The contribution of carbon and water in modulating wood formation in black spruce saplings

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One sentence summary: During wood formation, water availability is the most important factor for cell production while carbon is more important to sustain the differentiation of the living cells.

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Summary

Non-structural carbohydrates (NSCs) play a crucial role in xylem formation and represent, with water, the main constraint to plant growth. We assessed the relationships between xylogenesis and NSCs in order to (1) verify the variance explained by NSCs and (2) determine the influence of intrinsic (tissue supplying carbon) and extrinsic (water availability and temperature) factors. During two years, wood formation was monitored in saplings of black spruce submitted to a dry period of about one month in June and exposed to different temperature treatments in a greenhouse. In parallel, NSCs concentrations were determined by extracting the sugar compounds from two tissues (cambium and inner xylem), both potentially supplying carbon for wood formation. A mixed-effect model was used to assess and quantify the potential relationships. Total xylem cells, illustrating meristematic activity, were modeled as a function of water, sucrose and pinitol (conditional R^2 of 0.79). Water availability was ranked as the most important factor explaining total xylem cell production, while the contribution of carbon was lower. Cambium stopped dividing under water deficit, probably to limit the number of cells remaining in differentiation without an adequate amount of water. By contrast, carbon factors were ranked as the most important in explaining the variation in the living cells (conditional R^2 of 0.49) highlighting the functional needs during xylem development, followed by the tissue supplying the NSCs (cambium) and water availability. This study precisely demonstrates the role of carbon and water in structural growth expressed as meristematic activity and tissue formation.

Keywords: Cambium, cell enlargement, cell wall thickening, drought, temperature, non-structural soluble sugars, source-sink relationships

INTRODUCTION

Mobile sugars (e.g. sucrose, glucose and fructose) and sugar alcohols (e.g. pinitol) play an essential role in sustaining plant growth and metabolism and plant signaling (Muller et al., 2011). The origin of non-structural carbohydrates (NSCs) (reserve *versus* recent photosynthetic products) and their importance for growth processes are currently under intensive investigation (Wiley and Helliker, 2012; Rocha, 2013). The C-incorporation during wood formation, mostly in the form of cellulose and other cell wall polymers, determines most of the biomass accumulated by trees. In red maple, the NSC used to build the xylem is less than 1-yr-old (Carbone et al., 2013), demonstrating a fast incorporation of C originating from the mobile sugars pool in the stem. Reserves are often used at growth resumption in early spring (Oribe et al., 2003; Begum et al., 2013), but most xylem is formed with newly synthesized NSCs (Hansen and Beck, 1990, 1994; Kagawa et al., 2006). The ray parenchyma cells in xylem could also act as a source of NSCs to sustain growth when assimilates coming from the leaf become scarce (Maunoury-Danger et al., 2010; Olano et al., 2013) such as during a water deficit. As the molecular networks driving cell division and expansion largely rely on the availability of carbohydrates to provide energy and biomass (Lastdrager et al., 2014), xylem formation is an ideal system to study source-sink relationships and C-dependence of the growth metabolism.

The cambium is the meristem that produces layers of phloem and xylem cells in stem, branches and roots with a periodic activity that results in the seasonal radial growth (Rossi et al., 2013). The machinery of growth requires C-resources for many processes, in particular to supply energy for division, generate water turgor pressure during cell expansion, and produce polysaccharides during cell-wall formation (Muller et al., 2011). Recent evidence strongly supports the association between the pattern of NSCs and wood formation: the rate of xylem growth follows the concentration of NSCs in cambium, reflecting the strong demand for C-compounds in the phases of cell enlargement and cell-wall thickening (Deslauriers et al., 2009; Simard et al., 2013). Specific carbon compounds are needed according to the stage of wood development: increases in volume during cell expansion (i.e. cell growth) need osmotically active C-compounds and water to generate a suitable wall-yielding turgor pressure in growing cells (Steppe et al., 2015) while cell maturation needs C-compounds as substrate to build cell walls (Koch, 2004). The mobile pool of sugars allocated to growing and differentiating cells thus represents a direct constraint to wood formation (Michelot et al., 2012; Simard et al., 2013). Two nearby mobile carbohydrate pools can directly sustain wood formation: cambium – assuming that the C-compound comes from sucrose unloaded from phloem, and xylem – assuming that the C-compound comes from ray parenchyma (Deslauriers et al., 2009; Giovannelli et al., 2011). A relationship between the forming xylem and the available soluble sugars in each pool could therefore significantly improve our understanding of the use of NSCs in secondary growth and the importance of their provenance (current photosynthesis versus reserve). Compared with the outermost xylem, much higher amounts of NSCs are found in cambium (Deslauriers et al., 2009; Simard et al., 2013) because of its proximity to the unloading sites in phloem.

In the absence of water deficit, correlations are reported between the growth of various organs and C-availability (in roots, young leaves, flowers, fruits and seeds), but these relationships can be modified or reduced under stress [see review by Muller et al. (2011) and Tardieu et al. (2011)]. Drought-related growth reductions are primarily caused by hydromechanical constraints rather than C-availability (Pantin et al., 2013; Steppe et al., 2015), which could explain the uncoupling between NSCs and cell growth. Characterization of the interacting role of C and water availability in xylem cell differentiation is therefore needed, as well as the main source of carbon for wood formation.

The objectives of this study were (1) to verify the relationships between xylem production and available NSCs and (2) to determine the influence of tissue supplying NSCs, water availability and temperature. In the study, xylem production was characterized by the living cells – the sum of the number of cells in cambium (C), enlargement (ENL) and cell wall thickening (CWT) – representing the cells with high metabolic activity, and by the total number of xylem cells formed – differentiating and mature cells – representing the sum of tree-ring growth during the growing season (Figure 1). The tissue supplying carbon represents the NSCs coming from cambium or the inner xylem (Figure 1). In relation to the objectives, we considered the following hypotheses:

- The amount of available NSCs located in cambium or xylem sustains metabolic activity of the living cells and influences the total number of cells produced
- Intrinsic (tissue preferentially supplying carbon) and extrinsic (water availability and temperature) factors represent constraints to NSC availability and xylem production.

RESULTS

Tree growth

Radial growth of the irrigated saplings showed a typical S-shaped curve for all temperature treatments (total cells, Figure 3). The increase in cell number was similar in non-irrigated and irrigated saplings before the treatment. At the end of the water deficit, the increase in total cells was observed to slow down or stop for several weeks in non-irrigated saplings, while growth continued undisturbed in irrigated saplings. On DOY 200-220, a new sharp increase in radial growth was observed in non-irrigated saplings, although the total cell number always remained lower than in irrigated saplings until the end of the growing season.

In respect to radial growth (i.e. the increase in xylem cells), apical growth occurred quickly with the maximum length being reached in 15-20 days (Figure 3). In both study years, this sharp increase was synchronous between the different treatments and started when irrigation was withheld. After the maximum apical length was reach, the observed deviations were caused by sapling difference height growth, which was higher in 2011 compared with 2010.

Similar annual trends of living cells were observed between the temperature and water treatments (Figure 3). The living cells were characterized by bell-shaped curves, with a depression at about 1/3 of the growing season for both water and temperature treatments. In May 2010, cambium division and cell enlargement had already started, as indicated by a number of cells varying between 10 and 20, depending on the temperature treatment (Figure 3). In May 2011 (around DOY 120), only the cambium was already active with 6–7 cells on average but no cell differentiation. Around DOY 160-170, the number of living cells decreased to a minimum of 9-10 and 12-15 in 2010 and 2011, respectively. These drops in the living cell were synchronized with the start of the apical growth of the saplings (Figure 3). After this drop, the number of living cells increased rapidly to its maximum, while the increase was much slower in saplings subjected to water deficit. Once annual activity had ended and the cambium stopped dividing, the number of living cells gradually decreased to the minimum value, corresponding to quiescence conditions of the cambium meristem. Between 4 and 6 cambial cells were observed in autumn, fewer than at the beginning of the season (Figure 3).

Correlations among NSCs

In cambium of irrigated saplings, the variation of sucrose was mainly correlated with D-pinitol (r=0.53), and marginally to raffinose (r=0.30) and fructose (r=0.23) (Table 1). Fructose varied according to glucose with a correlation coefficient of 0.88. Fructose was marginally correlated with D-pinitol (r=0.31). In xylem of irrigated samplings, the correlations between the soluble sugars were slightly different. Sucrose was correlated with raffinose (r=0.36) but not with D-pinitol (Table 1). The variation of D-Pinitol in the xylem was positively correlated with glucose (r=0.65) and fructose (0.71) (Table 1). As for cambium, the variation of fructose and glucose in xylem was highly similar (r=0.98).

In general, the correlations between the soluble sugars were mostly similar in saplings submitted to water deficit. In cambium, sucrose was correlated with D-pinitol (r=0.54) and fructose (r=0.21) but not with raffinose. However, D-pinitol and raffinose showed a positive correlation (r=0.23). Sucrose was negatively correlated with fructose and glucose, as showed by the correlation coefficients of -0.22 and -0.28 for, respectively.

Variation in NSC

NSCs measured in cambium or in xylem had similar trends irrespective of the treatments (Figure 4). Therefore, the means represent all temperature treatments confounded [see Deslauriers et al.

(2014) for full NSCs time series and statistical analyses]. In cambium, sucrose was up to 30 times more abundant than the other NSCs (Figure 4), followed by pinitol and fructose. In both years, the withholding of irrigation caused a small decrease in sucrose and fructose and a sharp increase in raffinose observed around the end of the water deficit period (Figure 4). Although the concentrations of NSCs were much lower in the inner xylem, analogous trends to that of cambium were observed in xylem.

Parallel variations were observed between NSCs and the number of living cells (Figure 3-4). When cambial activity started, between DOY 120 and 130, the amount of NSCs in cambium was high. A decline was observed between DOY 150 and 170, which was more pronounced in 2010 with concentrations close to zero. A second decline was observed in mid-July (DOY 208 in 2010 and DOY 196 in 2011).

Mixed-effects model

The results from the null models (total and living cells) showed that random effects were significant, particularly at tree level with high ICC (Table 2). The random effects were therefore included in the mixed-effects model. Random slopes and interactive effects of temperature × water treatments were not significant and so excluded from the final model. A full model was built and considered the best model for the total xylem cells and living cells, respectively, in terms of minimum AIC, AICC and BIC. Good performance of the two full models is also reflected by their high conditional R^2 (over 0.79 for total cells and 0.49 for living cells).

We found that the increase in number of total xylem cells during the growing season can be modeled as a function of sucrose, pinitol and water treatments, as well as random effects. For the other model, the living cells can be modeled as a function of raffinose, sucrose, pinitol, fructose, the tissue where the sugars were extracted, water and temperature treatments, as well as random effects. Even if significant, fixed effects only accounted for a small portion of variance, as shown by the lower PCV (Table 2) and marginal R^2 (Table 3). The majority of the variance was explained by the random effects, as indicated by the lower PCV and large difference between the marginal and conditional R^2 (Table 3). This could be explained by the high variance in the measured cell numbers, especially for the total xylem cells (Figure 3). Nevertheless, the number of total xylem cells and living cells changed positively or negatively according to the water treatment and specific sugar compounds (Figure 5).

Both full models demonstrated that the water treatment was a significant variable to account for the variations in cell number, particularly for the predicted total xylem cells ($P<0.0001$, Table 2) compared to living cells ($P<0.05$, Table 2). In general, we found a decline in the variance of total xylem cells and living cells explained under the water deficit treatment compared with the irrigated treatment, as shown by the marginal R^2 when tissue and temperature were fixed (Table 3).

In terms of difference among temperature treatments, most of the temperature treatments were not significant (Table 2). Therefore, temperature did not influence the variations in total xylem cells and slightly explains the variation in the living cells ($P<0.05$ for T+2 treatment only). The variance of total xylem cells and living cells explained under the different thermal treatments was generally higher in T0, T+6 Daytime and T+6 Nighttime compared to T+2 and T+5 as shown by the marginal R^2 (Table 3). Compared with 2011, higher temperatures were registered in 2010, mainly due to a warmer spring (Figure 2), with the greenhouse conditions being above the long-term average calculated for the boreal stands.

The decomposition of variance (DOV) also precisely indicated the contribution of each fixed effect to both dependent variables (Figure 6). The DOV indicated that water availability was a crucial factor influencing the total number of xylem cells (73%), while sucrose (23%) and tissue (23%) were both the most influent to predict the number of living cells. Of the NSCs concentrations, pinitol (10.5%) and sucrose (7.9%) were significantly related to the total xylem cells while all the carbon variables (sucrose, fructose, raffinose and pinitol, accounting for 59% in total) were in turn significantly associated with the living cells (Figure 6). The tissue was found to be a significant variable explaining the predicted living cells, as suggested by a higher percentage of DOV. Interestingly, more variance in the predicted living cells can be accounted for by the NSCs extracted from cambium than xylem, as indicated by higher marginal R^2 for cambium under constant water and temperature treatments (Table 3). However, the variable tissue was not significant for the predicted total xylem cells, as shown by a lower percentage of DOV (0.14%) and more or less similar variance explained between cambium and xylem when under the same water and temperature treatments (Table 2).

Discussion

In this study, xylogenesis was modeled as a function of NSCs availability and other intrinsic (tissue) and extrinsic (water availability and temperature) factors. Similar variations between xylogenesis and NSCs were also observed in conifers [larch and spruce (Simard et al., 2013)] and broadleaves [poplar (Deslauriers et al., 2009)]. However, this is the first time that the relationship has been mathematically quantified. We found that the NSCs measured in the cambium zone were more important in explaining the variation in the number of living cells during the growing season. This indicates the preference of mobile sugars probably coming from the recently fixed carbon unloaded from phloem transport for the metabolic needs of growth. In general, the variance explained in wood formation slightly decreases under water deficit implying some uncoupling between NSCs and growth, while the warming had very few significant effects.

The role of available carbon during xylogenesis

Given that wood formation was disentangled in total xylem and living cells, we were able to elucidate the physiological mechanisms underlying the contribution of each NSCs variable and water with both models (Figures 5-7). For living cells (all metabolically active), all the sugar compounds were found to explain their variation with higher DOV for sucrose (22.66%) and fructose (16.76%), followed by raffinose (13.19%) and pinitol (6.7%).The most important NSCs explaining the total xylem cells were sucrose (7.88%) and pinitol (10.43%) only, as non-living cells were included in the total xylem cells to account for the entire tree-ring development over time (figure 7).

Sucrose was the main compound forming the NCS pool in cambium [see Figure 3 and Deslauriers et al. (2014)]. This sugar is mainly broken down by invertase, forming hexose (glucose and fructose), and by sucrose synthase, forming fructose and UDP-glucose (Koch, 2004). Large concentration gradients of sucrose and hexose can be observed between meristematic (high sucrose) and differentiation zones (high hexose) in root tips (Scheible et al., 1997; Freixes et al., 2002). Even though no spatial gradient of carbohydrate was measured in this study, the amounts of both total xylem (meristematic activity) and living cells (metabolic activity) were positively coupled with sucrose. Sucrose was the most important carbon compound influencing the variability observed in living cells (Figure 5, middle panel) probably because these were composed of a large number of tracheids undertaking secondary cell wall formation (Deslauriers et al., 2014). Indeed, a large quantity of UDP-glucose is required during the process of wall formation (CWT, Figure 1) as the number of cells in this phase represents an irreversible sink for building the material composing the cell walls (cellulose, hemicellulose and lignin), a process that lasts several weeks in conifers (Deslauriers et al., 2003; Gruber et al., 2010; Cuny et al., 2014).

Moreover, the amount of living cells was explained by fructose (16.76%). As glucose was highly correlated with fructose (correlation coefficient varying between 0.88 and 0.98), the contribution and role of this sugar is expected to be similar to fructose. These results are in line with Freixes et al. (2002) who found a positive correlation between root elongation and hexose concentration in Arabidopsis. The hexose concentration is a good proxy for rapid expansion (Muller et al., 2011). A large amount of hexose are produced from vacuolar invertase to increase cell osmotic potential and generate the appropriate turgor pressure for cell expansion (Koch, 2004). Fructose can also be used for ATP production (Koch, 2004) necessary for cell metabolism during the growth processes or active transport of several compounds across membranes. The results for fructose therefore support the fact that sugar allocation and partition could also be linked with respiratory processes as the number of cambium and differentiating cells are related with CO_2 emitted by the stem during wood formation (Lavigne et al., 2004; Gruber et al., 2009).

The osmotically active sugars, D-pinitol and raffinose were also found significant. D-pinitol explained both total xylem (DOV of 10.43) and living cells (DOV of 6.70) but with a negative and positive influence, respectively (Figure 5, upper panel). Cyclitols, such as D-pinitol, are normally implicated in cell osmoregulation in order to maintain cell turgor in the case of stress [i.e. cold, drought or salinity, see review by Orthen et al. (1994)], which could explain the negative influence of this sugar on the total xylem cell, especially under water deficit. However, it cannot be excluded that D-pinitol might also contribute to the overall water potential [maintaining turgor (Orthen et al., 1994; Johnson et al., 1996)] and explain the positive influence on the living cells (Figure 5, lower panel). The growing cells first accumulated pinitol and hexose in order to regulate cell osmosis to generate the turgor to enlarge and then shifted to more complex sugars (i.e. raffinose) when the water potential drops (Deslauriers et al., 2014). Raffinose drastically increases during water deficit because this sugar mainly acts as osmoprotector and ROS scavenger (Nishizawa-Yokoi et al., 2008; dos Santos et al., 2011). The negative effect of this sugar on the living cells (Figure 5, lower panel) is therefore perfectly in line with its main role.

The role of tissue in supplying NSCs

Our mixed-effects model showed that NSCs extracted from cambium tissue were more important as a source of NSCs to predict the amount of living cells (DOV of 22.61%), as shown by the higher variance explained by cambium (Table 3). However, the variable tissue was not significant in explaining the variation in total xylem cells possibly because non-living cells were included to account for the tree-ring increase over the growing season. Compared with the outermost xylem, much higher amounts of NSCs are found in cambium (Deslauriers et al., 2009; Simard et al., 2013), because of its proximity to the unloading sites in phloem. The recently-produced NSCs are thus preferred in terms of carbon allocation (Carbone et al., 2013; Steppe et al., 2015) as phloem transport relies on the turgor pressure gradient created by the difference between sugar loading and unloading processes (De Schepper et al., 2013), represented here by the breakdown of sugars by the living cells (Figure 1 and 7). The bimodal pattern in the number of living cells characterized by a slight depression in June is in agreement with previous observations realized on conifer species (Rossi et al., 2009), which were supposed to be associated with the internal competition for carbohydrates among meristems and the allocation priority towards primary growth. This hypothesis is confirmed by reduction in sucrose measured in cambium, especially in 2010, at the time of apical growth (DOY 160). In cambium, NSCs can also come from the hydrolysis of maltose during the process of starch degradation in bark at the beginning of the growing season (Oribe et al., 2003; Begum et al., 2013). Hence, our mixed-model results are in line with CASSIA dynamic growth model results, suggesting that cambial growth was sensitive to current-year carbon production (Schiestl-Aalto et al., 2015).

Girdling experiments – blocking down the phloem translocation of photosynthates – have also demonstrated the foremost importance of current photoassimilate flux to sustain stem growth (Daudet et al., 2005). While some girdling studies indicated that NSC reserves in the stem were not sufficient to sustain growth below the girdle in the short or in the long term (de Schepper et al., 2010; Maier et al., 2010), others demonstrated that stem reserves could be used to restart growth (Daudet et al., 2005; Maunoury-Danger et al., 2010). This assumption imposes source – sink relationships between xylem ray parenchyma cells and living cells (Olano et al., 2013). In the stem, ray parenchyma cells are the connective tissues between the different compartments (from the phloem to heartwood) and have many roles such as reserve storage (Gartner et al., 2000) and respiration (Wolfgang, 1985; Spicer and Holbrook, 2007). This could explain why less variance in the predicted living cells was represented by the NSCs extracted from the xylem tissues, even during drought. C storage indeed has priority over growth, reflecting a safety strategy in trees growing in cold or dry conditions. Hence, our result demonstrates that water is much more limiting than inner soluble sugars, in agreement with Palacio et al. (2014).

Effect of water deficit and warming

The water deficit had a significant effect on both models, with a higher percentage of DOV on total cells (73%) compared with living cells (15%). Fewer tracheids were produced at the end of and after the period of water deficit. This was reflected first by the smaller populations of living cells and second by a temporary plateau in the total number of cells (Figure 2). The mixed-model results show that water availability was ranked as the most significant factor explaining total xylem cell production, before any carbon factors (Figure 6). This modulation of growth under low water is crucial to limit the number of living cells undertaking differentiation which need water to fully complete their development. Otherwise, many differentiating cells would be locked in enlargement, without enough water to generate the suitable wall-yielding turgor pressure required for cell growth (Steppe et al., 2015). This key result confirms that radial growth, expressed here as the number of cells produced, is not source-limited (Körner, 2003; Rocha, 2013; Palacio et al., 2014) because cambium is inhibited at a lower level of water stress than photosynthesis (Muller et al., 2011; Balducci et al., 2013; Fatichi et al., 2014). Similar conclusions were obtained with CASSIA dynamic growth model (Schiestl-Aalto et al., 2015). For the living cells however, water availability was ranked in fifth position after carbon (sucrose, fructose, raffinose) and tissue (cambium). The apparent contradiction between these results could be explained by the low number of cambial cells with respect to differentiating cells, especially wall-thickening cells, and by the sugar compounds needed to build and maintain the turgor pressure of enlarging cells (Pantin et al., 2013; Steppe et al., 2015). In other words, once temperature and water availability are sufficient for cambial cells to divide, sugar compounds are allocated to start expansion (i.e. attracting water molecules) and afterwards, to thickening the cell walls.

The variance explained in both total xylem and living cells (as shown by the marginal R^2 when tissue and temperature treatment were fixed) decreased under water deficit, showing some uncoupling between NSCs and growth, especially for the living cells. This reduction could represent an alteration of NSCs dependence on growth, caused by the reduced water availability (Muller et al., 2011): (1) Because wood formation practically stopped during water deficit, NSCs could be less required for wood formation processes (i.e. sink-limitation), which in turn, could reduce the variance explained; (2) In response to water deficit, the flow of available carbon, especially in the form of sucrose, could be further directed to osmoregulation (i.e. forming raffinose) at the expense of growth. Although the quantities are minor, these carbohydrates are unavailable for osmotic purposes when sequestered in cell structures (cell wall and vacuoles) (Pantin et al., 2013); (3) The accessibility and utilization of NSCs during water deficit is questioned (Sevanto et al., 2014) because the movement of NSCs decreases under low water content (Woodruff and Meinzer, 2011) and translocation of carbohydrates in phloem becomes more limited under water stress (Woodruff, 2014).

The contribution of temperature in explaining total xylem cells was non-significant while only T+2 was significant in explaining the number of living cells (2.63%), showing a marginal effect of temperature in our experiment. Although temperature is important in determining the potential growth rate (Pantin et al., 2012), little difference was found in wood formation (Balducci et al., 2013) possibly because the onset of growth was similar between treatments and the temperatures were well above the threshold limit for growth (Rossi et al., 2008) due to the greenhouse conditions. Lower amount of hexose (Deslauriers et al., 2014) and starch in the ray parenchyma cells (Balducci et al., 2013) was measured under warmer growth temperature, confirming the results of Way and Sage (2008) in black spruce trees. Although acclimation occurs, the increase in respiratory rate at higher temperature (Atkin and Tjoelker, 2003; Turnbull et al., 2004), may reduce the hexose pool in cambium and xylem. Even if this suggests a lower carbon availability for growth (particularly for cell enlargement), more studies are needed to characterize the role of temperature, especially under natural conditions.

Conclusion

Our analyses provide, for the first time, quantitative relationships linking NSCs and xylem development and including the influence of the nearby supply of carbon, water availability and temperature. The mixed-model elaborated in this study confirmed earlier observations about the parallel variation of sugars and differentiating xylem cells and is in line with the dynamic growth model. These results have important implications at large ecological scale because cambial activity is responsible for the permanent sequestration of carbon in the woody tissues (Cuny et al., 2015). The understanding of how carbon and water availability influence secondary growth is fundamental to improve growth models (Fatichi et al., 2014) and, in this sense, we provide empirical knowledge on important C compartments, xylem and cambium, and their respective roles in structural growth, including meristematic activity and tissues formation. However, our investigation was based on black spruce sapling, which might have different dynamics of carbon requirements and allocation compared to older trees, or trees of other species, especially if deciduous. Despite the mechanisms of xylem growth remain the same across ontogenetic states and species, caution in generalization of the results of this study for all trees must be taken due to the potential variations in the timings and rates of carbon source (photosynthesis) and sinks (wood formation).

METHODS

Experimental design

The experiment was performed in a greenhouse complex located in Chicoutimi (QC), Canada (48º 25’ N, 71º 04’ W, 150 m above sea level) equipped with an automatic warming and cooling system that controls the environmental parameters. Four-year-old black spruces [*Picea mariana* (Mill.) B.S.P.)] were transplanted into 4.5 l plastic pots with a peat moss, perlite and vermiculite mix, and left in an open field during a growing season and the following winter. In April 2010 and 2011, the saplings were taken into three different sections of the greenhouse for the experiment and fertilized with 1 g l^{-1} of NPK (20-20-20) dissolved in 500 ml of water (Balducci et al., 2013). Three hundred saplings were installed in every section in both years, with a buffer zone of one additional row of saplings at the borders. Saplings were 48.9 ± 4.7 cm tall with a diameter of 8.0 ± 2.0 mm at the root collar, and were provided with drip trickles for irrigation.

In one section of the greenhouse, the temperature was set to mimic the current thermal condition of the region (T+0). It was therefore maintained as close as possible to the external air temperature, except on days of the year (DOY) 142 – 152 in 2010, when a technical problem occurred. The two other sections were subjected to specific thermal regimes (Figure 2). In 2010, the treatments (called T+2 and T+5) consisted of a temperature 2 and 5 K higher than T+0, respectively (Balducci et al., 2013). In 2011, the treatments (called T+6 Daytime and T+6 Nighttime) were 6 K warmer than T0 during the day (T+6 Daytime, from 07:00 to 19:00) or during the night (T+6 Nighttime, from 19:00 to 07:00) (Figure 2).

Two irrigation treatments were applied: (1) irrigated, consisting of maintaining the soil water content at approximately 80% of field capacity; (2) water deficit, in which irrigation was withheld from mid-May to mid-June, the period when cambium is vigorously dividing (Rossi et al., 2006). At the end of the water deficit period, the soil water content of non-irrigated saplings was less than 10%, while that of irrigated saplings varied between 40 and 50%. The predawn leaf water potential (Ψ_{pd}) of irrigated saplings was maintained close to -0.5 MPa in both years. During the water deficit, Ψ_{pd} of non-irrigated saplings dropped in response to the decrease of soil water availability, reaching the lowest values on DOY 172 (-2.7±0.2 MPa) and DOY 180 (-1.3±0.7 MPa) in 2010 and 2011, respectively. One week after the resumption of irrigation, Ψ_{pd} of irrigated and non-irrigated saplings were similar, showing that the saplings were able to recover an optimal water status after the stress (Balducci et al., 2013; Deslauriers et al., 2014).

Xylem growth

From May to September 2010 and 2011, stem disks were collected weekly at 2 cm from the root collar of 36 randomly-selected saplings (6 saplings × 2 water regimes × 3 thermal treatments). At the same time, the apical growth (mm) was measured on each sapling with a precision digital calibre to the nearest1 mm. The collected stem disk 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., 2006).

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× to distinguish the developing xylem cells. For each section, (i) cambial, (ii) enlarging, (iii) cell-wall thickening and (iv) mature cells were counted along three radial files. 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 (Deslauriers et al., 2003). The progress of cell wall lignification was detected with cresyl violet acetate that reacts with the lignin (Rossi et al., 2006). 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

Every two weeks, 18 of the 36 saplings used for xylem analysis were selected for NSCs assessment (3 saplings × 2 water regimes × 3 thermal treatments). 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 and stored at -20 ºC. Dehydration was performed with a 5-days lyophilisation. The cambium zone, probably including some cells in enlargement, was manually separated by scraping the inner part of the bark and the outermost part of xylem with a surgical scalpel (Giovannelli et al., 2011). After having removed the cambium, the wood was milled to obtain a fine powder.

Soluble carbohydrates extraction followed 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 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 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 supernatants 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* (200 mg/3ml) and one of *CH* (200mg/3ml). The solution was evaporated until 1.5 ml and filtered through a 0.45 µm syringe filter into a 2 ml amber vial.

An Agilent 1200 series HPLC with a RID and a Shodex SC 1011 column and 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 (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 curves had R^2 of 0.99 and F-values close to one, indicating that each sugar had a 1:1 ratio 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 unmanipulated sorbitol. The loss percentages were then calculated and added to the final results (Deslauriers et al., 2014).

Statistical analysis

A mixed-effects model was used to fit the hierarchical data, in which an autocorrelation error structure in the repeated measurements over time was extended to Level 1 [AR(1)] that was nested within the measurements of different NSC concentrations (Level 2) extracted from different tissues (Level 3, figure 1) of the randomly selected trees (Level 4). The trees were nested under different temperature treatments (Level 5) and water treatments (Level 6). In addition, there was a nested error structure, i.e., correlation among trees within temperature and water treatments. The mixed-effects model can effectively deal with this nested error structure to account for correlation associated with clustered data. Mixed-effects model techniques estimate fixed and random parameters simultaneously and give unbiased and efficient estimates of fixed parameters (Pinheiro and Bates, 2000).

Prior to the modeling analysis, the normality was checked and square root of data transformation was then applied to the number of living cells and total xylem cells to meet the assumption of normality. Note that hereinafter the transformed data were used in the mixed-effects model analysis. Variance inflation factors [VIF, Belsley et al. (1980)] were also calculated to detect multicollinearity among the predictors including sucrose, pinitol, glucose, fructose and raffinose. VIFs were generally lower than the accepted value of 4 (O'Brien, 2007) except for fructose that had a VIF > 10 due to collinearity with glucose, so was then removed from the model. Fructose was kept as this sugar is the product of both sucrose synthase and invertase.

Fixed effects included different NSC concentrations (raffinose, sucrose, pinitol, fructose), measured tissue (xylem and cambium), temperatures (control T+0, versus T+2, T+5, T+6 Daytime and T+6 Nighttime) and water availability (water deficit and irrigated), and the interaction temperature × water. Random effects included trees and repeated measurements over time. In order to verify the two hypotheses, a mixed-effects model was built starting from a null model and then gradually extended to the higher levels (Singer, 1998). Therefore a mathematical function can theoretically be expressed by both fixed and random effects as follows:

$$Y_{i(jklmt)} = \beta_0 + (\beta_1 + \mu_{1k})R_{i(jklmt)} + (\beta_2 + \mu_{2k})S_{i(jklmt)} + (\beta_3 + \mu_{3k})P_{i(jklmt)} + (\beta_4 + \mu_{4k})F_{i(jklmt)} + \beta_5 O_{jklm} + \beta_6 T_{lm} + \beta_7 W_m + \beta_8 T_{l(m)} W_m + \mu_{0k} + \varepsilon_{i(jklmt)}$$

where,

$Y_{i(jklmt)}$ is the *ith* (*i*=1 to 3) measured cell number from the *jth* tissue (*j*= 0 and 1, indicating xylem and cambium, respectively) in the *kth* tree (*k*=1 to 12) under the *lth* temperature treatment (l=0 to 4, indicating T+0, versus T+2, T+5, T+6 Daytime, and T+6 Nighttime, respectively) and the *mth* water availability (m=0 and 1, indicating water deficit and irrigated, respectively) on day t;

β_0 is the overall mean; $\beta_1, \beta_2, \ldots$ and β_8 are the corresponding fitted parameters;

$R_{i(jklmt)}$, $S_{i(jklmt)}$, $P_{i(jklmt)}$, and $F_{i(jklmt)}$ is the *ith* measured NSC concentrations (raffinose, sucrose, pinitol, and fructose, respectively) from the *jth* tissue in the *kth* tree under the *lth* temperature treatment and the *mth* water treatments on day t.

$T_{l(m)} \times W_m$ is the temperature*water interactive effects;

μ_0 is random intercept, and μ_1, μ_2, μ_3 and μ_4 are random slopes,

where $\begin{pmatrix} \mu_0 \\ \mu_1 \\ \mu_2 \\ \mu_3 \\ \mu_4 \end{pmatrix} \sim N\left[\begin{pmatrix} 0 \\ 0 \end{pmatrix}, \begin{pmatrix} \tau_{00} & \tau_{01} \\ \tau_{10} & \tau_{11} \end{pmatrix}\right]$, τ_{00} and τ_{11} are the elements representing the variance components for the intercept and slope, respectively; τ_{10} is the covariance component representing correlation between the intercept and slope.

$\varepsilon_{i(jklmt)}$ is the random error associated with the *ith* measurements from the *jth* tissue of the *kth* tree under the *lth* temperature treatment and the *mth* water treatments on day t, $\varepsilon_{i(jklmt)} \sim N(0, \sigma_\varepsilon^2 \Omega)$, where

$\sigma_\varepsilon^2 \Omega = \frac{\sigma_\varepsilon^2}{(1-\rho^2)} \begin{bmatrix} 1 & \rho & \rho^2 & \ldots & \rho^{n-1} \\ \rho & 1 & \rho & \ldots & \rho^{n-2} \\ \rho^2 & \rho & 1 & \ldots & \rho^{n-3} \\ . & . & . & \ldots & . \\ \rho^{n-1} & \rho^{n-2} & \rho^{n-3} & \ldots & 1 \end{bmatrix}$, ρ is the autocorrelation coefficient, $|\rho| < 1$.

The mixed-effects model was built according to the conventional building process (Singer, 1998); the detailed mathematical or statistical approaches used can be found in (Huang et al., 2014). A marginal R^2, which was calculated on the fixed effects only, and a conditional R^2, which was calculated on both fixed and random effects (Nakagawa and Schielzeth, 2013) were also provided to assess the overall performance of a mixed-effects model.

$$\text{[a]}\quad R^2_{marginal} = \frac{\delta_f^2}{\delta_f^2 + \delta_{tree}^2 + \delta_{ar(1)}^2 + \delta_\varepsilon^2 + 0.25}$$

$$\text{[b]}\quad R^2_{conditional} = \frac{\delta_f^2 + \delta_{tree}^2 + \delta_{ar(1)}^2}{\delta_f^2 + \delta_{tree}^2 + \delta_{ar(1)}^2 + \delta_\varepsilon^2 + 0.25}$$

Where, δ_f^2 is the variance calculated from the fixed effect components of the mixed model and was estimated through multiplying the design matrix of the fitted effects with the vector of fixed effects estimates, i.e., predicting fitted values based on fixed effects alone, followed by calculating the variance of these fitted values; δ^2_{tree} is the variance at tree level; $\delta^2_{ar(1)}$ is the variance for the first order autocorrelation term; δ^2_ε is the variance for the error term; 0.25 is the distribution-specific variance linked by square root.

To provide information regarding the variance explained at each level, the proportion change in variance (PCV) was calculated according to (Merlo et al., 2005):

$$\text{[c]}\quad PCV_{tree} = 1 - \frac{\delta_{tree}^2}{\delta_{tree(null)}^2}$$

$$\text{[d]}\quad PCV_{ar(1)} = 1 - \frac{\delta_{ar(1)}^2}{\delta_{ar(1)(null)}^2}$$

$$\text{[e]}\quad PCV_{residual} = 1 - \frac{\delta_{residual}^2}{\delta_{residual(null)}^2}$$

Where, $\delta^2_{tree(null)}$, $\delta^2_{ar(1)(null)}$ and $\delta^2_{residual(null)}$ is the variance from the null model, respectively; δ^2_{tree}, $\delta^2_{ar(1)}$ and $\delta^2_{residual}$ is the variance from the full model, respectively.

The assumption of normality of the residuals was also verified. In addition, the decomposition of variance (*DOV*) (Huang et al., 2013; Huang et al., 2014) was performed to further quantify how much variance in the predicted square root of total xylem and living cells can be attributed to different fixed-effects predictors using SAS Proc GLM (type III). All analyses were conducted with SAS (Version 9.3, SAS Institute Inc. Carry, NC, USA).

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List of authors contributions

AD and SR elaborate the concept behind the experiment, AD wrote the text helped by all the other authors, JGH elaborate and realized the mixed model effect, MB and LB made the sampling and the NSCs and wood formation analysis respectively.

Table 1. Pearson correlation coefficients between NSCs. In irrigated sampling (left part), the correlations were performed between the soluble sugars measured in cambium (grey background, n=188) and xylem (white background, n=223). In water deficit sampling (right part), the correlations were performed between the soluble sugars measured in cambium (grey background, n=163) and xylem (white background, n=215). Asterisks indicate significant correlation coefficients P<0.0001 (***); P<0.001 (**); P<0.05 (*). Suc – sucrose; Pin – pinitol; Fru – fructose; Glu – glucose; Raf – raffinose.

	Irrigated					Water deficit				
	Suc	Pin	Fru	Glu	Raf	Suc	Pin	Fru	Glu	Raf
Suc	–	0.53***	0.23*	0.02	0.30***	–	0.54***	0.21*	0.04	0.08
Pin	0.20**	–	0.31***	0.05	0.08	0.15*	–	0.28**	0.12	0.23*
Fru	-0.08	0.71***	–	0.88***	0.28***	-0.22**	0.57***	–	0.89***	0.08
Glu	-0.15*	0.65***	0.98***	–	0.17*	-0.28***	0.46***	0.97***	–	0.00
Raf	0.36***	0.09	0.25***	0.26***	–	0.27***	0.09	0.04	0.02	–

Table 2. Statistics of the null and full models built for the predicted total xylem (left) cells and living cells (right). Standard error (SE) is reported in parentheses; P<0.0001 (***); P<0.001 (**); P<0.05 (*); ICC, Intra-class correlation; PCV, Proportion change in variance.

Dependent variables	**Total xylem cells**		**Living cells**	
Model	Null model (×10^{-2})	Full model (×10^{-2})	Null model (×10^{-2})	Full model (×10^{-2})
n	6357	6264	6360	6267
Fixed effects				
Intercept (β_0)	591.61 (8.74)***	620.00 (28.18)***	368.17 (1.90)***	331.43 (7.50)***
Raffinose (β_1)		-0.21 (1.42)		-1.45 (0.43)***
Sucrose (β_2)		0.47 (0.20)*		0.27 (0.06)***
Pinitol (β_3)		-1.85 (0.51)***		0.51 (0.19)**
Fructose (β_4)		-0.02 (1.13)		1.35 (0.36)***
Tissue (xylem, β_5 *versus* cambium)		6.52 (23.86)		28.27(6.42)***
Temperature *(warm versus T+0)*				
Temperature (T+2) (β_6)		45.86 (25.78)		12.02 (5.40)*
Temperature (T+5) (β_6)		34.79 (25.72)		7.20 (5.42)
Temperature (T+6 daytime) (β_6)		-11.04 (25.70)		1.82 (5.44)
Temperature (T+6 nighttime) (β_6)		-24.03 (25.66)		-0.34 (5.46)
Water (water deficit, β_7 *versus irrigated*)		-70.26 (17.06)***		-11.06 (3.62)*
Random effects				
Residuals (additive dispersion)	111.81 (2.41)***	108.98 (2.37)***	24.11 (0.60)***	23.94 (0.59)***
Tree (tissue ×temperature ×water)	528.91 (29.18)***	493.02 (27.66)***	20.42 (1.44)***	17.57 (1.28)***
Repeated measurement (tree) [AR(1)]	21.63 (1.48)***	21.18 (1.50)***	31.53 (1.58)***	30.69 (1.58)***
ICC (measurements)	3.27 %		41.45%	
ICC (tree)	79.85 %		26.85%	
PCV (measurements)		2.08%		2.66%
PCV (tree)		6.79%		13.96%
PCV (residuals)		2.53%		0.71%
Model fit				
-2 Log Likelihood	20871.7	20395.4	9556.1	9335.1
AIC	20879.7	20423.4	9564.1	9363.1
AICC	20879.7	20423.4	9564.1	9363.2
BIC	20871.7	20395.4	9556.1	9335.1

Table 3. The marginal and conditional R^2 (%) explained by the full model for total xylem cells and living cells under different treatments. The treatments are expressed as the combination of tissue (xylem *versus* cambium), water treatment (irrigated *versus* water deficit) and temperature treatment (T+0 *versus* T+2, T+5, T+6 Daytime and T+6 Nighttime)

Treatment combinations	**Total xylem cells**		**Living cells**	
	Marginal R^2	Conditional R^2	Marginal R^2	Conditional R^2
Cambium – Irrigated				
T+0	1.20	79.58	4.79	52.06
T+2	0.27	79.39	2.66	50.99
T+5	2.07	79.76	2.91	51.11
T+6 Daytime	1.02	79.54	3.63	51.48
T+6 Nighttime	1.55	79.65	4.79	52.06
Cambium – Water deficit				
T+0	0.82	79.50	4.34	51.83
T+2	2.81	79.91	2.57	50.94
T+5	0.84	79.50	2.62	50.97
T+6 Daytime	0.94	79.52	4.06	51.69
T+6 Nighttime	0.68	79.47	4.34	51.83
Xylem – Irrigated				
T+0	1.10	79.56	0.85	50.08
T+2	0.25	79.38	0.20	49.75
T+5	0.44	79.42	0.19	49.75
T+6 Daytime	0.17	79.37	0.06	49.68
T+6 Nighttime	1.01	79.54	0.85	50.08
Xylem – Water deficit				
T+0	0.03	79.34	0.48	49.90
T+2	0.43	79.42	0.06	49.68
T+5	0.03	79.34	0.10	49.70
T+6 Daytime	0.61	79.46	0.05	49.68
T+6 Nighttime	0.44	79.42	0.48	49.89

Figure legends

Figure 1. Differentiating cells during wood formation (C, cambium; ENL, enlargement; CWT, cell wall thickening) and the mature cell (M). The living cells in the analyses included C, ENL and CWT while the total cell represents ENL, CWT and M cells. The available NSCs were measured in the cambium and xylem. PH; living phloem.

Figure 2. Temperature measured during the experiments in 2010 (left) and 2011 (right). In 2010 the temperature treatments were T+0 (blue line), T+2 (red line) and T+5 (black line). In 2011, the temperature treatments were T+0 (blue line), T+6 Daytime (red line) and T+6 Nighttime (black line). The grey bands represent the period when irrigation was withheld during both years.

Figure 3. Dynamics of wood formation in 2010 (left) and 2011 (right) represented as the mean. The living cell represents the sum of cambial cells, cells in enlargement and in wall formation. The total cell represents tree ring growth as the sum of cells in enlargement, in wall formation and mature cell. In 2010 the temperature treatments were T+0 (blue line), T+2 (red line) and T+5 (black line). In 2011, the temperature treatments were T+0 (blue line), T+6 Daytime (red line) and T+6 Nighttime (black line). The grey background indicates the period when irrigation was withheld. SD; standard deviation among the measured values in the corresponding graph.

Figure 4. Mean soluble sugars in cambium and xylem measured during 2010 and 2011.The vertical bars represent the standard deviation among the measured trees (all temperature treatments confounded). Open circles represent the irrigated plants, and filled circles water deficit plants. The grey bands indicate the period when irrigation was withheld. Note the different ranges of the vertical axes between cambium and xylem.

Figure 5. Surface plot (after smoothed spline interpolation based on n=3348 for irrigated and n=2937 for water deficit). The relationships are illustrated between the predicted total xylem cells (square root) and sucrose and pinitol (upper panel), and between the predicted living cells (square root) and sucrose and fructose (middle panel), and raffinose and pinitol (lower panel). The unit for sucrose, pinitol, fructose, raffinose is mg g^{-1} dry weight (mg g^{-1} d wt).

Figure 6. Mixed model decomposition of variance (DOV, percentage %) in predicting total xylem cells (dark green) and living cells (light green).

Figure 7. Illustration of the observed wood formation processes expressed as the total xylem cells (upper panel) and living cells (lower panel) in the presence (red) and absence (blue) of water deficit (horizontal grey bars). Illustration of wood formation at the beginning of wood formation is shown to illustrate the measured processes. The implications of the DOV's result are explained to stress the contribution of the factors in wood formation (see discussion for full explanation).

Figure 1

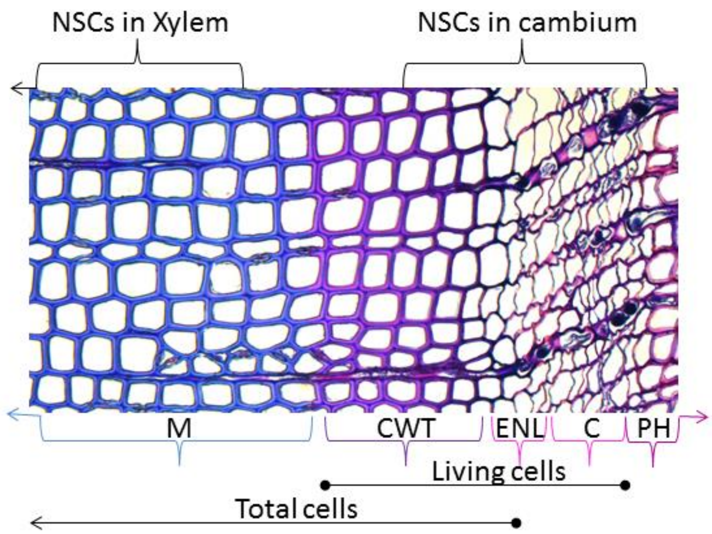
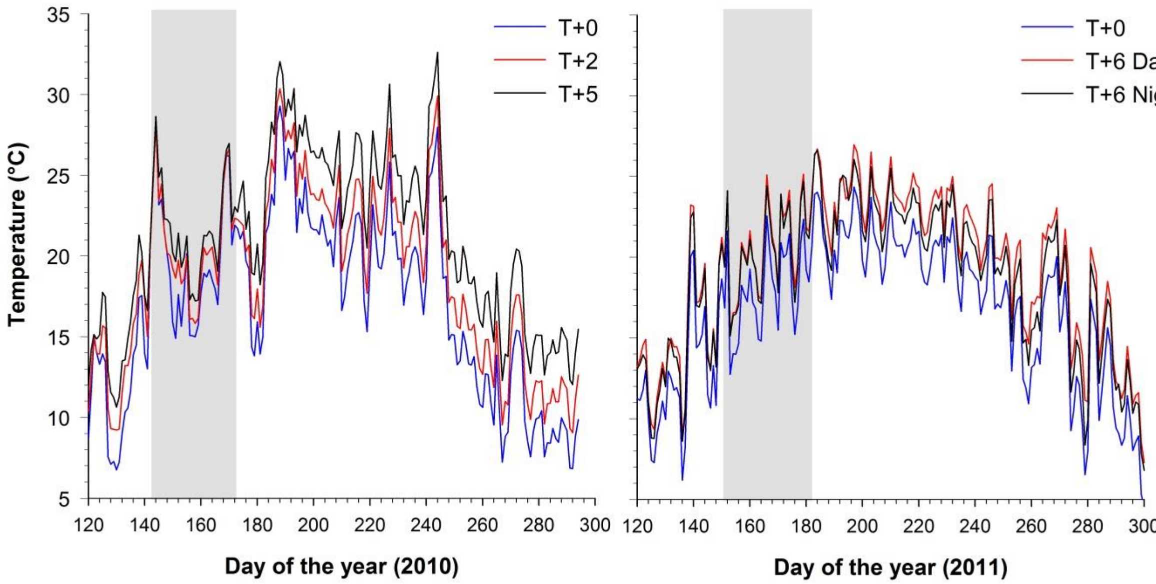

NSCs in Xylem
NSCs in cambium
M
CWT
ENL
C
PH
Living cells
Total cells

Figure 2


T+0
T+2
T+5
T+0
T+6 Daytime
T+6 Nighttime
Temperature (°C)
35
30
25
20
15
10
5
120
140
160
180
200
220
240
260
280
300
Day of the year (2010)
Day of the year (2011)

597 Figure 3

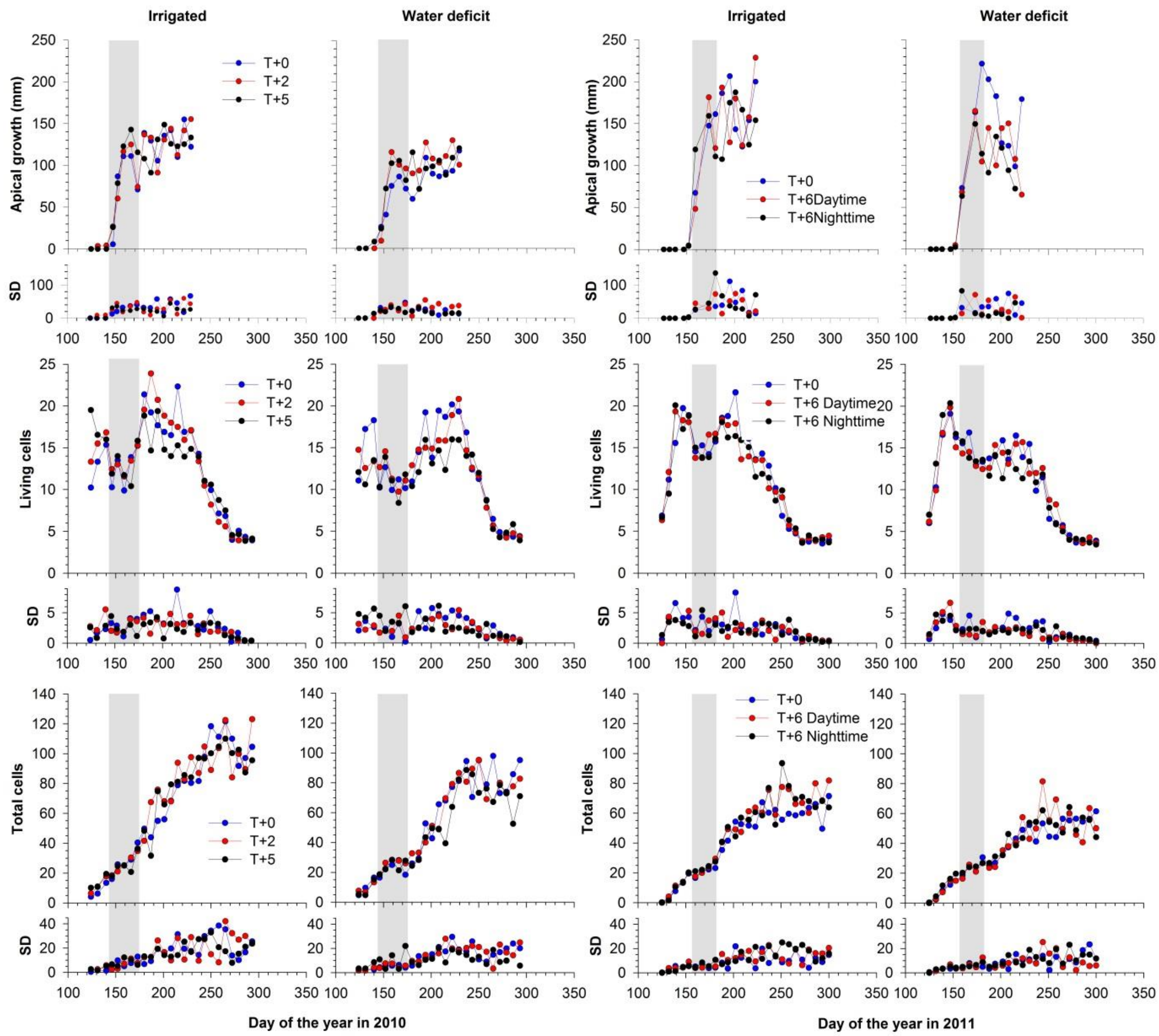


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Figure 4

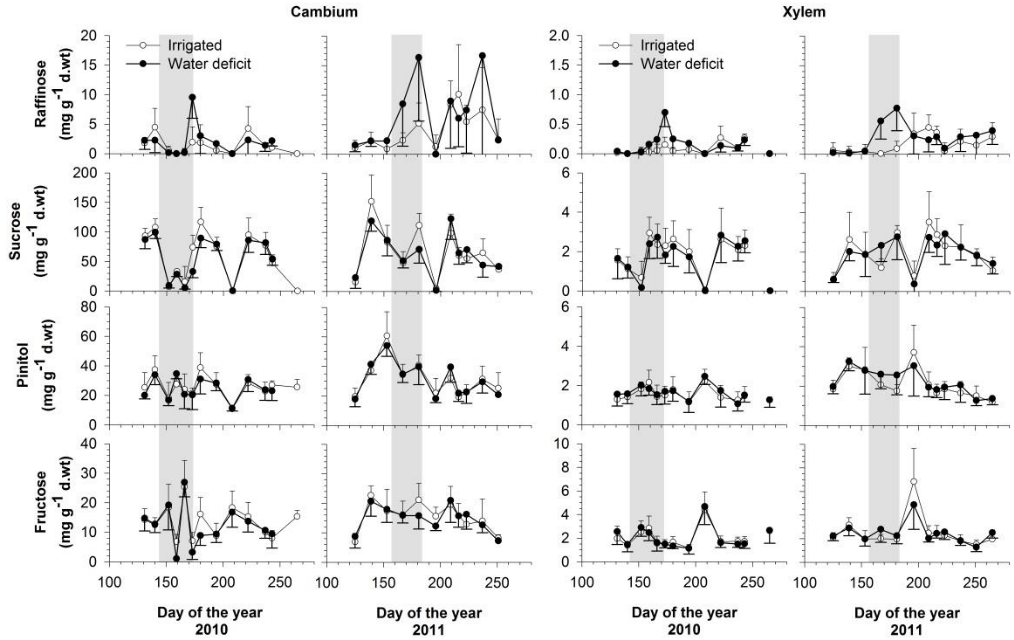

Cambium
Xylem
Irrigated
Water deficit
Raffinose (mg g-1 d.wt)
Sucrose (mg g-1 d.wt)
Pinitol (mg g-1 d.wt)
Fructose (mg g-1 d.wt)
Day of the year 2010
Day of the year 2011
Day of the year 2010
Day of the year 2011

Figure 5

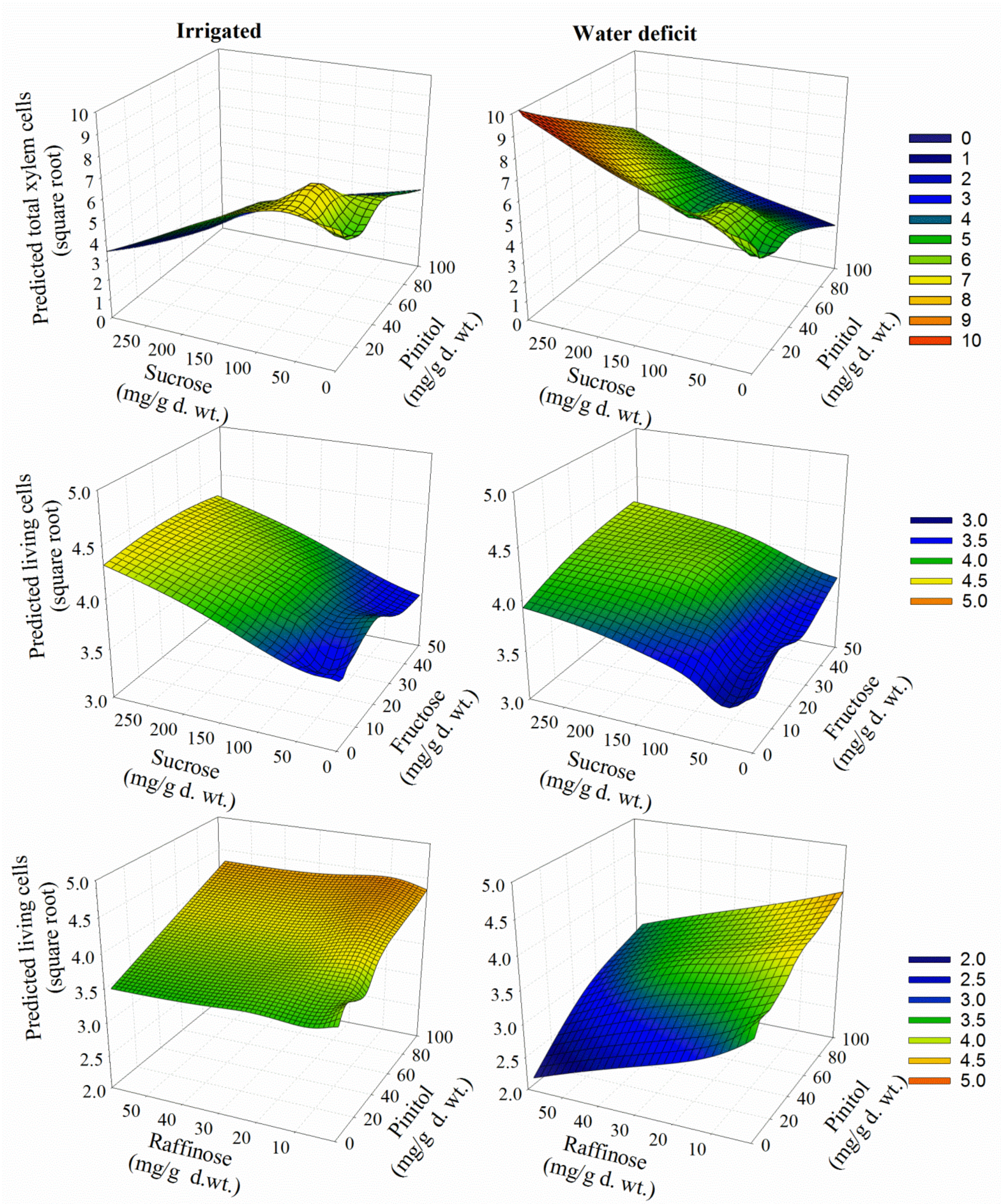
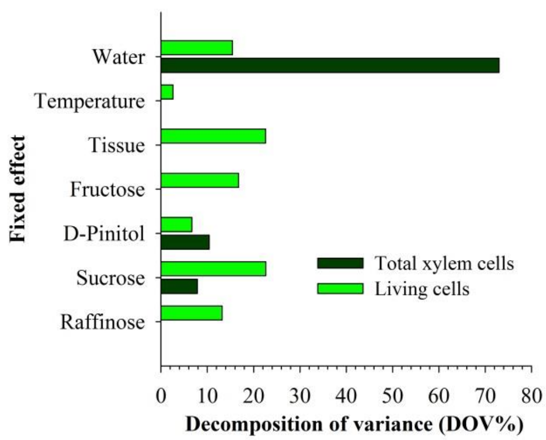
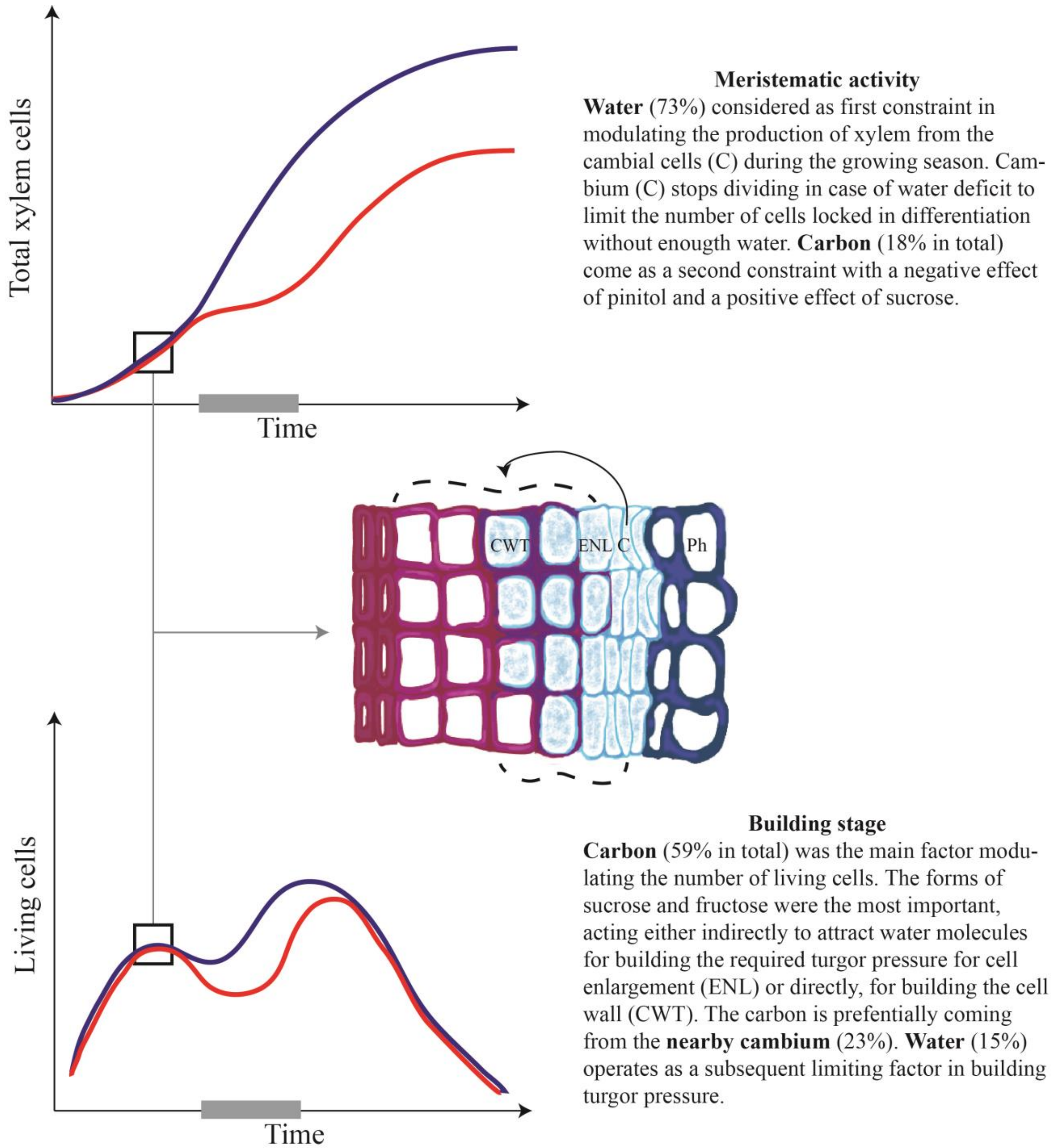

Irrigated
Water deficit
Predicted total xylem cells
(square root)
Sucrose
(mg/g d. wt.)
Pinitol
(mg/g d. wt.)
0
1
2
3
4
5
6
7
8
9
10
Predicted living cells
(square root)
Fructose
(mg/g d. wt.)
3.0
3.5
4.0
4.5
5.0
Raffinose
(mg/g d.wt.)
2.0
2.5
3.0
3.5
4.0
4.5
5.0

Figure 6



Water
Temperature
Tissue
Fructose
D-Pinitol
Sucrose
Raffinose
Fixed effect
Total xylem cells
Living cells
0
10
20
30
40
50
60
70
80
Decomposition of variance (DOV%)

Figure 7


Total xylem cells
Time
Meristematic activity
Water (73%) considered as first constraint in modulating the production of xylem from the cambial cells (C) during the growing season. Cambium (C) stops dividing in case of water deficit to limit the number of cells locked in differentiation without enougth water. Carbon (18% in total) come as a second constraint with a negative effect of pinitol and a positive effect of sucrose.
CWT
ENL
C
Ph
Living cells
Time
Building stage
Carbon (59% in total) was the main factor modulating the number of living cells. The forms of sucrose and fructose were the most important, acting either indirectly to attract water molecules for building the required turgor pressure for cell enlargement (ENL) or directly, for building the cell wall (CWT). The carbon is prefentially coming from the nearby cambium (23%). Water (15%) operates as a subsequent limiting factor in building turgor pressure.

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