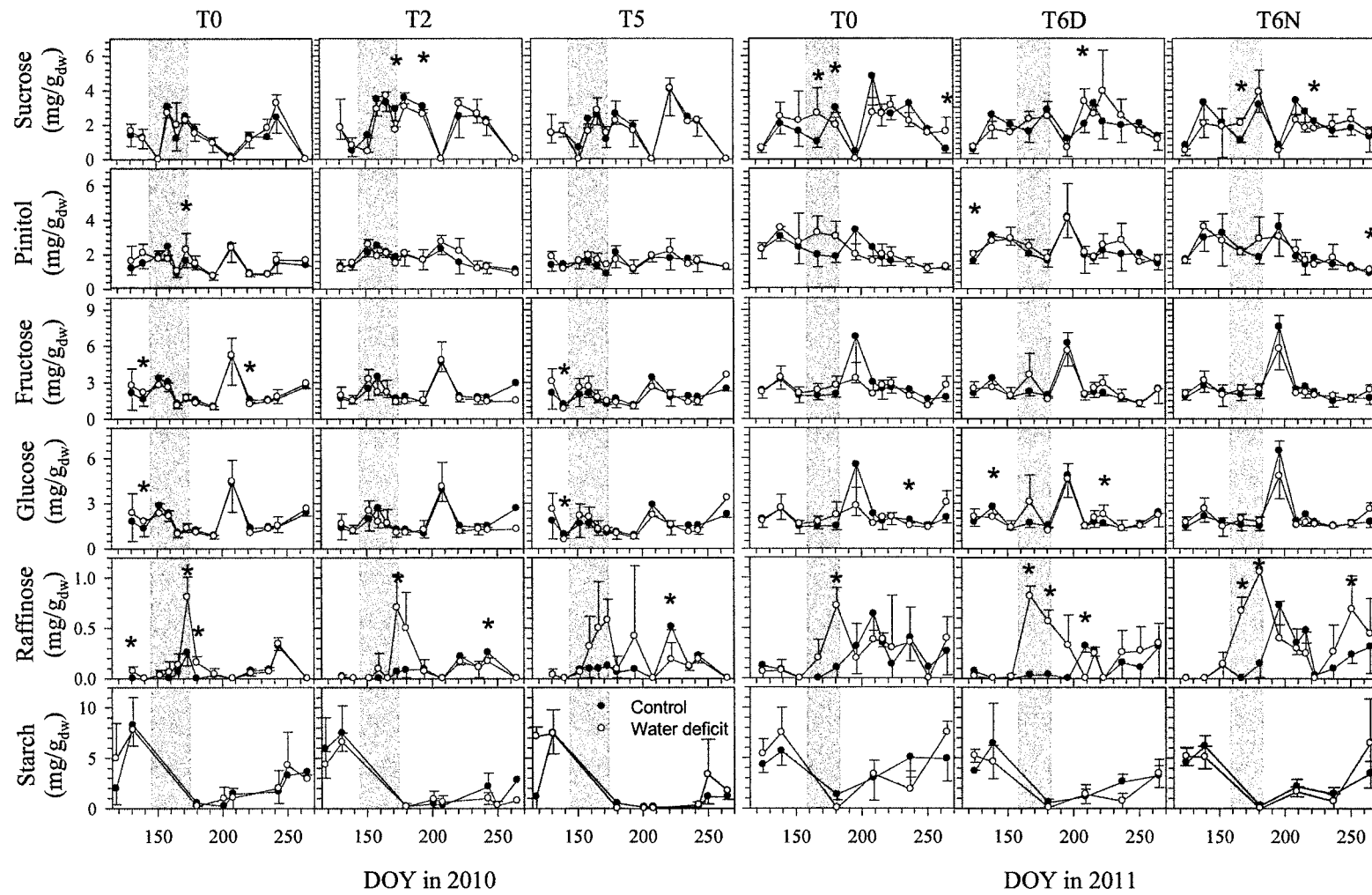


Figure 5. Soluble sugars in mg/g<sub>dw</sub> in xylem for 2010 and 2011. See figure 3 for details

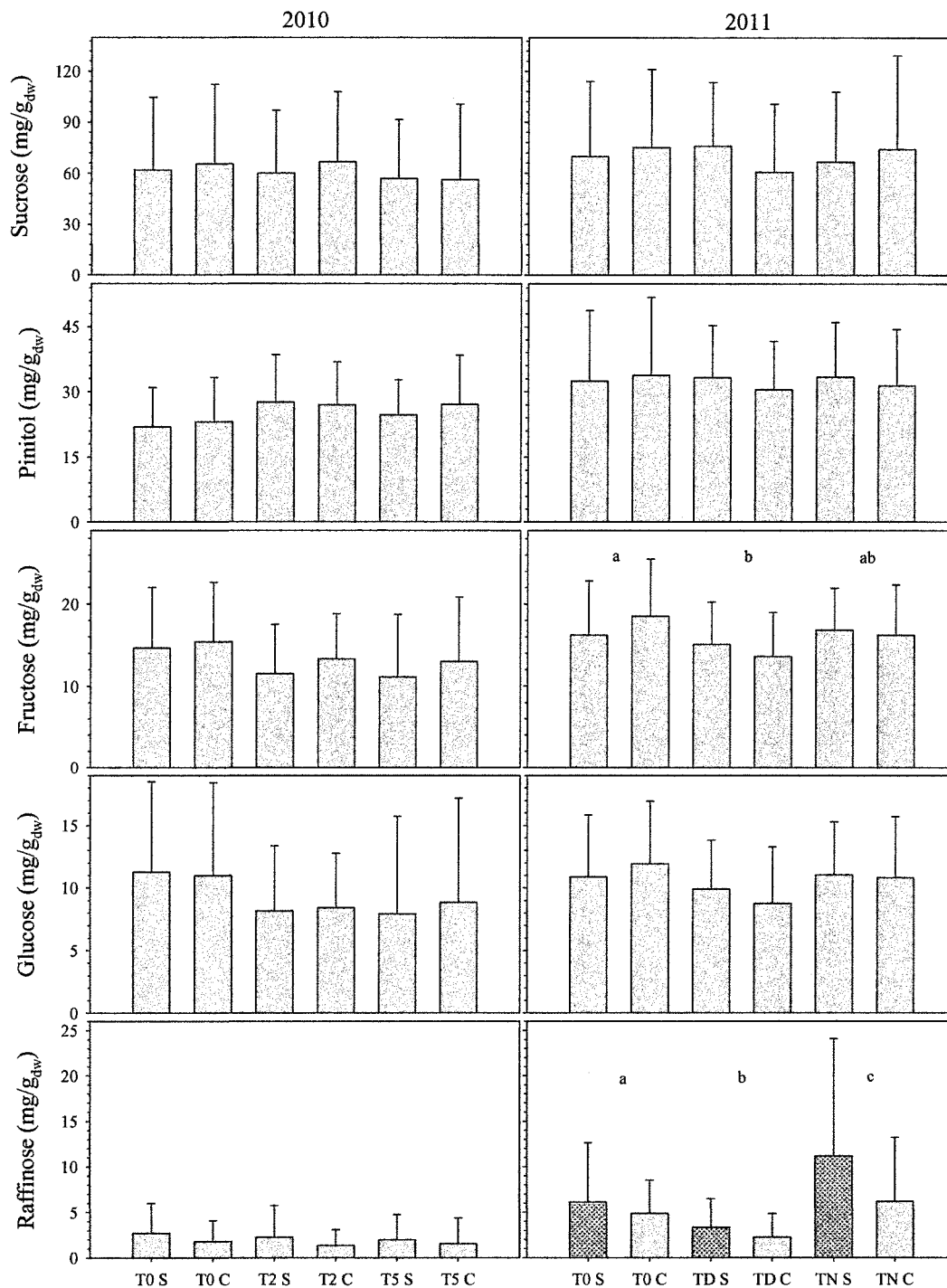


Figure 6. Means (mg/g<sub>dw</sub>) of carbohydrates in the cambium by temperature and water treatments. Temperature treatments are represented as Figure 3. Water treatments are represented by S (water deficit) and C (control). Letters represent significance for temperature treatment and shade bars for water treatment with  $p \leq 0.05$

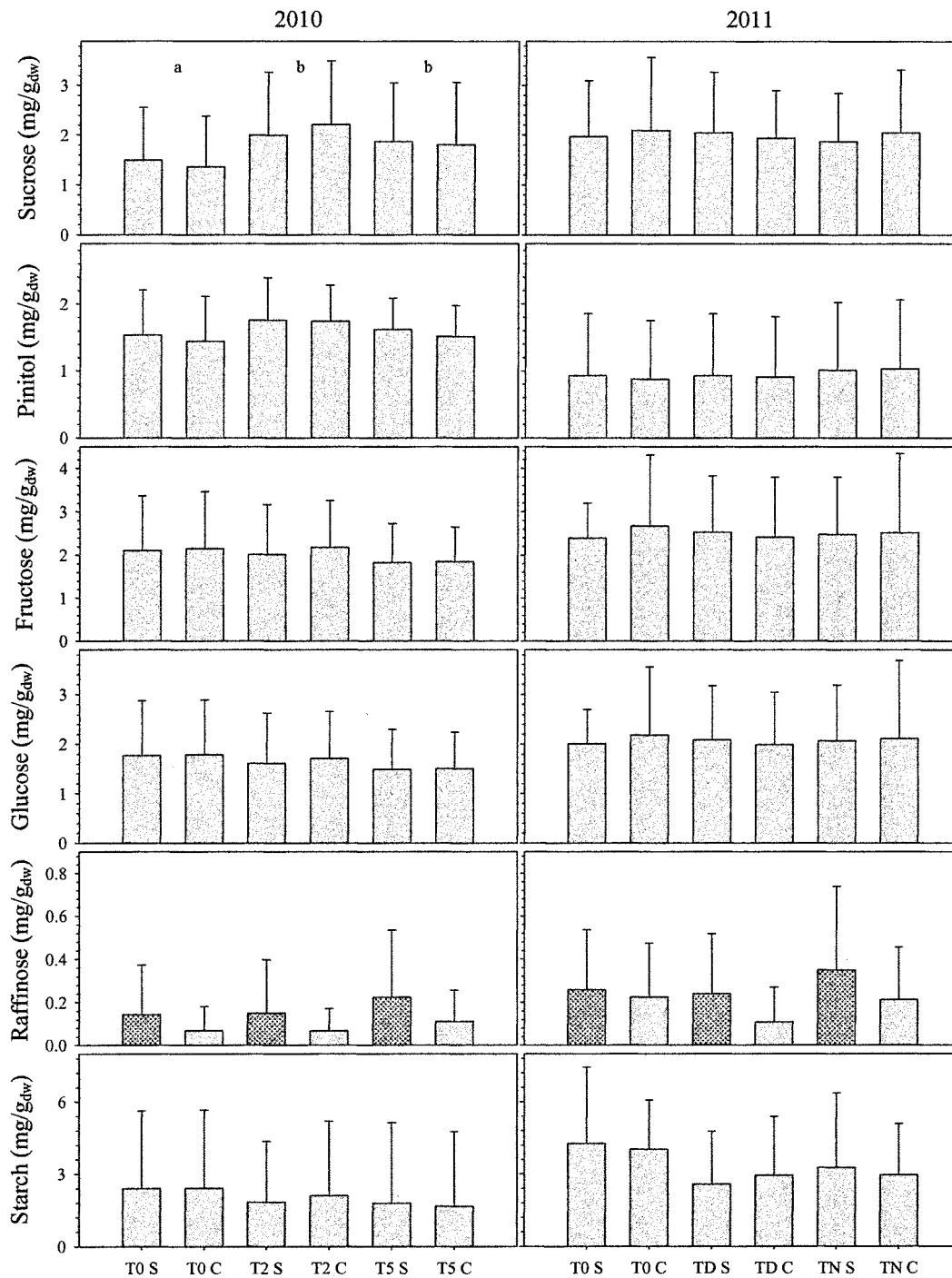


Figure 7. Means (mg/g<sub>dw</sub>) of carbohydrates in the xylem by temperature and water treatments. Temperature treatments are represented as Figure 3. Water treatments are represented by S (water deficit) and C (control). Letters represent significance for temperature treatment and shade bars for water treatment with  $p \leq 0.05$

For starch, Kruskal-Wallis test with values transformed by  $\log(x+1)$  showed significant effect of temperature treatment (table 5). No difference between irrigation regimes were observed, thus, results were not shown here.

Table 5. Mean starch values are expressed as mean and standard deviation ( $\text{mg/g}_{\text{dw}}$ ). Kruskal-Wallis test between temperature treatments in 2010 and 2011. Chi-square ( $\chi^2$ ) values are significant at  $p \leq 0.05$  (\*)

Year	Temperature treatment			$\chi^2$
	T0	T2	T5	
2010	2.41±3.24	1.97±2.81	1.72±3.23	7.06*
	T0	T6D	T6N	
2011	4.15±2.60	2.75±2.32	3.10±2.62	6.00*

## Relation between carbohydrates and growth

### *Canonical correlations*

Canonical correlations were performed between a dataset concerning wood formation (number of cells in cambium, enlargement and cell wall formation) and another one concerning sugar concentration (raffinose, sucrose, glucose, pinitol and fructose) in both cambium and xylem (table 6-7). As the sugar variation during the years was similar for the temperature and water regimes, the data of the different treatments were pooled together.

In the xylem, the correlations between the two data sets were all significant with values of 0.67, 0.46 and 0.39 between the first, second and third canonical axis. Although every canonical axes were correlated, the first one accounted for 64.4% of the variability. The second and third axes had a weight of 21.3% and 14.2% respectively. In the xylem, the first canonical axis represented, in order of importance, the cambium (correlation of 0.97) and the number of cell in enlargement (0.88) while the second canonical axis mostly represented cell wall formation only (0.97). The first canonical axis for both data sets was influenced by pinitol (0.81), fructose (0.32) and raffinose (-0.34). Instead, the second canonical axis was positively influenced by sucrose (0.66) and negatively influence by glucose, fructose and pinitol (correlation of -0.64, -0.51 and -0.36 respectively). Despite its low weight, the third canonical axis mainly represented the number of cell in enlargement and was influenced by glucose and fructose (0.70 and 0.71 respectively). Therefore, the canonical correlation indicated that pinitol, sucrose and fructose located in the xylem positively influenced cambial activity and cell enlargement while raffinose played a negative role. The lignification process was mostly influenced by sucrose.

For cambium, the correlations between the two data sets were only significant for the first two canonical axes with a correlation of 0.67 and 0.62 between the first and second canonical axis. The first one accounted for 54.8% of the variability while the second had a weight of 41.8%. The first canonical axis mainly represented, in order of importance the cambium (0.86), the number of cell in enlargement (0.86) and then cell wall formation (0.53). The first canonical axis was highly positively correlated with sucrose, (correlation coefficient of 0.93). Pinitol also had a positive correlation, but of less amplitude with a

coefficient of 0.62. Compared with xylem, the correlation coefficient for raffinose was very low (-0.04). The variables defining the second canonical axis differed from their sign and represented the number of cell in enlargement (-0.57) and cell wall formation (0.43). Nevertheless, the hexose pool was correlated with the second canonical axis, representing mainly cell enlargement while cell wall formation was still positively related with sucrose.

Table 6. Canonical correlation in xylem. The canonical correlations between the first, second and third canonical variables were 0.67, 0.46 and 0.39, respectively. The first, second and third canonical variables accounted for 64.4%, 21.4% and 14.2% of the variability (cumulative 100%). The approximate F-test indicated that these three components were significant ( $p \leq 0.0001$ )

Variable	Canonical axis 1		Canonical axis 2		Canonical axis 3	
	Correlation	Canonical coefficient	Correlation	Canonical coefficient	Correlation	Canonical coefficient
Cambium	0.97	0.65	0.05	0.02	-0.25	-0.10
Enlargement	0.88	0.59	0.23	0.11	0.42	0.16
wall formation	0.21	0.14	0.97	0.45	0.12	0.05
Raffinose	-0.34	-0.23	0.04	0.02	-0.39	-0.16
Sucrose	0.16	0.11	0.66	0.31	-0.12	-0.05
Glucose	0.10	0.07	-0.64	-0.30	0.70	0.27
Pinitol	0.81	0.54	-0.36	-0.17	-0.05	-0.02
Fructose	0.32	0.22	-0.51	-0.24	0.71	0.28



Table 7. Canonical correlation in the cambium. The canonical correlations between the first, second and third canonical variables were 0.67, 0.62 and 0.22, respectively. The first, second and third canonical variables accounted for 54.8%, 41.8% and 3.4% of the variability (cumulative 100%). The approximate F-test indicated that the first two three components were significant ( $p \leq 0.0001$ ) and the third was not significant ( $p = 0.1310$ )

Variable	Canonical axis 1		Canonical axis 2		Canonical axis 3	
	Correlation	Canonical coefficient	Correlation	Canonical coefficient	Correlation	Canonical coefficient
Cambium	0.86	0.58	-0.18	-0.11	-0.47	-0.10
Enlargement	0.78	0.52	-0.57	-0.35	0.25	0.05
Wall formation	0.53	0.35	0.43	0.26	0.74	0.16
Raffinose	-0.04	-0.02	0.44	0.27	-0.38	-0.08
Sucrose	0.93	0.62	0.23	0.14	-0.06	-0.01
Glucose	0.20	0.13	-0.75	-0.47	0.09	0.01
Pinitol	0.62	0.42	0.10	0.06	-0.67	-0.14
Fructose	0.42	0.28	-0.47	-0.29	0.17	0.03

## DISCUSSION

The carbohydrates found in the stem of the black spruce seedlings during the growing season consisted of sucrose, pinitol, fructose, glucose, raffinose and starch. Concentrations in the xylem only accounted for about 7% of the sugars in the cambium. Sucrose represented 54% of the total sugars in the cambium while the sucrose, pinitol and glucose each represented about 23% of the total sugars in the xylem, with a higher abundance of fructose, corresponding to 28% of the total carbohydrate.

Water deficit and the increase of temperature affected soluble sugars content in different ways and time. Except for sucrose and starch, no clear pattern along the growing season was found. Correlations between cell development and selected carbohydrates were presented.

### **Identification and quantification of carbohydrates**

The NSC found in the black spruce corresponded with those described in the literature for trees (Giovannelli *et al.*, 2011; Bertrand and Bigras, 2006). No unknown compound was identified on the HPLC chromatogram and all NSC were separated enough to avoid merging peaks. Only raffinose could have been miscalculated because of its low quantity, near the detection limit.

HPLC chromatogram showed that the amount of carbohydrates found in the stem was low, especially for the xylem. Xylem and cambium had a mean of total NSC of 1.61 mg/g<sub>dw</sub> and 24.62 mg/g<sub>dw</sub> respectively from May to September, the demand in energy of

growing cells of cambium explaining here the larger amount of carbohydrates then in the xylem, a mostly non active part of the stem. In contrast, needles of white spruce contained about 32 mg/g<sub>dw</sub> while the entire stem contained about 21 mg/g<sub>dw</sub> of soluble sugars from mid-July to the end of September (Dhont *et al.*, 2011). However, stems are known to have lower amount of carbohydrates than branches and leaves (Hoch *et al.*, 2003).

As the sampling was performed during the growing season, a lower amount of NSC was found. Indeed, in northern species, the amount of sugar is usually in higher quantity during the cold season independently if the tree is a broadleaf or a coniferous species (Ashworth *et al.*, 1993; Richardson *et al.*, 2013). NSC in winter play a role in cold hardiness, especially for glucose, fructose and raffinose (Bertrand and Bigras, 2006; Lee *et al.*, 2012; Zuther *et al.*, 2004). In the summer, photosynthetic products are rapidly used for growth, respiration and transport (Antonio *et al.*, 2008a, b; Bolouri-Moghaddam *et al.*, 2010) and are thus found in lower quantities.

### **Trends and correlation**

The sucrose in the cambium seemed to follow the same pattern over the two years of experiment with a “W” shape. Lower amounts were found at the beginning and at the end of the season as well as in July. Starch-sucrose conversion could explain the amount found in spring and in autumn (Schaberg *et al.*, 2000) as sucrose is converted to starch during the end of the growing season and inversely, starch is converted to sucrose at the beginning of the growing season. The pattern of starch observed in the xylem (figure 4) could support this theory (*i.e.* relatively high abundance in spring and autumn with a decline during the

summer). The brutal drop followed by a high peak in July was not mentioned in other studies. This period of low sucrose corresponded with a period of high cell development and cell wall lignification (figure 1). It is possible that almost all the sucrose present was taken for those activities. It appears that sucrose is the most important carbohydrate in the production of cambium and also plays an essential role as a signal in the induction of xylem and phloem elements (Jeffs and Northcote, 1967).

Fructose and glucose were strongly correlated between them and they both followed the same pattern along the growing season and part of the stem. They were generally correlated to sucrose but did not always follow the same pattern, like pinitol. Difficulties to observe a clear pattern for the majority of the NSC in the stem were also reported in other species like red spruce (*Picea rubens* Sarg.) (Schaberg *et al.*, 2000) or white spruce (*Picea glauca* (Moench) Voss) (Hoch *et al.*, 2003). It seems that the temporal fluctuation of the amount of NSC show a clearer trend in the needles and roots rather than in the stem. In fact, in the xylem, carbohydrates and other fat compounds of parenchyma cells act mostly as reserve material. They are used for maintaining respiration when no photosynthetic products are available (Wolfgang, 1985). NSC are actually mostly used for the bud break, new shoot growth and root development (Wolfgang, 1985).

### **Treatments effects**

Raffinose and sucrose were the sugars more affected by the water deficit in both cambium and xylem. Although differences were not always significant, stressed seedlings had higher amount of raffinose, especially from the middle to the end of the water deficit

period, with more pronounced effects at the higher temperatures. For sucrose, the irrigated seedlings had, more often, higher values than non-irrigated ones except for 2011 in T6D and T6N where stressed plants had higher amount of sugar. These sugars have been associated as protectant of stressed plants and the more complex is the molecule, the more effective it become against cold, heat or dryness (Santarius, 1973). In leaves, they are known for their role as antioxidant and osmoprotectant (Nishizawa-Yokoi *et al.*, 2008; Pinheiro *et al.*, 2011), and to form protective compounds or to stabilize membranes and proteins (Santarius, 1973). In spruces, raffinose is observed during winter and is nearly inexistent during the growing season (Bertrand and Bigras, 2006; Wang and Zwiazek, 1999; Wiemken and Ineichen, 2000). Therefore, the presence of this sugar was directly associated with water deficit. In somatic embryo of black spruce, raffinose was observed only in those affected by desiccation (Bomal *et al.*, 2002), which agrees with our results. Sucrose content also increased more than 3-fold during a desiccation period in somatic embryo (Bomal *et al.*, 2002) while in our study, no substantial increase was observed in the stem of black spruce after the water stress. The explanation of this could be that sucrose content was already up to six times higher in the control treatment in our study compared to the study in somatic embryo. It would be then possible that it was relatively less affected. Also, raffinose is synthesized from sucrose by galactinol synthase and raffinose synthase with subsequent addition of activated galactose fractions donated by galactinol (Sengupta *et al.*, 2012). This could maybe explain the general lower value of sucrose in the water deficit when raffinose was high in the cambium. Finally, the earlier reactivity of raffinose in

higher temperature during water deficit could be a synergetic effect of both water and thermal stresses.

In this study, starch was affected by increases of temperature, mostly at the end of the growing season. In fact, starch quantity tended to decrease as the temperature raised in both 2010 and 2011. Many hypotheses can be suggested to explain these results such as a decrease of photosynthesis and/or an increase in whole plant respiration (Bronson and Gower, 2010; Way and Sage, 2008a, b). Way and Sage (2008a) observed that seedlings growth at high temperature had a bigger ratio of dark respiration to net carbon dioxide assimilation rate, proposing a larger starch attribution to respiration. Thus, diminution in the warmer night treatment can be explained by a higher respiration and a higher utilisation of the starch accumulated during the day (Turnbull *et al.*, 2002; Turnbull *et al.*, 2004). Higher temperature during the day enhances export rate and utilisation of sucrose in the plant, lowering sucrose allocation for starch production (Hussain *et al.*, 1999).

Temperature had a small significant effect on fructose and raffinose but had in general no or low effect on the other carbohydrates in the two years of the study. Young trees are often more resistant to stresses than older trees: a higher metabolism and a better photosynthetic system seems to decrease mortality of the seedlings compared to older trees exposed to the same stress (Kull and Koppel, 1987; Rossi *et al.*, 2009a).

### **Canonical correlation in the cambium**

In cambium, canonical correlation identified the most important sugars for xylem development. The first canonical axis was mainly correlated with sucrose (0.93) and for the

developmental phases with, in order, the number of cells in the cambium (0.86), in enlargement (0.78) and in lignification (0.53). These results put in evidence the positive correlation between sucrose and the number of cells in the cambium and in enlargement mostly. The sucrose, coming either from starch degradation (Begum *et al.*, 2013) or directly from the leaves, thus stimulate cambium division and cell expansion. Cell wall formation was also correlated, but in a less significant way (0.53), with the first canonical axes and mainly related with sucrose. Sucrose is cleaved by sucrose synthase forming fructose and UDP-glucose (Koch, 2004). The UDP-glucose product is then implicated in the formation of diverse cell wall polysaccharides (Albrecht and Mustrup, 2003; Doblin *et al.*, 2002) and its amount could directly limit the cellulose synthesis (Roach *et al.*, 2012). The formation of cell wall is considered a developmental, rather than metabolic process. Time is thus more important than rate in the deposition of wall material in the latewood tracheid (Uggla *et al.*, 2001). Cambium shows greatest quantities of sugars as it is composed of live cells in development and because of its incorporation into the transport system (Uggla *et al.*, 2001; Antonova and Stasova, 2008).

As the variation of sucrose and pinitol were highly correlated (table 4), pinitol was also correlated with division and enlargement. In many plants species (Ford, 1984; McManus *et al.*, 2000; Streeter *et al.*, 2001) and in trees (Ericsson, 1979; Streit *et al.*, 2013), pinitol has been described as an important carbohydrate, especially under different stress conditions such as water, salt and low temperature (Orthen *et al.*, 1994) acting as an osmolyte (Reddy *et al.*, 2004). This sugar is also important for osmoregulation in order to maintain the cell turgor potential (Johnson *et al.*, 1996; Orthen *et al.*, 1994). As no difference in the pinitol

concentration was observed between control and water deficit seedlings, it was not excluded that pinitol might also contribute to the overall water potential (maintaining turgor) needed to enlarge the cell. Being a boreal species experiencing very low temperatures in winter, the protective role of pinitol in black spruce might be more oriented through cold acclimation than water stress. In larch, the pinitol concentration was four-times higher at high altitude as a result of a long term adaptation to lower temperature (Streit *et al.*, 2013).

The hexose pool could also contribute to division and enlargement processes but in a smaller amount. Glucose is mainly related to the second canonical axis (-0.75) that is linked with cell enlargement (-0.57). Fructose was linked with the first (0.42) and the second canonical axes (-0.47) that were both correlated with cell division and enlargement. However, this sugar, a product of sucrose cleavage by invertase or sucrose synthase, is more used for respiration and eventually ATP production (Koch, 2004) necessary for cells metabolism during growth processes or active transport of several compounds across membrane. This explains that the living number of tracheid in the stem was positively correlated with the stem CO<sub>2</sub> efflux (Lavigne *et al.*, 2004; Gruber *et al.*, 2009) during the growing season. Therefore, the result for fructose could support the fact that the sugar allocation and partition was linked not only with growth but also with metabolic processes.

As mentioned before, raffinose is an important compound for tissue protection as it binds with water molecules. Raffinose was only positively related with the second canonical axis (0.44) and therefore, negatively linked with cell enlargement (-0.55). This proposes that an increase in raffinose decreases the number of cells observed in the



enlargement phase. This result is in agreement with the decrease in the number of cell in enlargement after the water stress (figure 2) and the increase in the raffinose content in the cambium.

### **Canonical correlation in the xylem**

Significant relationships were also found for the xylem. However, especially for the first canonical axis, those were more difficult to interpret. The main reason is that one has to assume that the non-structural available soluble sugars in the parenchyma cells of wood have to migrate throughout the rays, to the site of cell differentiation near the cambium and act as a source. This assumption thus imposes sink-source relationship between the rays parenchyma cells and xylem cell differentiation (Olano *et al.*, 2013). In the cases of water stress, the possible carbon allocation coming from the ray parenchyma cells should decrease or be null in order to protect the rays cells from desiccation and/or protect/repair xylem embolism (Nardini *et al.*, 2011). Moreover, sink competition can occur in the case of water deficit as sugars are needed for osmoregulation. Therefore, the interpretation of these results should take into account these important but complex assumptions.

The first canonical axis was mainly related with cell division (0.97) and enlargement (0.88) that was mainly link with pinitol (0.81). As for cambium, we assumed that pinitol might also contribute to the overall water potential (maintaining turgor) needed to enlarge the cell. Apart from their more general role in osmotic adjustment, the mode of function of cyclitols is well understood only in the case of stress (Orthen *et al.*, 1994). Raffinose was negatively linked with the first canonical axis (-0.34) indicating again the negative effect of

this sugar on cell division and enlargement (*i.e.* water stress effect). Cell wall formation explained most of the second canonical axis (0.97) and was positively related with sucrose (0.66) which is in agreement with the results found for cambium. However, this growth process was negatively related with glucose (-0.64), pinitol (-0.36) and fructose (-0.51) possibly illustrating complex sink competition for growth processes and metabolic needs of parenchyma cells.

## CONCLUSION

The aim of this study was to establish the nature and the variation of the non-structural carbohydrates in black spruce seedlings and their behaviors under warming and water deficit along the growing season. We also wanted to demonstrate that carbohydrates are directly related to xylogenesis, from the cambial production to the mature cells. We hypothesised that temperature warming and water deficit stresses would result in diminishing carbohydrate availability for xylogenesis and thus, diminishing cambial cell development and growth. Results of the study, merged with cell growth observations of the same trees allowed us to understand a little more about those links between sugars and secondary growth.

Results demonstrated that soluble carbohydrates content in black spruce seedlings were mostly drove by water deficit and that reactions were amplified when seedlings were exposed to high temperatures. Sucrose, raffinose and, at some point, starch were the more affected. Those sugars are the most complex of the non-structural carbohydrates found in the black spruce and their rapid response to stresses prove their protection role for the plants.

Correlations between carbohydrates and xylogenesis were found. We can assume that secondary growth is dependant of the carbohydrates, mostly on sucrose, for its capacity of transport toward the phloem and cleaving into structural compounds. Raffinose and pinitol were also correlated to secondary growth of cambium and xylem but we think that here,

their presence is more associated to their function of osmoprotectant and for maintaining the turgor into the cells.

But either if carbohydrates were correlated to water deficit and temperature rise, and that xylogenesis was found correlated to carbohydrates, small differences observed in cells development were not correlated to the treatments. In that case, we could suggest that even if carbohydrates availability is lower in case of the stressing events studied here, there were still enough sugars to maintain normal cambial growth and development during the growing season. We could hypothesise that northern species that need more carbohydrates to protect themselves from the cold during the winter are accumulating a larger amount of carbohydrates than necessary during the growing season. It would thus be possible to observe more mortality during the winter or in the growing season after the stressing events because of a lack of carbohydrate availability.

This kind of project rise more questions than it answers. But the work here was among the first steps to understand the physiology of the black spruce, one of the most important forest species in the northern part of the North American. Because of the correlations made here, it would be essential to study the effect of temperature rise and water deficit during one growing season on a two year scale, to observe if there are differences between the treatments in carbohydrates availability for a longer period of time. It was also impossible to go any further in the exact role of those carbohydrates in the black spruce with this study and more precise experience should be done with hormones, enzymes, protein and other compound to fully understand their functions, especially under environmental changes. More studies should also be done on older trees and on other parts of the tree. Stems are

great to measure the secondary growth but are less chatty when it comes to photosynthetic compounds.

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