

**Impact of warming and drought on carbon balance related to wood formation
in black spruce**

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Running title: Soluble sugars under warming and drought

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15 **ABSTRACT**

16 **Background and Aims** Wood formation in trees represents a carbon sink that can be modified in
17 case of stress. The way carbon metabolism constrains growth during stress periods (high
18 temperature and water deficit) is now under debate. The amounts of non-structural soluble sugars
19 (NSCs) for xylogenesis in black spruce saplings were assessed under high temperature and
20 drought to determine the role of sugar mobilization for osmotic purposes and its consequences
21 for secondary growth.

22 **Methods** Four-year-old saplings of black spruce were submitted to different thermal conditions
23 with respect to the outside air temperature (T₀) in 2010 (2 and 5 K higher than T₀) and 2011 (6
24 K warmer than T₀ during the day or night) with a dry period of about one month in June of each
25 year. Wood formation combined with starch, NSCs and leaf parameters (water potential and
26 photosynthesis) was monitored from May to September.

27 **Key Results** Except for raffinose, the amounts of soluble sugars were not modified in the
28 cambium even if gas exchange and photosynthesis were greatly reduced during drought.
29 Raffinose increased more than pinitol under a Ψ_{pd} of less than -1 MPa because this compound is
30 more apt than polyol at replacing water, capturing free radicals, and its degradation into simple
31 sugar is easier. Warming decreased the starch storage in xylem as well the available hexose pool
32 in cambium and xylem, probably because of an increase in respiration.

33 **Conclusions** Radial stem growth was reduced during drought due to the mobilization of NSC for
34 osmotic purposes and the lack of cell turgor. In cambium and xylem, plant water status during
35 wood formation can influence the NSCs available for wood formation.

36 **Keywords:** Cambium, drought, non-structural soluble sugars, raffinose, starch, warming,
37 xylogenesis

38

39 INTRODUCTION

40 Climatic models predict increases in temperature in the boreal forest of up to 3 °C over the next
41 50 years, with the greatest increases occurring in winter and spring, at resumption of plant
42 growth (Plummer *et al.*, 2006). Changes in precipitation regime are also predicted with more
43 extreme events, especially during winter (increase in precipitation) and summer (drought).
44 However, temperatures are not expected to change linearly during the day: between 1950 and
45 1998, unlike the daily maximum, the daily minimum increased significantly, indicating that the
46 nights were warmer (Bonsal *et al.*, 2001). These modifications could affect gas exchanges (Way
47 and Sage, 2008b) in the plant and consequently the production of photosynthates (i.e. soluble
48 sugars), as well as degradation of starch which are necessary during growth process.

49 Within the stem of conifers, the formation of wood represents a powerful carbon sink that is
50 linked with the non-structural carbohydrate (NSC) in cambium and xylem (Deslauriers *et al.*,
51 2009, Simard *et al.*, 2013). As reviewed by Pantin *et al.* (2012), cell growth involves the
52 movement of water and solute into the cell, generating sufficient turgor pressure for irreversible
53 growth as well as an accumulation of biomass into new structures. Under drought, growth can be
54 inhibited before photosynthesis, which can temporarily increase NSC (McDowell, 2011, Muller
55 *et al.*, 2011) or not (Gruber *et al.*, 2012, Duan *et al.*, 2013). Thus, growth constraints during
56 drought are related to turgor but unrelated to carbon availability (Woodruff and Meinzer, 2011).
57 Under high temperature, even if a previous study indicated that total NSC remained unchanged
58 (Duan *et al.*, 2013), nocturnal warming can have a significant impact on the plant metabolism:
59 nocturnal warming increase respiration (Turnbull *et al.*, 2004, Turnbull *et al.*, 2002) leading to a
60 faster degradation of the transitory starch [i.e. starch stored during the day in chloroplasts and

broken down at night for export (Lu *et al.*, 2005)], thus decreasing carbon to support sucrose synthesis and growth at night and during the following day.

Under high temperature and water deficit however, the flow of available carbon could be further directed to osmoregulation at the expense of growth (Pantin *et al.*, 2013). During drought conditions, a high amount of non-structural sugars accumulates in all tissues in order to protect living tissues, especially from ROS (Reactive Oxygen Species), and to avoid cavitation (Regier *et al.*, 2009). Adaptive responses of plants to disturbances also include solute accumulation, such as inorganic ions (K^+ , Cl^- , Na^+), organic components (proline, serine, malate, etc.) and other soluble sugars (raffinose, sucrose and pinitol) that play an important role in active osmotic adjustments (Aranjuelo *et al.*, 2011, Liu *et al.*, 2008). Among the soluble sugars, the role of raffinose in plant cell protection as an osmoprotectant or antioxidant is very well-known (Nishizawa-Yokoi *et al.*, 2008, dos Santos *et al.*, 2011). In many herbaceous (Streeter *et al.*, 2001, Ford, 1984, McManus *et al.*, 2000) and tree species (Ericsson, 1979, Streit *et al.*, 2013), pinitol has been described as an important polyol, especially under stress conditions such as drought, salinity or low temperature (Orthen *et al.*, 1994), acting as an osmolyte (Reddy *et al.*, 2004). The variation of cyclitols, such as D-pinitol, and soluble sugars has recently been assessed in conifers (Gruber *et al.*, 2011, Simard *et al.*, 2013, Streit *et al.*, 2013) and related with secondary growth of the stem as well as cell protection. Consequently, the main challenge could be to understand how plant growth may be influenced by changes in concentration of each single sugar in response to drought and warming.

Here, we want to verify how an increase in temperature and drought will modify the amount of soluble sugars available for xylogenesis in the stem of black spruce [*Picea mariana* (Miller B.S.P.)]. We tested the hypothesis that during a period of water deficit, the pool of available

sugars in cambium and xylem will be directed towards cell osmoregulation more than growth, with an amplified effect under increasing temperature. Two temperature regimes were applied (one per year) with warming occurring only during daytime or nighttime, or in both periods. According to the forecasts for the next century, the increase in temperature will not be uniform, but minimal temperatures will be more affected by warming than maximum temperature (Bonsal *et al.*, 2001). Thus, we also tested the hypothesis that a heterogeneous warming will influence nighttime respiration and the availability of mobile sugars and starch reserves in plants. The seasonal dynamics of soluble sugars content under supra-optimal growth temperatures and water deficit was therefore monitored in cambium and xylem of four-year-old black spruce saplings to assess the major changes in non-structural carbohydrates (NSCs) concentration within both tissues and to evaluate their consequences for growth.

96 **METHODS**

97 **Study area and experimental design**

98 The study took place in a greenhouse complex located at the University of Quebec in Chicoutimi
99 (48° 25' N, 71° 04' W, 150 m above sea level, QC, Canada). The mean temperatures in 2010 and
100 2011 were 5.2 °C and 2.2 °C, respectively. The higher mean in 2010 was caused by a particularly
101 mild winter and spring with a mean January-May temperature of -0.2 °C compared with -4.5 °C
102 in 2011. The average temperatures in summer 2010 and 2011 were 18.1 °C and 17.6 °C,
103 respectively.

104 Two independent experiments were performed in a greenhouse divided in three independent
105 sections and automatically controlled with misting and window-opening systems for cooling.
106 Approximately 300 saplings of black spruce were installed in every section both years. Plants
107 were four-year-old saplings transplanted in 4.5 L plastic pots with a peat moss, perlite and
108 vermiculite mix, and left in an open field during the entire previous growing season and winter.
109 In April of each year, the saplings were taken inside the greenhouse for the experiment and
110 fertilized with 1 g l⁻¹ of NPK (20-20-20) fertilizer dissolved in 500 ml of water. Only the
111 vigorous trees were selected for the experiment, while the others were used in the buffer zone at
112 the borders. Overall, the saplings were 48.9 ± 4.7 cm in height, with a diameter of 8.0 ± 2.0 mm
113 at the collar. Each sapling was equipped with drip trickles to perform the irrigation. Different
114 irrigation and temperature regimes were applied in each section. The control (named T0)
115 corresponded to outside temperature, while the two other sections were subjected to specific
116 thermal regimes. In 2010, T2 and T5 experienced a temperature 2 and 5 K higher than T0,
117 respectively (Balducci *et al.*, 2013). In 2011, TD and TN were 6 K warmer than T0 during the
118 day (TD, from 07:00 to 18:59) or during the night (TN, from 19:00 to 06:59), respectively

(Figure 1). For irrigation, the soil water content was maintained at over 80% of field capacity in the control, while the other saplings were submitted to a water deficit from about mid-May to mid-June in 2010 and from June to the beginning of July in 2011, when cambium is vigorously differentiating (Rossi *et al.*, 2006b). The water deficit period correspond to DOY 142-173 in 2010 and DOY 158-182 in 2011. At the end of the water deficit period, the soil water content of non-irrigated saplings was less than 10% while irrigated saplings had a soil water content varying between 40 and 50%.

Xylem growth

Each week from May to September, stem disks were collected 2 cm above the root collar from 36 randomly-selected saplings [6 saplings \times 3 thermal conditions \times 2 water regimes, (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 μm thickness were cut with a rotary microtome (Rossi *et al.*, 2006a). 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 total radial number of cells including (i) cambial, (ii) enlarging, (iii) cell-wall thickening, and (iv) mature cells were counted along three radial files and averaged according to Rossi et al. (2006a).

Water relations, gas exchange and CO₂ assimilation

Plant water status was followed by measuring pre-dawn (Ψ_{pd}) leaf water potential from May to August on branches of the first whorl of 3 saplings per treatment (3 thermal conditions \times 2 irrigation regimes) with a pressure chamber (PMS Instruments, Corvalis, OR). Stomatal conductance (g_s , $\text{mol m}^{-2} \text{s}^{-1}$) and maximum photosynthesis rate (A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$) were

measured from 10:00 to 13:00 under saturating irradiance conditions ($1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, (Bigras and Bertrand, 2006)] using a portable photosynthesis system (Fig. 1) [Li-6400, LI-COR Inc., Lincoln, NB) and processed according to Balducci et al. (2013). In the greenhouse, the saplings were grown at $400 \mu\text{mol m}^{-2} \text{s}^{-1}$. In order to avoid light stress the saplings were acclimated for 15-20 min at $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$ before the measurements.

NSC extraction and assessment

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

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

The soluble carbohydrates extraction followed the protocol proposed by 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 lower than the HPLC detection limit. 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 (0.01g/ml) was also added as an internal standard at the first extraction. In each extraction, the homogenates were gently vortexed for 30 minutes and centrifuged at 10,000 rpm for 8 minutes. The three resulting supernatants were evaporated and recuperated with 12 ml of nano-filtered water. This solution was then

164 filtered by the solid phase extraction (SPE) method using a suction chamber with one column of
165 *N+* quaternary amino (200 mg/3ml) and one of *CH* (200 mg/3ml). The solution was evaporated
166 until 1.5 ml and filtered through a 0.45 µm syringe filter to a 2 ml amber vial.

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

174 The sugar loss during extraction was calculated by comparing the concentrations of sorbitol
175 added to the sample at the beginning of the extraction, to those of free-sorbitol. The loss
176 percentages were then calculated and added to the final results.

177 Xylem powder was used for starch extraction, performed according to Chow and Landhäusser
178 (2004). The extraction consisted of adding 5 mL of 80% ethanol to 50 mg of powder at 95 °C.
179 The solution was vortexed for 30 minutes and centrifuged, and the supernatant was removed.
180 This step was repeated twice. The starch was solubilised with NaOH 0.1M and acetic acid 0.1M
181 and digested with an alpha-amylase solution at 2000U/mL and amyloglucosidase 10U/mL. PGO-
182 color reagent and H₂SO₄ 75% were added to the solution 24 hours later. Starch was assessed
183 using a spectrophotometer at 533 nm (Chow and Landhäusser, 2004).

184 **Statistical analysis**

Because of asymmetric distributions in the water potential data (few points with a $\Psi_{pd} < -1$ MPa) across treatments, Spearman's rank correlations were used to assess the monotonic relationship between the Ψ_{pd} and sugar concentrations of sucrose, pinitol and raffinose [water deficit (W), temperature (T), day of the year (DOY)] (Quinn and Keough, 2002). For each sugar and starch, the effect of temperature and water deficit was tested by General Linear Models (GLM procedure in SAS) with a factorial model with three (df=3) as the error term for testing the treatments effect [water deficit (W), temperature (T), day of the year (DOY)] (Quinn and Keough, 2002). Differences between treatments were found with the Tukey test. Starch data were transformed into their log in order to respect the homogeneity of variance.

To verify the effect of treatment on radial growth response, comparisons of fitted curves were performed. The Gompertz logistic function (NonLINear regression, SAS) was fitted to the total number of cells for the 6 combinations of water and temperature treatments for each year and compared (Potvin *et al.*, 1990). The Gompertz function was defined as:

$$y = A \exp \left[-e^{\beta - \kappa t} \right]$$

where y is weekly cumulative sum of cells, t the time computed in day of year (DOY), A the upper asymptote (maximum of growth expressed as cell number or tree-ring width), β the x -axis placement parameter and κ the rate of change of the shape (Deslauriers *et al.*, 2003). In the Gompertz function, the inflection point (t_p) corresponds to the culmination of growth rate. The placement of the inflection point on the horizontal axis (t_p , DOY) occurs where the second derivative is equal to 0, i.e. when $t_p = \beta/\kappa$ (Rossi *et al.*, 2006b). A weighted mean absolute cell formation rate (r , cell.day⁻¹) was also calculated as (Deslauriers *et al.*, 2003):

$$r = \frac{A \kappa}{4}$$

RESULTS

Xylem cell production

Sapling radial growth was characterized by a sharp increase starting around DOY 120-130, followed by a plateau indicating the end of radial growth and resulting in a typical S-shaped curve (Figure 2). Significant differences were found between the radial growth curves in 2010 and 2011 (group effect, $p < 0.0001$) (Table 1, supplementary material). Successive pairwise comparisons revealed a significant difference between the water treatments for each year ($p < 0.0001$), thus reducing the rate (r) and total number of formed cells (A) in the non-irrigated saplings. Temperature treatment in 2010 and 2011 led to different results. Although the number of cells decreased with increasing temperature (T2 and T5) for both irrigated and non-irrigated saplings in 2010, the effect was not significant ($p = 0.59$). A temperature effect was found in 2011 ($p = 0.025$) but with contradictory results between the irrigation treatments: TD and TN treatments increase the total number of cells (A) in the irrigated saplings whilst both decrease A in the non-irrigated saplings.

Leaf water relations, gas exchanges and photosynthesis

During 2010, the leaf Ψ_{pd} of non-irrigated saplings dropped dramatically in response to the decrease of soil water availability, reaching the lowest values on DOY 172 (-2.7 MPa) without evident differences between thermal regimes. In 2011, leaf Ψ_{pd} of non-irrigated saplings was at -0.5 MPa at T0 and ranged from -1.09 MPa for TD to -2.28 MPa for TN. One week after the resumption of irrigation, the leaf Ψ_{pd} of non-irrigated saplings were similar to those observed in irrigated ones, showing that the saplings were able to recover an optimal water status after a period of water deficit. These conditions persisted for the rest of the summer. At the end of the

water deficit period in 2010, A_{\max} of irrigated saplings was 10 (units) fold higher than non-irrigated ones (2.57 and $0.27 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, for irrigated and non-irrigated saplings, respectively). The differences in A_{\max} were more pronounced under warmer temperature than T0. Average values of stomatal conductance (g_s) ranged from 0.06 to $0.00 \text{ mol m}^{-2} \text{ s}^{-1}$, for irrigated and non-irrigated saplings, respectively. Similar patterns were observed in 2011, at the end of water deficit period A_{\max} of irrigated saplings was also 10 (units) fold higher than non-irrigated ones (5.37 and $0.51 \mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$, for irrigated and non-irrigated saplings, respectively). Average values of g_s ranged from 0.13 to $0.03 \text{ mol m}^{-2} \text{ s}^{-1}$, for irrigated and non-irrigated saplings, respectively.

Variation of carbohydrates in cambium and xylem during the growing season

Cambium

Each soluble sugar varied in a similar way with respect to the temperature and irrigation regime in cambium (Figure 3). Sucrose was from 2 to 30 times more abundant than the other sugars, followed by D-pinitol (Table 1, Figure 3). In both years, the amount of sucrose in the cambium was high at the beginning of stem growth, with a concentration of about $100 \text{ mg/g}_{\text{dw}}$ and then showed several decreases and increases in concentration. In 2010, the variation of fructose and glucose showed an irregular pattern, while in 2011, they increased at the beginning of tree-ring formation and gradually decreased towards the end of the growing season (Figure 3). D-pinitol concentration followed the seasonal trend of sucrose, but did not drop to almost zero like sucrose (Figure 3). The concentration of raffinose was always very low in cambium during the growing season, with the exception of high values recorded in non-irrigated saplings between DOY 160 and 180 in both years, as well as in TN saplings at the end of tree-ring formation (Figure 3).

251 *Xylem*

252 In xylem, fructose was the most abundant soluble carbohydrate followed by sucrose and D-
253 pinitol with an amount lower than 3 mg/g_{dw} (Table 1). Concentrations of sucrose in the xylem
254 were generally high at the beginning and end of the growing season (Figure 4). As in cambium,
255 sucrose almost disappeared in July (DOY 160-170), reaching concentrations close to zero.
256 Variations of D-pinitol, fructose and glucose showed no specific seasonal trend. The
257 concentration of raffinose was always near zero mg/g_{dw} throughout the growing season except
258 for the high values observed mainly in non-irrigated saplings during and after water deficit.

259 Starch in xylem did not follow the same pattern as the other sugars and showed a pronounced
260 seasonal trend. It was more abundant at the beginning of the growing season and dropped to
261 almost zero on DOY 180, then stayed low until the end of summer when starch reserves started
262 to build up again (Figure 4).

263 **Effects of plant water status, temperature and water deficit on soluble sugars**

264 The concentration of sucrose, D-pinitol and raffinose in cambium was influenced by the plant
265 water status (Figure 5, Table 2). Sucrose and D-pinitol concentrations changed according to leaf
266 water potential. For irrigated saplings at T0, sucrose and D-pinitol (Figure 5) increased with
267 decreasing leaf Ψ_{pd} with significant regression (except for sucrose in 2010) (Table 2). However,
268 under water deficit (leaf $\Psi_{pd} < -1$ MPa), sucrose and D-pinitol did not increase proportionally.
269 Contradictory results were observed for the temperature treatments. In 2011, increasing daily
270 temperature did not affect the relationship between leaf Ψ_{pd} and the measured quantities of
271 sucrose and D-pinitol whilst no relationships were found at increasing night temperature in 2011.

In 2010, no significant correlation was observed at T2 and T5 (Figure 5, Table 2) and the signs of the correlation were mostly positive, as for the night temperature in 2011.

Only raffinose showed an increase in concentration with a decreasing of predawn water potential (Ψ_{pd}) under water deficit. With leaf Ψ_{pd} values higher than -1 MPa, no clear relationships were observed for any irrigation or temperature treatments because the concentration was mostly close to zero. For saplings growing under water deficit however, an increased raffinose concentration was observed in both years with a more significant correlation in 2011. Therefore, with values of leaf Ψ_{pd} lower than -1 MPa, the variation of raffinose in the cambium was mostly affected by leaf Ψ_{pd} and year of growth.

Besides the plant water status, temperature and irrigation treatment had an effect on the mean sugar concentration and starch quantities. A general linear model (GLM) was run to compare the effect of irrigation, temperature, irrigation \times temperature and DOY (Table 1). Results were all significant ($p < 0.001$) for DOY, meaning that a significant difference occurred in the seasonal pattern of sugars concentration. The treatment cross effect (irrigation \times temperature) was never significant, except for sucrose in cambium (2011), meaning that sugars across the different temperature treatments varied in parallel in the irrigated and non-irrigated trees.

For the irrigation treatment, only raffinose showed a significant increase in the non-irrigated plants, in both cambium and xylem in 2010 and 2011. According to Figures 3 and 4, raffinose started to increase at the end of the water deficit period in 2010 and in the middle in 2011. The highest increase was observed for TN in 2011, with a value of 11.2 mg/g_{dw}. No other differences in sugar concentration were observed for the irrigation treatment.

The temperature treatments, applied during the whole growing season, had several significant effects on the NSC concentration (Table 1). In 2010, the sucrose concentration in cambium decreased in the temperature treatment T5 ($p=0.03$). In the xylem, higher concentrations of sucrose were found in T2 followed by T5 and T0 ($p<0.001$). However, no effects of day or night temperature were observed in 2011 for sucrose concentration in either cambium or xylem. D-pinitol also had a divergent response between 2010 and 2011. In 2010, higher concentrations were found in both cambium and xylem for T2 and T5 treatments ($p<0.05$) compared with T0, but no such increase occurred for TD or TN.

For both years, similar results were found for glucose and fructose in cambium (Table 1). They significantly decreased with increasing temperature T2, T5, TD but not in TN. Values in TN were slightly higher than in T0. Glucose and fructose also decreased in the xylem with increasing temperature but the results were significant only for 2010 ($p<0.05$). For raffinose, a significant effect of temperature was found in cambium and xylem in 2011 ($p<0.05$). Day and night temperature treatments produced a contrasting effect in cambium when compared with T0: an increase of raffinose concentration was observed in TN and a decrease in TD. In the xylem, a difference was observed only between TD (decreasing effect) and TN (increasing effect).

Increased temperature during the growing season caused a significant decrease of starch reserves in the xylem (Table 1). In 2010, the starch in T0 was significantly higher ($p<0.001$) compared with T2 and T5. The same results were found in 2011 where starch was found in higher quantities in T0 ($p<0.001$). In 2011, the lowest starch quantities were found in TD for the non-irrigated saplings ($2.71 \text{ mg/g}_{\text{dw}}$). The differences were mainly caused by a lower starch deposition after the summer starch depletion.

315

316

317 **DISCUSSION**

318 Under water stress, the behaviour of black spruce was typical of an isohydric species, with early
319 stomatal closure that prevented desiccation while photosynthesis was shut down. Despite this,
320 similar patterns were observed in the sugars concentration within the stem under water deficit
321 and warming, except for raffinose. According to Sala *et al.* (2012), time (short *vs* long stress
322 period) and scale (specific tissues *vs* whole plant) have to be taken into account when
323 interpreting carbon dynamics of trees under stress. In the case of fast acting drought, carbon
324 reserves are relatively untouched and carbohydrate availability depends more on water potential
325 and phloem functioning than on photosynthate production (Sevanto *et al.*, 2014) since water
326 molecules are essential in many reactions of starch degradation (i.e. hydrolysis of maltose) and
327 sucrose hydrolysis for the production of hexoses. At short time scale, radial growth slowed down
328 or even stopped for about two weeks during water deficit meaning that the population of cells in
329 differentiation was lower, which in turn decreased the need for carbohydrate. A decrease in
330 respiration during drought could also decrease the carbon consumption leading to a surplus of
331 total carbon (Duan *et al.*, 2013). As hypothesized, during a fast acting drought, osmoregulation
332 was far more important for survival than wood formation. However, the osmoregulatory
333 response was directly dependent on raffinose concentration. In both cambium and xylem,
334 raffinose was the key sugar for osmoregulation until a leaf Ψ_{pd} of -3.6 (the minimum we
335 measured on a living sapling). Beyond that value, carbohydrate unavailability could compromise
336 both osmoregulation and hydraulic conductivity leading to plant death (Sevanto *et al.*, 2014).
337 Contrary to our hypothesis, osmoregulation was not affected by increased temperature as the
338 raffinose concentration was essentially driven by the leaf water potential (i.e. global plant water
339 status) while ambiguous patterns were observed for sucrose and pinitol. At longer time scale (i.e.

over the whole wood formation period), warming affects the hexose pool and starch recovery after the summer minimum, which could eventually compromise the growth and metabolism of the sapling the following year.

Seasonal trend

The observed intra-annual trends of increase and decrease in soluble sugars during wood formation were probably caused by carbon partitioning to sustain growth in different parts of the trees and starch to sugar conversion. Similar patterns of sucrose were found over the two years of experiment, with an alternation of low and high quantities in both cambium and xylem. Fructose and glucose were strongly correlated and both followed the same pattern over the growing season in both xylem and cambium. Seasonal low (sucrose) and high values (glucose, fructose and pinitol) were found around July 20 (DOY 200) in all treatments and years. The increase in the hexose pool and decrease in sucrose could correspond with the beginning of starch mobilisation at mid-summer in order to refill the reserves within the storage compartment. According to Witt and Sauter (1994), the concentration of glucose and fructose in ray cells showed peaks in certain periods of the year, such as during starch mobilisation in April and in the phase of rapid starch deposition during the summer. Although not reported in the literature, the relatively low quantities of sucrose found in June and July could also be linked with growth activities of primary meristems and roots. The maximum period of needle growth corresponded with the decrease of sucrose in June (data not shown). An accumulation of NSC was observed in coarse roots of *Pinus sylvestris* at the end of July (Gruber *et al.*, 2012) and used for root growth after the end of aboveground growth period (Hansen and Beck, 1994). In older trees, a parallel change between the dynamics of wood formation and the available pool of sugar in cambium was reported in larch and spruce (Simard *et al.*, 2013). In the xylem however, difficulties in

observing a clear pattern in the stem were found in other species like red spruce (*Picea rubens* Sarg.) (Schaberg *et al.*, 2000) and white spruce (*Picea glauca* (Moench) Voss) (Hoch *et al.*, 2003).

Sugar variations under water deficit and warming

Under mild water deficit, a proportional increase in pinitol and sucrose concentration was observed with a decreasing leaf Ψ_{pd} , but this relationship was not maintained with leaf Ψ_{pd} lower than -0.8 MPa. The osmoregulatory roles of pinitol and sucrose thus seem to be limited to a definite range of water potential. This pattern was not followed by raffinose. The concentration of this sugar, a member of the raffinose family oligosaccharides (RFOs), increased proportionally with decreasing Ψ_{pd} potential. We postulate that in stem of black spruce, living cells first accumulated pinitol and hexose in order to regulate cell osmosis and they only began to produce complex sugars (oligosaccharides) when the level of stress increased ($\Psi_{pd} < -0.8$ MPa), which directly prevented cell oxidation caused by stress. In this study, raffinose was the only sugar affected by water stress (Table 1, Figure 5). In 2011, high night temperature also led to an increase in raffinose with respect to T0 but this was caused by the lower water potential reached during the TN temperature treatment.

According to Ford (1984), tropical legumes accumulated pinitol with decreasing leaf water potential. In our experiment however, the pinitol concentration did not continue to rise with a more negative water potential (Figure 5) showing a substantial independence from water stress (Table 1). Pinitol concentration increased in Maritime pine seedlings with decreasing osmotic water potential (Ψ_s) in roots, with the minimum value of Ψ_s reaching -0.8 MPa (Nguyen and Lamant, 1988). During water stress, pinitol could replace water molecules, because of its alcohol

function (Nguyen and Lamant, 1988) and also act as a hydroxyl radical scavenger as drought favours the development of oxygen free radicals (Orthen *et al.*, 1994). In black spruce, leaf water potential of -2.5 MPa can severely injure black spruce because of little osmotic adjustment capacity (Johnsen and Major, 1999, Marshall *et al.*, 2000). The response of sucrose, being similar to pinitol, demonstrated that the effect of this sugar was also Ψ_{pd} limited. Nishizawa-Yokoi *et al.* (2008), found in *Arabidopsis thaliana* leaves, that the increase in intra-cellular levels of galactinol and raffinose had no effect on level of glucose, fructose and sucrose.

Raffinose has important roles such as osmoprotection and ROS scavenging (dos Santos *et al.*, 2011, Nishizawa-Yokoi *et al.*, 2008) associated with several types of stress responses (i.e. drought, cold, salinity and warming). Galactinol synthase is the enzyme catalyzing the first step of RFOs by forming galactinol from UDP-galactose and myo-inositol. Raffinose is then formed by the addition of a galactinol unit to sucrose, which liberates a myo-inositol molecule, a reaction catalyzed by raffinose synthase (Castillo *et al.*, 1990). As sucrose is an essential sugar for the biosynthesis of raffinose, sucrose could be directed through this pathway and thus its concentration fails to increase at low Ψ_{pd} . Raffinose molecules are more apt at capturing free radicals (O_2 - for example) through the many hydroxyl functions (OH), which have high reducing power. In comparison, pinitol molecules have fewer OH functions. Cyclitols, such as sorbitol (Ahmad *et al.*, 1979) and pinitol, do not easily diffuse through cell membranes and thus accumulate in the cells causing an osmotic pressure change. Sugars are better molecules for replacing water in membranes to maintain the space between the phospholipids' molecules, thus avoiding membranes fusion. Finally, for plant metabolism, raffinose degradation into simple sugars (glucose, fructose and galactose) may be faster, more useful and less harmful than pinitol degradation such as for trehalose (Wingler, 2002). Thus, the fact that pinitol changed in a

definite range of Ψ_{pd} , could be caused by its own molecular structure and eventual degradation, despite having a similar role to raffinose.

In this study, confusing results were found for sucrose depending on the temperature treatment and type of tissue (xylem versus cambium) which make interpretation more difficult. Despite this, results for cambium in 2010 were in agreement with literature reports for plants: an increase of pinitol and decrease of sucrose with increasing temperature (Liu *et al.*, 2008, Guo and Oosterhuis, 1995). In 2011, sucrose slightly decreased at high night temperature but increased at TD in the non-irrigated plants.

At higher temperature, the decrease in the hexose pool was probably caused by an increase in respiration, with glucose and fructose more involved through glycolysis and the pentose phosphate pathway. According to Amthor (2000), with increasing temperature, maintenance respiration increased more than respiration due to growth. In *Arabidopsis* cells culture, increasing the temperature induced a change in the proportion of both ATP and NADPH that were used for maintenance (Cheung *et al.*, 2013). The hypothesis that hexose was used for metabolic needs was also verified over the growing season in both 2010 and 2011: mean values of glucose and fructose were lower at higher temperature with respect to T0 (Table 1). Both glucose and fructose could be transformed to hexose-phosphate before entering glycolysis. These results are in agreement with Way and Sage (2008a), who found that glucose and fructose concentrations (% of dry mass in needles) were lower for black spruce growing at higher (30 °C/24 °C day/night) compared with lower temperature (22 °C/16 °C day/night), suggesting a rise in respiration.

Starch variations under water deficit and warming

Starch tended to decrease as the temperature rose in both 2010 and 2011. Diminution of the starch amount in ray cells during the warmer night can be explained by higher respiration rate induced in plants growing under high temperature. Thus, a high respiration rate could require an elevated amount of glucose to use in the glycolysis which in turn could derive from 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). Contemporarily, the impact of severe drought on carbon reserves was confirmed in young Norway spruce trees. Severe events induced a use of the aboveground starch reserves as starch was only completely depleted in roots when the trees were dead (Hartmann *et al.*, 2013).

Consequence for NSCs availability during xylogenesis

We found that water availability (i.e. water potential) during the growing season has an effect on the NSCs availability in both cambium and xylem. Under limited water availability, even if carbon was not depleted, NSC availability for wood formation in stem was significantly reduced due to their mobilization for osmotic purposes (Pantin *et al.*, 2013): growth differences between the irrigated and water deficit saplings were most probably caused by (1) hydromechanical limitations due to lack of cell turgor for growth and (2) the mobilization of NSC for osmotic adjustment in order to protect the living cells. However, more studies are needed to link the available NSCs in cambium and xylem parenchyma with the phases of wood formation and to determine the effect of water deficit on this link.

In eucalyptus, total non-structural carbohydrates also remained unchanged under high temperature (Duan *et al.*, 2013). In this study, ambiguous results were obtained with temperature increase. The number of woody cells produced slightly decreased with temperature increase in

2010 and 2011, except for the irrigated trees in 2011. In the long term however, increased temperature could impair the carbon reserves in the stem, which are fundamental in the case of stresses like drought, herbivore damage or heating. Although small plants may need less carbon to cope with stresses because of their lower biomass (Sala *et al.*, 2012), the decrease in starch refilling and the use of more hexose in both cambium and xylem at higher temperature could, in the long term, affect the growth and survival of young plants.

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618 **Table list**

619 **Table 1.** Soluble sugars (mg/g of dry weight) found in cambium and xylem for the different
620 water and temperature treatments. In 2010, T2 and T5 experienced a temperature of 2 and 5 K
621 higher than T0, respectively. In 2011, TD and TN were 6 K warmer than T0 during the day (D)
622 or night (N), respectively. The effect represents the significant probability ($P=0.05$) between
623 treatment [water deficit (W), temperature (T), day of the year (DOY)]. Results were all
624 significant ($p<0.001$) for DOY (not shown).

625 **Table 2.** Spearman correlations coefficients between the non-structural soluble sugars (mg/g_{dw})
626 and the pre-dawn leaf water potential (Ψ_{pd} , MPa). Asterisks represent significance with $*<0.05$,
627 $**<0.01$.

628 **Table 1 supplementary.** Growth fitting during 2010 and 2011 in the greenhouse experiment. A,
629 β and κ represent the fitted parameters of the Gompertz function, r indicates a weighted mean
630 absolute rate (r , cell.day⁻¹) and t_p the placement of the maximum growth rate on the time axis
631 (DOY). Comparisons are presented between Gompertz growth response curves, fitted to the
632 number of cells (data for the fitting of 2010 are from Balducci et al. 2013). F-values were
633 calculated among all groups, between temperature treatments and between irrigation regimes.

634 **Table 2 supplementary.** Leaf parameters of black spruce saplings before, during and after the
635 water deficit period at three thermal conditions in 2010 and 2011. Symbols/abbreviations and
636 units: pre-dawn leaf water potential (Ψ_{pd} , MPa), CO₂ assimilation (maximum photosynthesis
637 rate, A_{max} , $\mu\text{mol m}^{-2} \text{s}^{-1}$) and gas exchange (stomatal conductance g_s , $\text{mol m}^{-2} \text{s}^{-1}$), respectively
638 (mean \pm SD). Time equal 1, 2 and 3 corresponds to before, during and after water deficit period,

639 respectively at specific day of the year (DOY). The complete time series for 2010 can be found
640 in Balducci et al. 2013.

641

642

Table 1.

Temp.	Year 2010									Year 2011								
	Irrigated			Water deficit			Effect (P)			Irrigated			Water deficit			Effect (P)		
	T0	T2	T5	T0	T2	T5	Water	Temp.	W×T	T0	TD	TN	T0	TD	TN	Water	Temp.	W × T
Cambium																		
Suc	65.46	66.63	56.36	61.89	60.02	56.95	0.26	0.03	0.51	75.29	60.67	74.14	69.89	76.15	66.74	0.87	0.56	0.03
Pin	23.02	27.01	27.15	21.94	27.58	24.69	0.27	0.001	0.53	33.78	30.49	31.37	32.51	33.26	33.39	0.37	0.64	0.38
Fru	15.37	13.33	13.00	14.63	11.51	11.11	0.05	<0.001	0.75	18.54	13.60	16.22	16.23	15.09	16.82	0.84	0.001	0.08
Glu	10.98	8.41	8.84	11.28	8.17	7.93	0.75	<0.001	0.70	11.92	8.77	10.8	10.88	9.91	11.06	0.80	0.004	0.25
Raff	1.78	1.34	1.52	2.72	2.25	1.97	0.02	0.42	0.81	4.90	2.26	6.19	6.10	3.34	11.16	0.009	<0.001	0.14
Xylem																		
Suc	1.36	2.21	1.81	1.49	2.0	1.88	0.96	<0.001	0.37	2.09	1.94	2.05	1.97	2.05	1.87	0.59	0.89	0.59
Pin	1.44	1.74	1.52	1.54	1.76	1.62	0.28	0.005	0.84	2.06	2.22	2.01	2.15	2.37	2.11	0.22	0.11	0.96
Fru	2.15	2.17	1.84	2.10	2.02	1.83	0.49	0.03	0.80	2.67	2.41	2.52	2.40	2.53	2.48	0.60	0.90	0.44
Glu	1.79	1.71	1.51	1.78	1.62	1.49	0.64	0.02	0.90	2.18	1.99	2.11	2.01	2.08	2.06	0.68	0.89	0.61
Raff	0.069	0.068	0.111	0.143	0.150	0.225	<0.001	0.05	0.76	0.223	0.109	0.211	0.257	0.238	0.350	0.003	0.03	0.35
Starch	2.59	1.93	1.53	2.63	1.60	2.06	0.69	0.01	0.39	4.17	3.09	3.09	4.46	2.71	3.42	0.79	0.002	0.67

Table 2.

Water Temp.	Year 2010						Year 2011					
	Irrigated			Water deficit			Irrigated			Water deficit		
	T0	T2	T5	T0	T2	T5	T0	TD	TN	T0	TD	TN
Pin	-0.52*	-0.13	0.05	-0.01	0.40	0.39	-0.45*	-0.71**	0.01	-0.62*	0.30	-0.28
Suc	-0.35	0.06	0.17	0.19	0.02	0.02	-0.73**	-0.51*	0.40	-0.40	0.31	-0.35
Raf	0.00	-0.09	-0.39	-0.29	-0.67**	-0.33	-0.27	-0.42	-0.16	-0.70**	-0.85**	-0.73**

Table 1 supplementary.

	Water Temp.	Year 2010						Year 2011					
		Irrigated			Water deficit			Irrigated			Water deficit		
		T0	T2	T5	T0	T2	T5	T0	TD	TN	T0	TD	TN
Curves fitting	A	122.2	109.8	109.7	98.6	90.8	87.8	65.1	78.9	73.28	62.6	60.8	59.1
	β	3.88	4.65	4.53	4.06	4.15	3.79	5.26	4.47	4.97	3.56	4.30	3.89
	$\kappa (10^{-2})$	2.15	2.73	2.61	2.30	2.41	2.16	3.13	2.58	2.92	2.05	2.51	2.35
	r	0.66	0.75	0.72	0.56	0.54	0.47	0.51	0.51	0.54	0.32	0.38	0.35
	t_p	180	170	173	177	172	175	168	173	170	174	171	166
Comparison between the fitted curves	Among groups												
	F			7.30							8.48		
	P			<0.0001							<0.0001		
	Among temperature treatments												
	F			0.82							2.21		
	P			0.59							0.025		
	Between water treatments												
	F			32.73							35.38		
	P			<0.0001							<0.0001		

Figure caption list

Figure 1. Daily temperature (°C) and difference of temperature in the three sections of the greenhouse for 2010 and 2011. Temperature treatments are control (T0), +2K (T2), +5K (T5), +6K during the day (TD) and +6K during the night (TN). The daily differences from T0 are calculated over the whole 24h period. The grey bands represent the water deficit period.

Figure 2. Effect of temperature and water deficit treatments on tree-ring formation, expressed as the number of cells formed each week in 2010 and 2011. After the water deficit period, the growth values are those of the surviving plants. Temperature treatments are control (T0), +2K (T2), +5K (T5), +6K during the day (TD) and +6K during the night (TN). Circles represent control and black dots represent water deficit plants. The grey bands represent the water deficit period.

Figure 3. Soluble sugars in the cambium (mg/g_{dw}) in 2010 and 2011. Temperature treatments are control (T0), +2K (T2), +5K (T5), +6K during the day (TD) and +6K during the night (TN). Circles represent control and black dots represent water deficit plants. The grey bands represent the water deficit period.

Figure 4. Soluble sugars in the xylem (mg/g_{dw}) for 2010 and 201. See figure 3 for details.

Figure 5. Variation of sucrose, pinitol and raffinose (mg/g_{dw}) in function of the pre-dawn water potential (Ψ_{pd} , MPa) in 2010 and 2011. Temperature treatments are presented as control (T0), +2K (T2), +5K (T5), +6K during the day (TD) and +6K (TN) during the night.

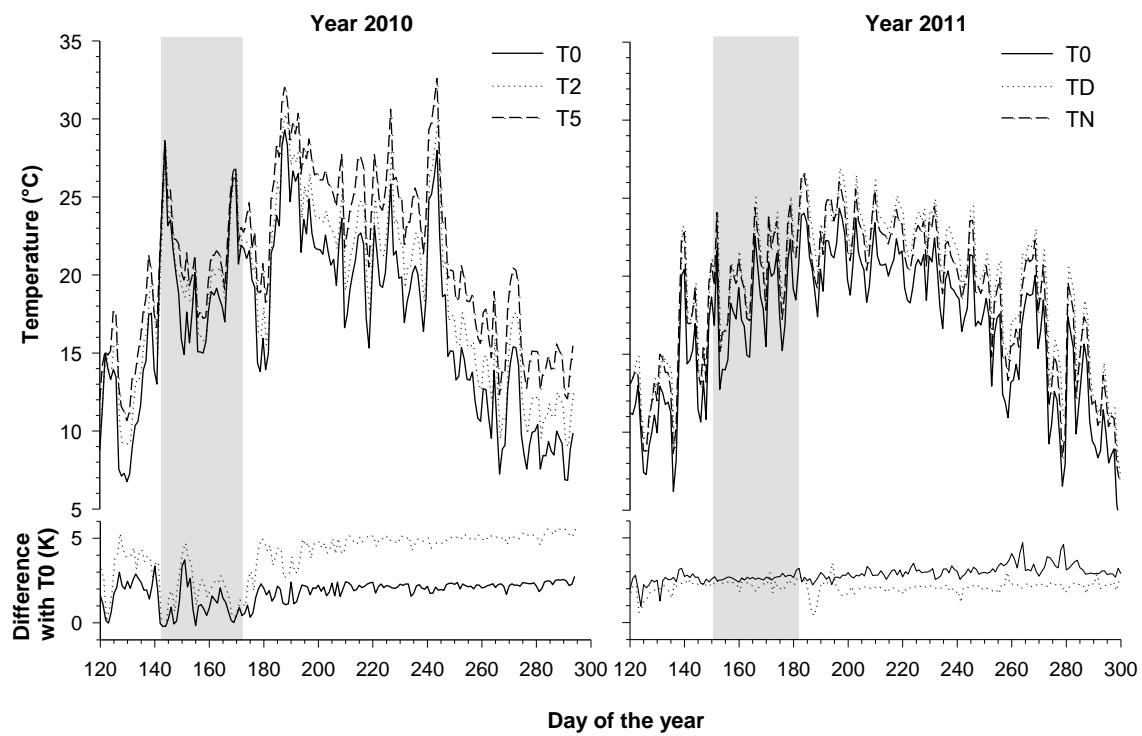


Figure 1.

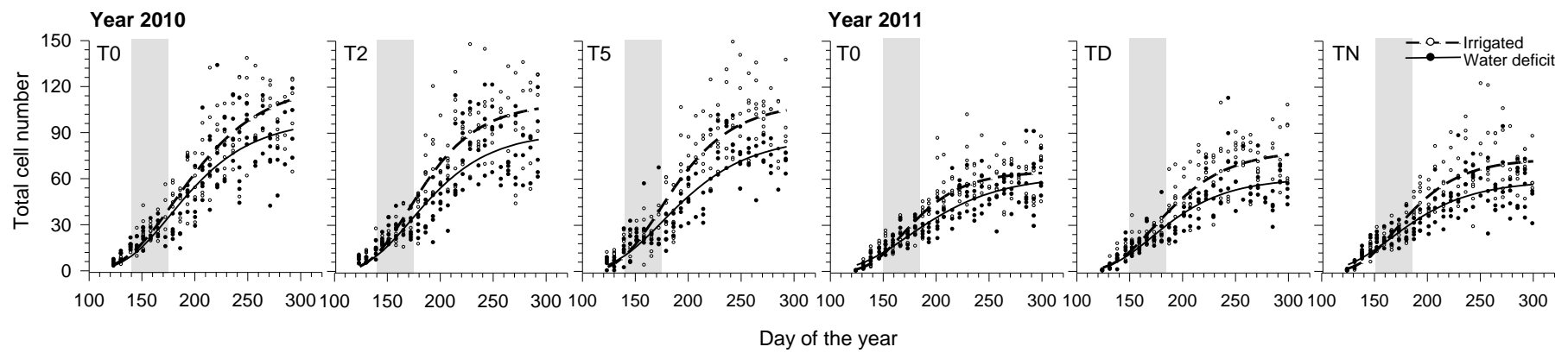


Figure 2.

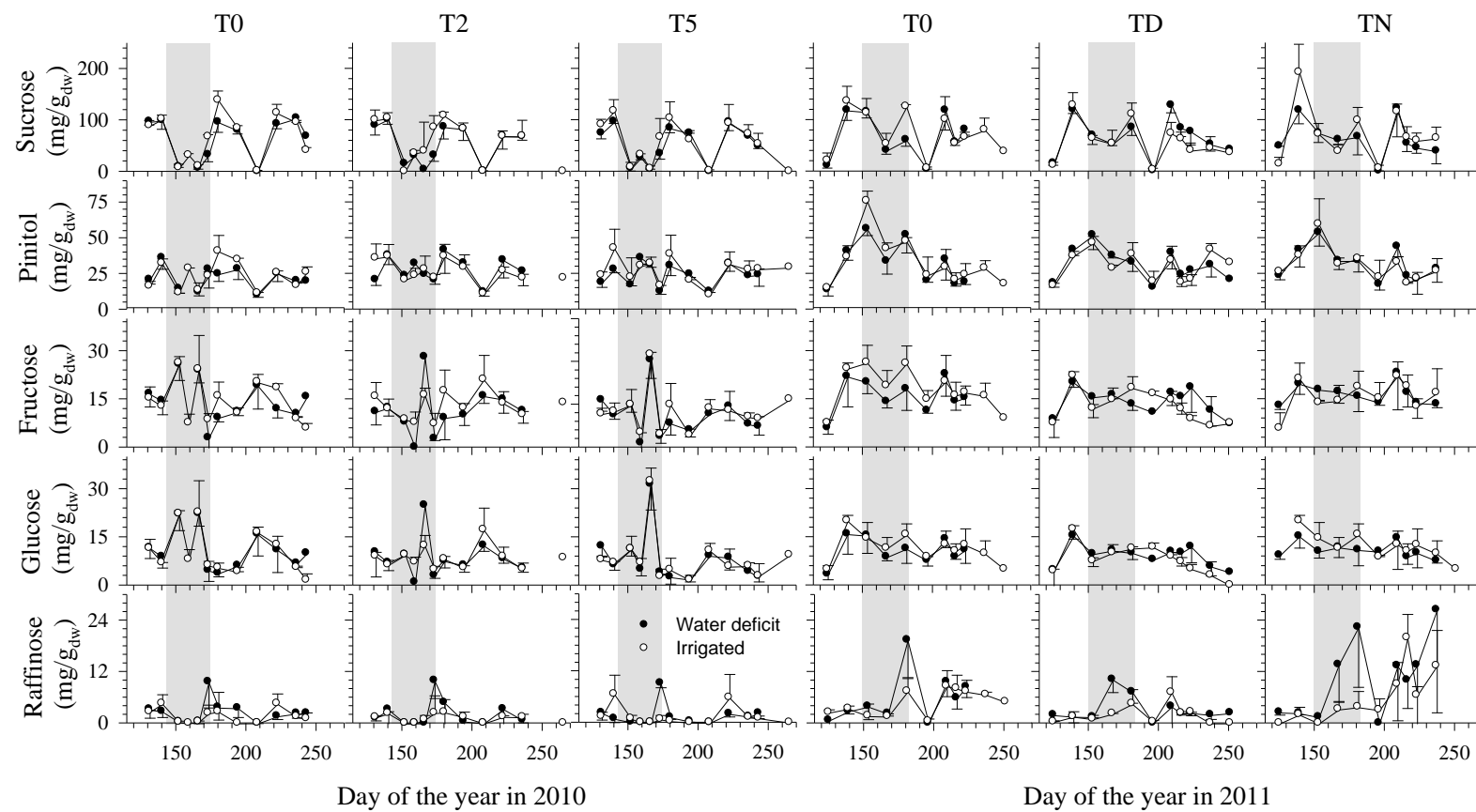


Figure 3.

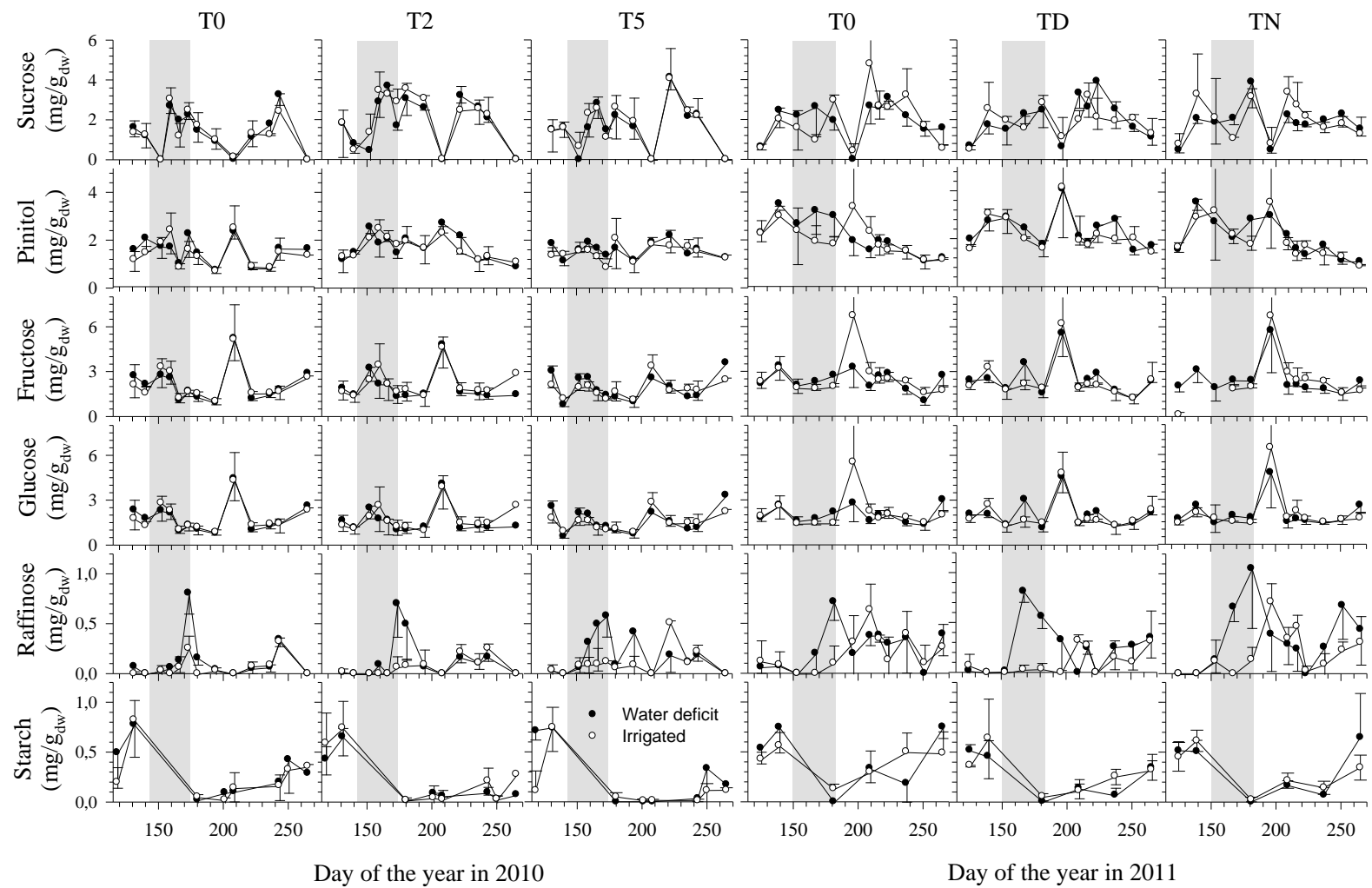


Figure 4.

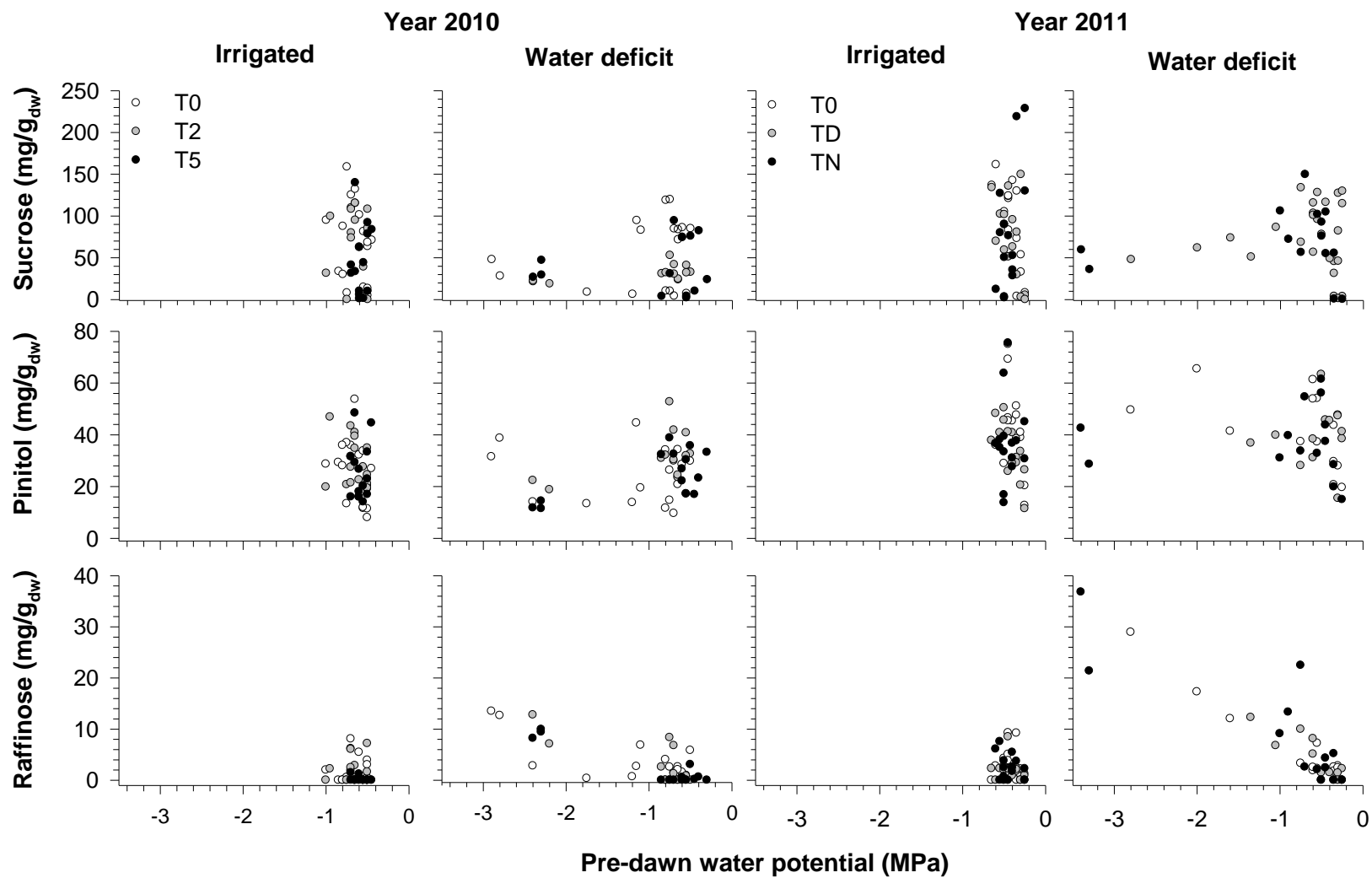


Figure 5

