SAMPLING CAMBIAL REGION AND MATURE XYLEM FOR CARBOHYDRATE ANALYSES

Alessio Giovannelli[1]*, Giovanni Emiliani[1], Maria Laura Traversi[1], Annie Deslauriers[2], Sergio Rossi[2]

[1] Laboratorio di xilogenesi, Istituto Valorizzazione Legno e Specie Arboree, IVaLSA-CNR, via Madonna del Piano, 50019 Sesto Fiorentino (FI), Italy

[2] Département des Sciences Fondamentales, Université du Québec à Chicoutimi, 555 Boulevard de l'Université, Chicoutimi (QC), Canada

* Address for correspondence:

Istituto Valorizzazione Legno e Specie Arboree, IVaLSA-CNR, via Madonna del Piano, 50019 Sesto Fiorentino (FI), Italy

Tel.:+39 0555 225 782, Fax: +39 0555 225 656, giovannelli@ivalsa.cnr.it

SUMMARY

This paper describes a procedure to analyse soluble carbohydrates and starch in xylem and cambium extracted by the scraping method. An application on woody logs of 2-years-old *Populus* × *canadensis* Moench 'I-214' during different phases of cambium activity is reported. Samples are freeze-dried in liquid nitrogen and, successively, the bark is removed. The cambium and the differentiating xylem are gently scrapped with a razor blade from the inner side of the bark and the outermost side of the stem, respectively. The xylem is milled until obtaining a powder. After extraction of the powder in a EtOH solution and centrifugation, the supernatant and the resulting pellet are used for assessing the contents of soluble sugars and starch, respectively. Soluble sugars are determined using the High-Performance Liquid Chromatography (HPLC). The amount of the cambial region obtained by scraping changed in respect to cambium phenology with higher amount of dry matter obtained during tree ring production. The HPLC method was particularly suitable to study the intra-annual sugars dynamic in woody forming tissues and mature xylem. In both tissues (cambial region and xylem), the major soluble sugars detected were glucose and fructose which represented together more 80% of the total soluble sugars during wood formation However, the total soluble sugars was higher in cambium compared with xylem, especially with the trees were actively growing. The scraping technique provided the possibility to sampling different tissues (mature and developing xylem, cambium and phloem) during the year, allowing the metabolic changes during tree-ring formation to be investigated.

Keywords: xylogenesis, cell differentiation, annual ring, soluble sugars, starch

INTRODUCTION

Tree rings are the results of a complex process involving the division of the cambial tissues and the differentiation of the newly formed cells (Rossi et al., 2006). This growth process requires a great amount of carbon and energy translocated from the source, the crown, to branches, stem and roots in form of sugars. Sugars in the growing tissues are utilised by the carbon sinks or converted in starch for storage. Although seasonal dynamics of wood formation has been analyzed thoroughly in the last decade both directly and indirectly (Giovannelli et al., 2007; Thibeault-Martel et al., 2008; Moser et al., in press; Rossi et al., 2009), the internal factors affecting tree-ring formation, such as cambium age, hormone distribution and carbohydrate availability (Rossi et al., 2008; Uggla et al., 1998; Schrader and Sauter, 2002), still remain to be clearly identified and precisely quantified. In particular, since the 1960s biologists have been trying to explain the influence of carbohydrates on cambial activity and early-latewood formation (Gordon and Larson, 1968; Wargo, 1976; Saranapää and Höll, 1989; Geisler-Lee et al., 2006). However, only a few studies on intra-annual carbohydrate availability for wood production in broadleaves are available (Piispanen and Saranpää, 2001, Barbaroux et al., 2003; Hoch and Körner, 2003). These studies were focused on carbohydrate content in the stem, but did not consider the meristem at the origin of wood: the cambium.

The main limitation in performing biochemical and molecular investigations in the cambial cells is that this tissue is usually difficult to collect. The cambial region consists of a cell layer in different developmental stages as the cambial zone (the true meristem) and the derivatives, which undergo expansion and secondary wall formation towards the

mature xylem and the phloem (Uggla and Sundberg, 2002). Even during the maximum expansion, cambium is constituted by 4-15 cells lacking in secondary wall (Rossi et al., 2007; Deslauriers et al., 2009), a very thin and soft tissue. Surprisingly, all variations in biochemistry, metabolic activity and gene expression across the cambial region occur within a very narrow region. Nevertheless, few details have been given about the available techniques for sampling and preparing cambium for biochemical analyses (Uggla and Sundberg, 2002; Magel et al., 1994; Micheli et al., 2002), leading to a number of difficulties in the accomplishment of such important experiments (Chaffey, 2002).

The arising question concerns how to extract suitably the cambial region in living trees during the year. In spring, the bark slippage is a well known marker of cambial reactivation. In this period, the bark can be easily removed from the stem and the cambial region is visible as a translucid film on the inner part of the bark or on the outermost mature xylem side. Although apparently easy to do, this sampling procedure involves difficulties connected to the sample size (radial thickness of cambial region), which depends on the cambium phenology and the whole plant water status. On the contrary, during dormancy, cambium is composed of few cells, which are strictly attached to the inner part of the bark. As a result, the cambial region is very difficult to sample when the vigorous growth is not occurring. This represents one of the major limitations when investigations should be performed in the time and should involve the different phases of cambium phenology.

According to the literature, the cambial region in trees is mainly extract from stem portions (consisting of bark, phloem, cambial region and mature xylem) through two

methods: (i) by scraping the macro-fractions (Micheli et al., 2002), or (ii) by cryo-sectioning the micro-fraction (Uggla and Sundberg, 2002) of individual tissues. The scraping method uses the unlignified enlarging xylem as a breaking zone to separate the mature xylem from the cambium, phloem and bark. The scraping method is generally used when a large amount of differentiating cambial tissues are required for biochemical and molecular analyses. Even if this method is less time consuming and expensive that cryo-sectioning, some limitation may occur as: (i) the sample is not homogeneous but composed of a multiplicity of different tissues (cambium, developing xylem and phloem), (ii) the amount of scraped tissue depends on the phenological stage of cambium (growth or dormancy), (iii) the ratio between the amount of cambium and differentiating tissues changes during in time (season) and space (along the stem), and (iv) the chemical composition of the extracted heterogeneous tissues is often unknown. Therefore, there is a need to test the scrapping method to verify the collected material. The aim of this paper is to describe a suitable procedure to analyse soluble carbohydrates and starch in xylem and cambium extracted by the scraping method and to present an application on broadleaves.

SAMPLING CAMBIUM AND XYLEM

Woody samples are extracted from the stem in form of logs of 10-15 cm in length, immediately immerged in liquid nitrogen and freeze-dried at a constant temperature of -50 °C under vacuum (0.15 mbar or less). During drying, the loss of intra and intercellular water in the cambial region causes the shrinkage of the elastic developing tissues leading to the appearance of many wide empty spaces within the growing tree ring, as observed at the scanning electron microscope (Figure 1A). Occasionally, breaks between xylem and cork can take place in correspondence to the differentiating cells (Figure 1B). Before handling the samples, the temperature of the logs is gradually increased to 22 °C in an environment with a relative humidity of 40% (Magel, 2002). Before dissection, the dried logs have to be maintained into sealed bags under vacuum and stored at room temperature. For collecting the cambial zone, bark is removed from the logs and cambium and the differentiating xylem are gently scrapped with a razor blade from the inner side of the bark and the outermost side of the stem, respectively (Figure 1C-D). The mature xylem are cut off from the log using a chisel (Figure 2A), reduced to a fine powder with a rotor mill (Figure 2B) and stored in falcon tubes under vacuum up to biochemical analyses.

BIOCHEMICAL ANALYSES

For both cambial region and xylem, 40 mg of dried powder are repetitively extracted three times in 5 ml of 80% EtOH solution, adjusted to pH 7, at room temperature. In each extraction, the homogenates are gently vortexed and, after 30 min, centrifuged at 10.000 rpm for 5 min. The supernatant and the resulting pellet are used for assessing the contents of soluble sugars [High-Performance Liquid Chromatography (HPLC)] and starch, respectively.

HPLC analysis

The surnatant is evaporated to dryness at room temperature with a Savant Speedvac Plus SC210A system and diluted with 2 ml of distilled water (pH 7). The solution obtained is then fractionated using liquid-solid extractions carried out by eluting samples through a reverse-phase cyclohexyl resin (pre-packed 3 ml Bond Elut CH cartridge, Varian CA, USA) and a quaternary-amine, strong anion-exchange resin (pre-packed 3ml Bond Elut SAX cartridge, Varian CA, USA). The cartridges are activated by 6 ml MeOH and conditioned by adding 6 ml of distilled water. The elution is performed using an additional 6 ml of distilled water, evaporated to dryness under vacuum and then diluted with 0.5 mm of distilled water. Analyses are conducted using a binary LC pump 250 (Perkin Elmer, USA) equipped with an automatic injection system (ISS101, Perkin Elmer). A Water Column Heater Module (Waters Division, Millipore, Milford, MA, USA), controlled by a Temperature Control Module (Waters), maintains the column at 80 °C. The column is an 8×300 mm Shodex Sugar SC 1011 (Showa Denko Europe GmbH, Germany) equipped with a Guard Pak Insert Sugar Pak II

(Waters). The mobile phase is water, Milli Q grade, at 0.5 ml min^{-1}. Identification and quantification of soluble carbohydrates is performed according to Romani et al. (1994) and the identity of soluble carbohydrates is confirmed using authentic carbohydrate standards (Sigma, USA) and adding an internal standard. In figure 3A, a typical chromatogram of 25 µl injection of 0.1% standard solution of raffinose, sucrose, glucose, galactose, fructose, mannitol and sorbitol is reported. Sorbitol, a sugar not detected in woody poplar tissues is used as internal standard and added to the crush material. The recovery is estimated for each carbohydrate. Thus 0.25, 0.50, 0.75, and 1.0 ml 1mg ml^{-1} carbohydrate solutions are fractionated and analysed as previously described, with recovery ranging from 92 to 99%. Calibration curves are performed for raffinose, sucrose, glucose, galactose, fructose, mannitol and sorbitol. Total soluble sugar content is obtained as the sum of the detected sugars (>0.1 µmol g^{-1}DW).

Starch content

Starch content is measured in the pellet remaining after extraction with 80% ethanol according to Gucci et al. (1991). The ethanol-insoluble residue is suspended in 1.5 ml acetate buffer (pH 5), boiled at 100°C for 1 hour in a sand bath and cooled at room temperature. After incubation at 55°C for 16 h with 150 µl amyloglucosidase from *Aspergillus niger* (Fluka), samples are diluted with distilled water to 5 ml and three 0.25-ml aliquots and each sample are assayed colorimetrically by using glucose oxidase (Sigma-Aldrich, Italy).

AN APPLICATION ON POPLAR

Material and methods

Woody samples of *Populus* × *canadensis* Moench 'I-214' were collected in 2007 in Casale Monferrato (AL, Italy). Plants were growing on an alluvial sandy-loam soil in one-year-old coppice with stumps arranged in 350-m-long rows with a distance of 2 m between rows and 0.5 m within rows. The average stem diameter at 0.30 cm from the collar was 17 mm.

Cambial phenology was studied by intra-annual analyses of wood formation on stem disks collected weekly according to Deslauriers et al. (2009). For the analysis of soluble sugars and starch, only the main phases of xylem growth were considered and corresponded to (i) dormancy [day of the year (DOY) 33], (ii) onset of xylem differentiation (DOY 110), (iii) maximum growth rate (DOY 152), (iv) decreasing (DOY 207) and (v) ending (DOY 236) of xylem differentiation.

Results

After freeze-drying, the bark was easily separated from the mature xylem except for the samples collected during dormancy (DOY 33). The amount of the cambial region obtained by scraping changed in respect to cambium phenology (table 1). Very low amounts of scrapped tissues were obtained during dormancy (0.05 g), due to the state of the cambium (a row of 3-4 cells). In most of the samples, this quantity was too low to perform HPLC analysis. Instead, during wood formation (DOY 110, 152 and 207), the scrapped tissues considerably increased with amounts varying between 0.16 and 0.23 g

of dry weight. These quantities represented about 1.8, 3.4 and 1.5 % of the collected dry material for onset, maximum growth rate and decreasing of cambium activity respectively. The high stem water content during onset and maximum growth rate also facilitated the extraction of the material by scrapping. At the end of wood formation, the quantity of scrapped tissues decreased at 0.2 g, representing less then 1 % of the collected dry material (20.3 g).

In figure 3B, a typical chromatogram of 20 µl woody tissue extracted of poplar, with sorbitol as internal standard is reported. In this exemple, it was possible to detected raffinose, sucrose, glucose, galactose, fructose and mannitol. In both tissues (cambial region and xylem), the major soluble sugars detected were glucose and fructose which represented together more 80% of the total soluble sugars during wood formation (figure 4). However, the variability of the soluble sugars found was greater in xylem, especially in winter. So, mannitol, raffinose and galactose accounted for about 15% of the total sugars within the xylem during winter (DOY 33). However, these carbohydrates disappeared completely during growth except for the galactose which was present only in small traces in the xylem.

Compared with the xylem, the cambial region had quantities of soluble sugars about 6-10 times higher (figure 5). Higher quantity of soluble sugars was found in cambium during the maximum growth rate (DOY 152), twice that observed at the onset or the end of wood formation, forming 16% of dry matter compared with about 8 % of dry matter respectively. In xylem, the total soluble sugar showed a high level in winter, a decrease during the onset of xylem differentiation (DOY 110) and a further increase in correspondence to the maximum growth rate (DOY 152). Afterwards, new minimums

were reached during decreasing and ending of xylem differentiation with values of less than 1% of dry matter. Starch was detected at different amount depending on the tissues in both the species (figure 5). As for the soluble sugars, cambial region had higher level of starch content than xylem. In cambial region of poplar, more than 80% of the total starch was detected at the end of wood formation (DOY 236) while within the xylem the highest level of starch was detected during dormancy (DOY 33) and at the onset of growth (DOY 110).

Discussion

Understanding of tree-ring formation involve knowledge on the growth processes in trees and investigations on the cambial meristems, which require specific technical procedures for sampling and preparing this thin and soft tissue. In this paper, we describe a suitable and rapid method to extract the cambial region and mature xylem from the stem for biochemical and molecular analyses. This technique provides the possibility to sampling different tissues (mature and developing xylem, cambium and phloem) along the stem and the circumference, allowing the metabolic changes during growth to be investigated.

The amount of cambial region scraped from the inner side of the bark and the outermost side of the xylem varied between the phases of dormancy and growth. Higher quantities were recuperated when the width of the developing zone and the degree of hydration of the stem were superior. On DOY 152 for example, the developing area was fully hydrated and, due to the lack of lignification and the apposition of cellulosic matrix, it was highly elastic and easy to scrap with a razor blade. Similar results was reported for

Picea abies, where the maximum width of the developing zone was recorded during early spring and it was associated to the high stem water content (Rosner et al., 2001). On the contrary, during winter (DOY 33), the cambial zone of poplar consisted of 4-5 narrow initial cells without differentiating xylem and phloem cells (Deslauriers et al., 2009). In these conditions, it was possible to scrap a very little amount of cambial region (< 50 mg), and only on the phloem side. Therefore, an analysis of soluble sugars in cambium with the scraping technique is easier only when the tissue is actively producing cells and with differentiating xylem. Otherwise, larger amount of fresh material have to be collected.

The rapid HPLC method used was particularly suitable for studies involving synthesis of sugars in woody forming tissues and mature xylem. The use of a single column enabled the quantification of five soluble sugars, namely, the sucrose pool (sucrose and raffinose), glucose, galactose, fructose and mannitol. These sugars represented more than 95% of total soluble carbohydrates in the woody tissues of poplar. With such a precise determination of sugars, it was possible to observe that the oligosaccharides concentration changed with respect to the tissue and cambium phenology. The period of wood formation was characterized by a high concentration of fructose and glucose both in cambial region and in xylem while an increase of sucrose was recorded at the end of xylem differentiation. Sucrose represents the major substrate for the synthesis of sugar polymers forming the microfibrillar phase of the cell. In the cytosol, sucrose is converted by sucrose synthase to fructose (used in the glycolisys) and the activated sugar uridine diphosphate-(UDP)-glucose which represent the substrate for the cellulose biosynthesis (Kock, 2004). The high level of glucose in the cambial region could be linked to the high β−1,4-glucan unit requirement during secondary wall formation.

In this work, more than 75% of the total starch measured in cambial region and wood ray parenchyma was detected during dormancy (DOY 33) and after the end of xylem differentiation (DOY 236). These results support the hypothesis that starch is directly used in differentiating tissues during wood formation as source of glucose for cellulose biosynthesis. Elle and Sauter (2000) found that the increased endoamylase activity, related with the starch degradation, leads to higher concentrations of malto-oligosaccharides, maltose and finally glucose.

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CAPTION LIST

Table 1. Weight (g) and water content of the sampled log and amount of scrapped tissue of the cambial region (g) collected in *Populus* × *canadensis*. The sampling days correspond to the main stages of cambial activity: dormancy (DOY 33), onset of xylem differentiation (DOY 110), maximum growth rate (DOY 152), decreasing (DOY 207) and ending (DOY 236) of xylem differentiation.

Figure 1. Analyses of a dried stem section of poplar collected on DOY 164 under a scanning electron microscope. A: Intact section of phloem (ph) developing xylem (dx) and mature xylem (mx) showing collapsed cambial regions (asterisk) due to the water extraction by vacuum. B: section with a break between phloem (ph) and mature xylem (mx) in correspondence of the developing xylem (dx). C: outermost part of mature xylem (mx) with scrapped (arrowhead) and intact (dx) developing xylem. D: inner part of the bark after separation from the xylem with cambial zone (cz) and phloem (ph).

Figure 2. Sampling (A) and milling (B) of the xylem of *Populus* × *canadensis.*

Figure 3. Chromatograms of (A) 25 μl injection of 0.1% standard solution of raffinose, sucrose, glucose, galactose, fructose, mannitol and sorbitol and (B) 20 μl of xylem of *Populus* × *canadensis*, with sorbitol as internal standard.

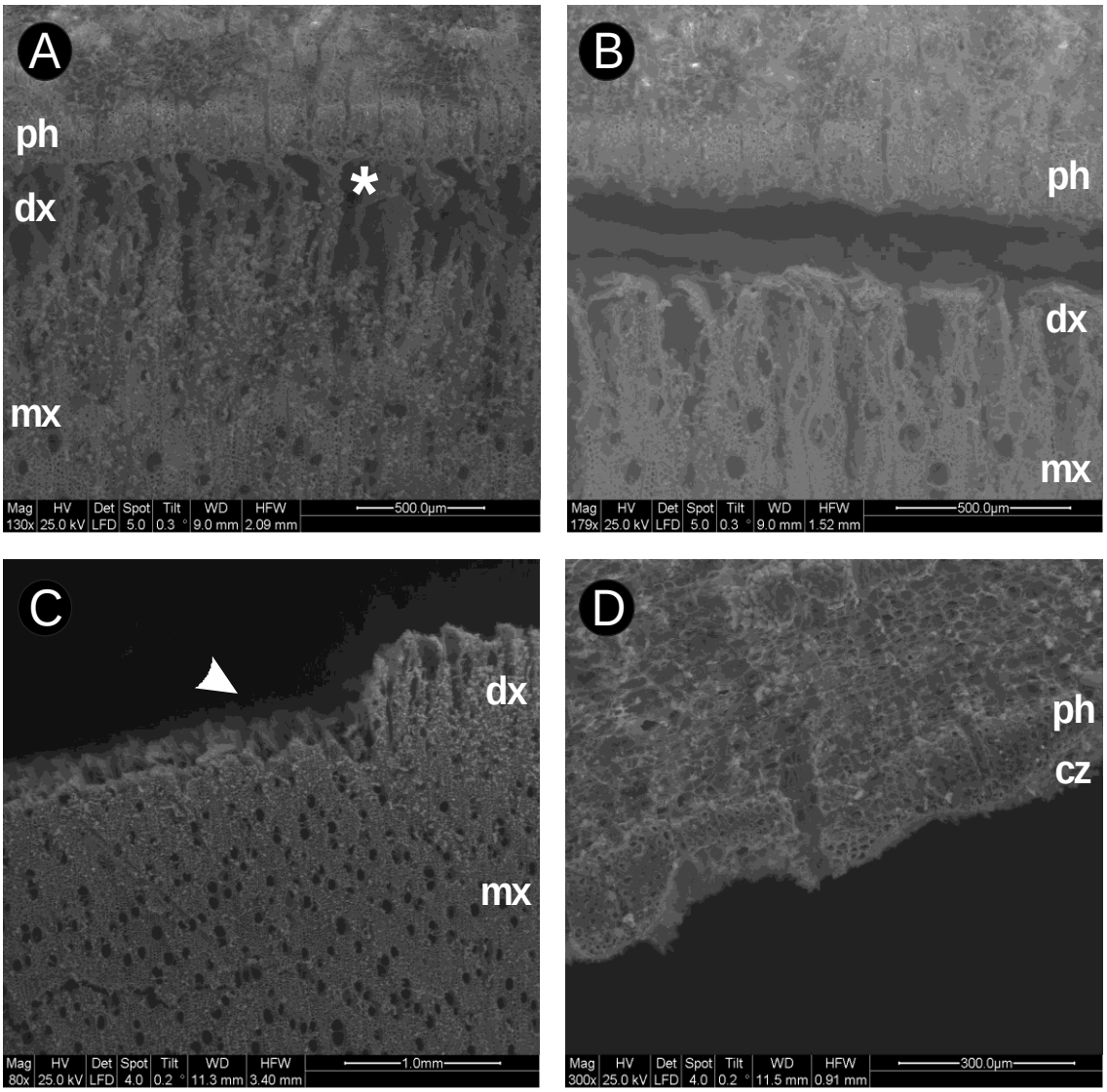
Figure 4. Proportion of soluble sugars in cambium and xylem of *Populus* × *canadensis* during the year. The sampling days correspond to the main stages of cambial activity: dormancy (DOY 33), onset of xylem differentiation (DOY 110), maximum growth rate (DOY 152), decreasing (DOY 207) and ending (DOY 236) of xylem differentiation.

Figure 5. Total soluble sugar and starch expressed as dry matter percent in cambium and xylem of *Populus* × *canadensis* during the year. The sampling days correspond to the main stages of cambial activity: dormancy (DOY 33), onset of xylem differentiation (DOY 110), maximum growth rate (DOY 152), decreasing (DOY 207) and ending (DOY 236) of xylem differentiation. Vertical bars indicate the standard deviation between six measurements. Asterisk represent missing data.

TABLE 1

DOY	Sampled log			Scrapped cambial region
	Fresh weight (g)	Dry weight (g)	Water content (%)	Dry weight (g)
33	27.2±2.5	9.2±1.2	180	0.05±0.001
110	25.6±2.8	8.6±1.2	195	0.16±0.005
152	26.0±1.3	9.1±1.0	186	0.31±0.004
207	36.4±1.3	15.2±1.6	138	0.23±0.002
236	42.7±10.0	20.3±4.5	109	0.20±0.006

FIGURE 1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Mag HV Det Spot Tilt WD HFW
179x 25.0 kV LFD 5.0 0.3 ° 9.0 mm 1.52 mm
500.0µm
C
dx
mx
Mag HV Det Spot Tilt WD HFW
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1.0mm
D
ph
cz
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300.0µm

375

FIGURE 2

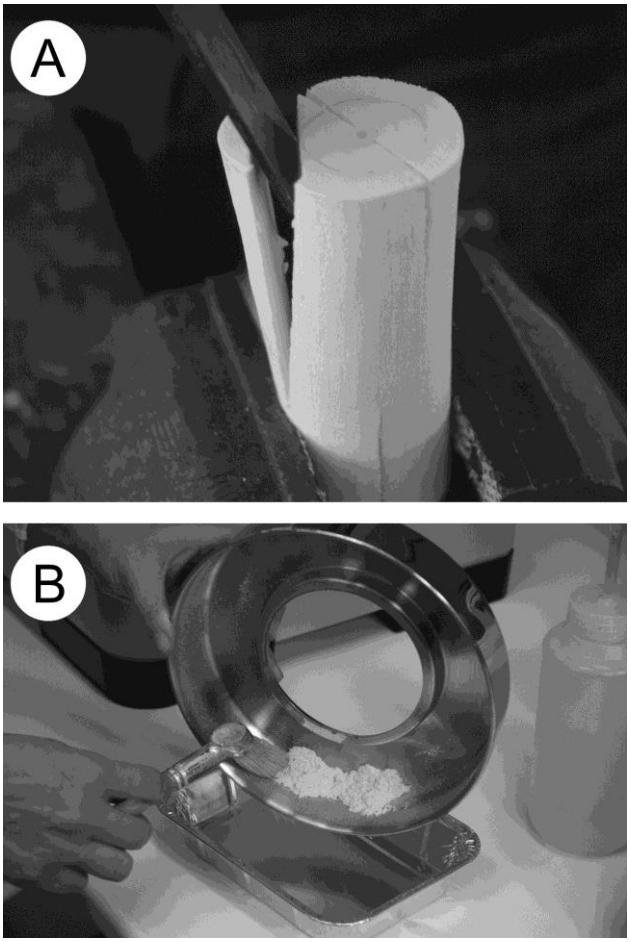
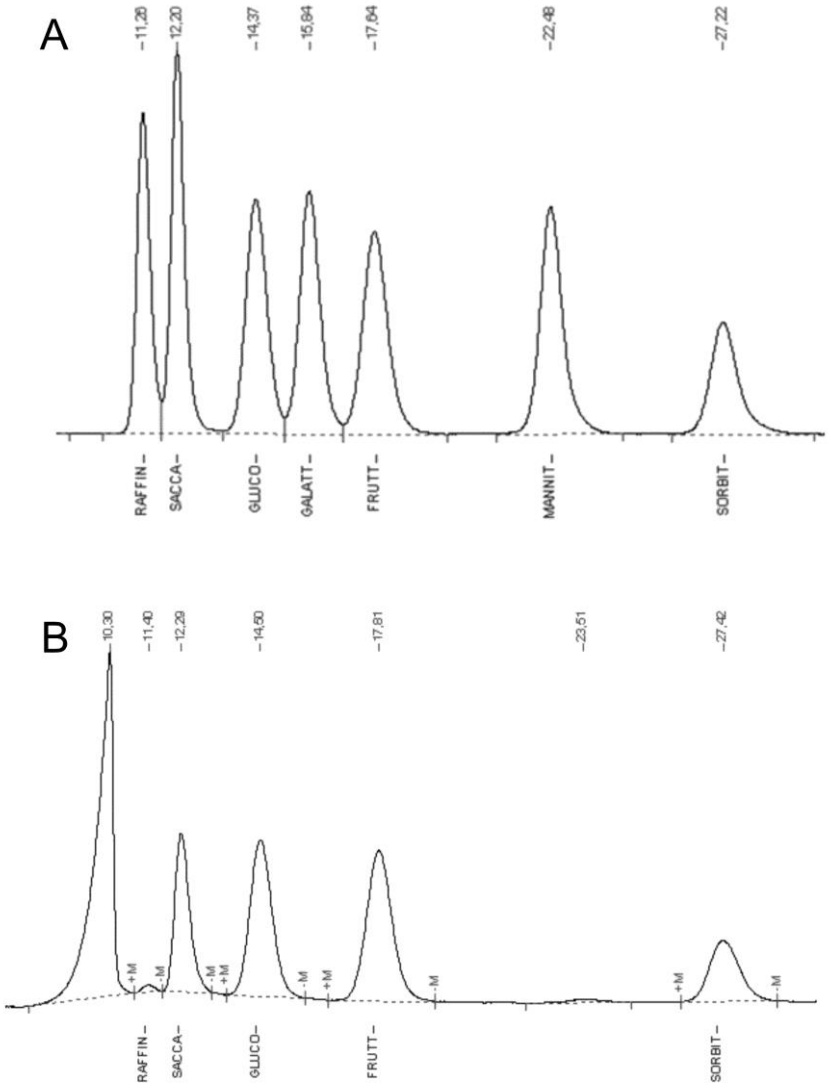


FIGURE 3


A
11,26
12,20
14,37
15,84
17,64
22,48
27,22
RAFFIN
SACCA
GLUCO
GALATT
FRUTT
MANNIT
SORBIT
B
10,30
11,40
12,29
14,90
17,81
23,51
27,42
RAFFIN
SACCA
GLUCO
FRUTT
SORBIT

380 **FIGURE 4**

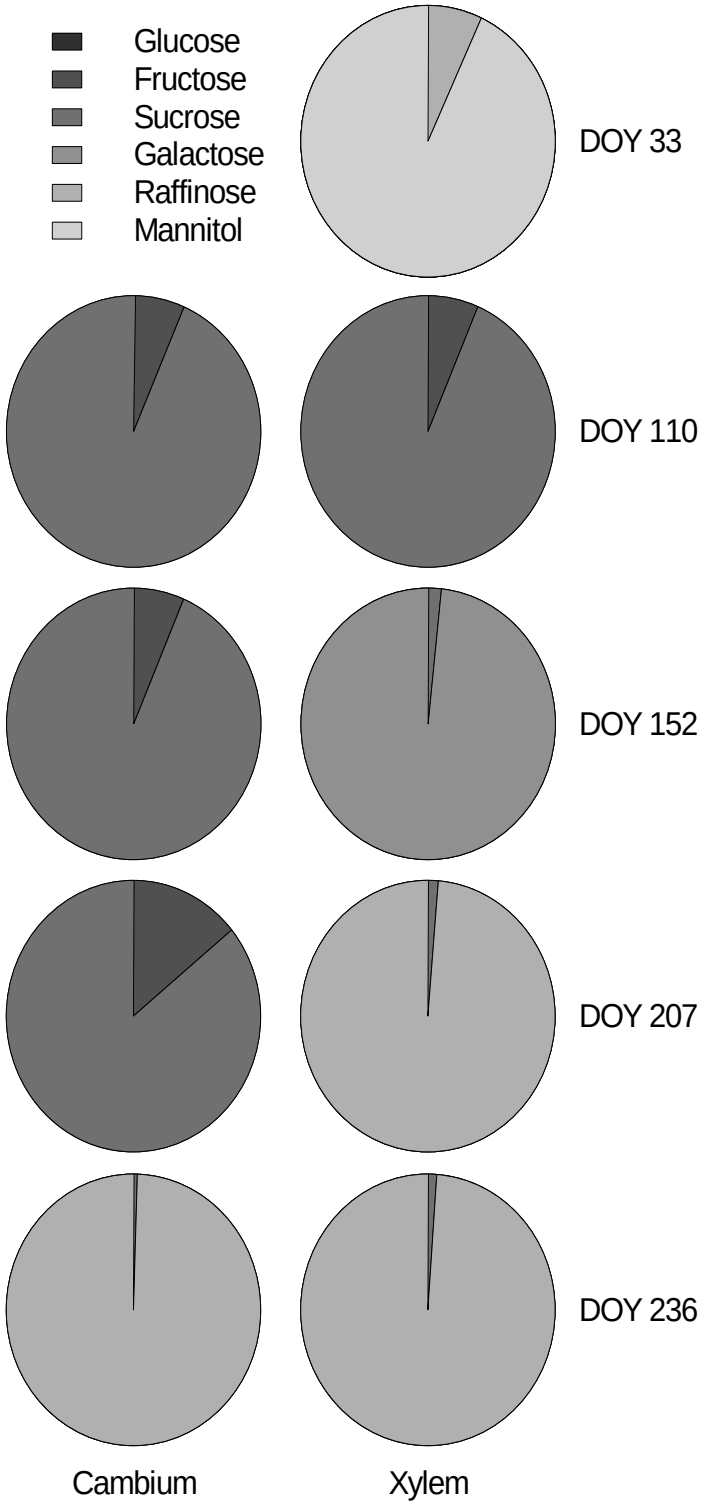
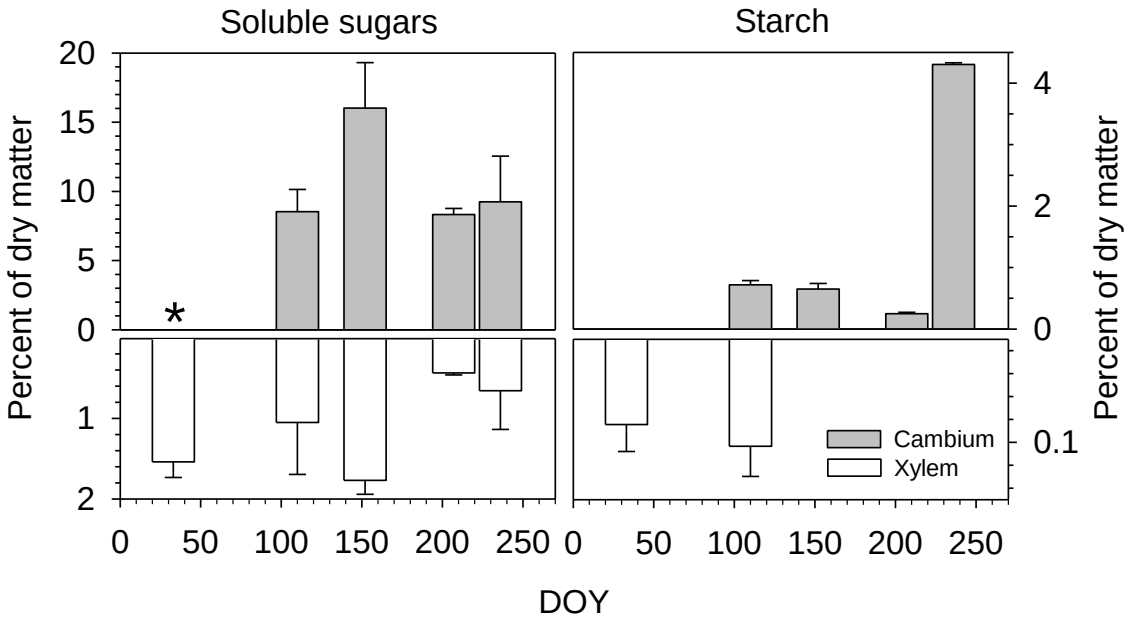

Glucose
Fructose
Sucrose
Galactose
Raffinose
Mannitol
DOY 33
DOY 110
DOY 152
DOY 207
DOY 236
Cambium
Xylem

FIGURE 5


Soluble sugars
Starch
Percent of dry matter
Percent of dry matter
20
15
10
5
0
1
2
4
2
0
0.1
*
Cambium
Xylem
0
50
100
150
200
250
0
50
100
150
200
250
DOY