RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2009; **23**: 2511–2518
Published online in Wiley InterScience (www.interscience.wiley.com) DOI: 10.1002/rcm.4092

RCM

Carbon stable isotope ratio of phloem sugars in mature pine trees throughout the growing season: comparison of two extraction methods†

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Received 26 February 2009; Revised 16 April 2009; Accepted 16 April 2009

The study presents a comparison of two phloem sugar extraction methods. The amount of phloem sugar extracted and the carbon isotope composition ($\delta^{13}C$) of the total extracts and of the main phloem compounds separated by high-performance liquid chromatography (sucrose, glucose, fructose and pinitol) are compared. These two phloem sap extraction methods are exudation in distilled water and a new method using centrifugation, which avoids the addition of any solvent. We applied both extraction methods on phloem discs sampled from 38-year-old *Pinus pinaster* trees in south-western France throughout the period from June 2007 to December 2008 on different time-scales: hourly, daily and monthly. We found that the centrifugation method systematically extracted ca. 50% less compounds from the phloem discs than the exudation method. In addition, the two extraction methods provided similar $\delta^{13}C$ values of the total extracts, but the values obtained by the exudation method were 0.6‰ more negative than those calculated from the mass balance using the individual constituents. Over the growing season, both extraction methods exhibited lower total sugar content and more ^{13}C-enriched phloem sap in summer compared with winter values. These findings suggest that both extraction methods can be applied to study the carbon isotope composition of phloem sap, and the centrifugation method has the advantage that no solvent has to be added. The exudation method, however, is more appropriate for the quantification of the amounts of phloem sugars.

The carbon isotope ratio ($\delta^{13}C$) of phloem sap carbohydrates from mature trees is a tracer of post-photosynthetic processes such as phloem sap loading and transport, cellulose deposition in tree rings and stem respiration.[1–3] Phloem plays a major role in the distribution of photoassimilates in plants from source organs (i.e. leaves) to sink organs (i.e. all non–photosynthetic organs of storage or transport, e.g. branches, main stem or roots). Phloem sap is mainly composed of sucrose, the main substrate for energetic metabolism of plants, but also of other metabolites, e.g. nitrogen compounds (e.g. amino acids, proteins, peptides, RNA), ions (K^+, Ca^{2+}) and signalling molecules (phytohormones, nitrogen oxides, oxygen-reactive species).[4] In conifers, phloem sap can also contain cyclitols, such as pinitol, which accumulates in response to drought stress.[5]

It is difficult to collect phloem sap from sieve tubes which are blocked as a result of callous formation after wounding.[6] Therefore, several methods have been developed to extract phloem sap and some studies have investigated available methods of sugar extraction; for instance, the aphid stylet method,[7] the bark incision or 'bleeding' method[8] and the exudation of phloem pieces.[9,10] According to Gessler *et al.*,[11] who compared some of these methods, the most adequate method for evaluating the carbon and oxygen isotope composition of the phloem sap of leaf, twig and stem of beech species is the exudation of phloem samples in demineralized water.

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†Presented at the 2nd Joint European Stable Isotope User Meeting (JESIUM), Presqu'île de Giens, France, 31 August–5 September, 2008.
Contract/grant sponsor: CARBOEUROPE project (Directorate General XII, European Commission, GOCE-CT-2003-505572), Aquitaine region project (''DURABILITE du SYSTEME de PRODUCTION FORET-BOIS d'AQUITAINE'') and INRA, division ''Forest, Grassland and Freshwater Ecology''.

A few studies on phloem sap have demonstrated dynamics in sugar content and carbon isotope composition on different time-scales. Gessler *et al*.[3] found diurnal variations of the carbon isotope composition of phloem sap, leaves and the trunk from the top to the base of the plant in *Ricinus communis* with ^{13}C-depleted values during the day compared with night-time values. This day-night cycle was suggested to result from isotopic effects during leaf transitory starch accumulation and remobilization at night.[12] However, Kodama *et al*.,[13] observed no diurnal variation in the $\delta^{13}C$ of phloem sap of *Pinus sylvestris* trunks, although some diurnal variations were found in leaves and twigs.

There is little information on the sugar content and carbon isotope composition dynamics of phloem sap in the stem compartment over at least a complete year. In general, studies report seasonal variations of phloem sap in different compartments, i.e. leaves, twigs, stems or roots and on different time-scales, i.e. over few months or over a growing season. Gholz and Cropper[14] examined the dynamics of the sugar and starch contents of tree compartments, from needles to roots, in mature pine trees over 2 years without any carbon isotope analysis. Keitel *et al*.,[15] showed significant variations of sugar content and $\delta^{13}C$ of phloem sap of *Fagus sylvatica* over a growing season. More recently, Maunoury *et al*.[16] studied the dynamics of the $\delta^{13}C$ of CO_2 respired by trunk of *Quercus petreae* in comparison with the dynamics of total organic matter and of soluble sugar carbon isotope composition of phloem on diurnal, daily and seasonal time-scales.

The objectives of the study were (i) to compare two methods of phloem sap extraction, i.e. exudation and centrifugation methods, and (ii) to analyze sugar content and carbon isotope composition of phloem sap of maritime pine trunks on several time-scales: hourly, daily and monthly, over a whole year. The centrifugation method is a new method that consists of centrifuging phloem discs and thus does not require the addition of any solvent to the phloem sample. Following extraction, we measured the total sugar content and its carbon isotope composition as well as the individual constituents, i.e. sucrose, glucose, fructose and pinitol, after separation by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Study site

The experiments were conducted at the Le Bray site, a 38-year-old even-aged stand of maritime pine (*P. pinaster*) located 20 km south-west from Bordeaux (latitude 44°43′1.6″N, longitude 0°46′9.5″W) in France. The understory vegetation consists mainly of purple moor-grass *Molinia coerulea* L. *Moench*. and the soil is a sandy hydromorphic humic podzol. The climate is temperate maritime characterized by cool wet winters and warm dry summers. The mean (1971–2000) annual temperature and precipitation are 13.2°C and 971 mm, respectively. Over the studied period (2007–2008), no severe soil water deficit occurred in the summer compared with the previous years (2002, 2003, 2005 and 2006). Additional phloem samples were collected in a mixed stand of *P. pinaster* and *P. sylvestris*, near the Le Bray site.

Phloem sampling

The phloem discs were sampled at a height of 1.3 m from the main stem of dominant trees. Three sets of three phloem samples located around each tree trunk were collected ($n = 9$ phloem discs per tree per sampling time). After removing the bark, each sample was punched with a cylindrical 12 mm cork-borer. The bark and the xylem pieces remaining on the sampled disc were immediately removed with a scalpel.

The phloem sap was extracted from the phloem discs with two different methods: the exudation method on two sets (for a duplication of analysis) and the centrifugation method on the last set, except for day-to-day variations for which the exudation method only was used (see below). These extracts were then analyzed for sugar content and carbon isotope composition.

These procedures were carried out at the Le Bray site from June 2007 to December 2008 to explore the temporal variations of phloem sugar content and carbon isotope composition. The hourly variations were assessed on 26 July 2007 by collecting phloem samples ten times between 06:00 and 22:00 from a single tree (all times are expressed in Greenwich Mean Time GMT), with a 0 to 24 h notation). The day-to-day variations were investigated from a set of phloem samples collected every day from five mature trees (mean diameter at breast height (DBH) of 45.58 ± 0.71 cm) for 3 weeks in June 2008 with the exudation method only. The seasonal variation was assessed from monthly samples taken from five other dominant pine trees (DBH of 43.19 ± 0.35 cm, mean height of 25 m) between 09:00 and 11:00 using both extraction methods. Another set of five mature pine trees was added to the initial sample set in June 2008.

Phloem sap extraction by exudation

The exudation of phloem consisted of sap retrieval from phloem sieve tubes by osmosis, introducing the set of three phloem discs in 6 mL of exudation solution (2 mL per phloem disc) for a few hours to allow the extraction of sugars. The phloem discs were then removed and dried at 65°C. The solution containing phloem exudates was stored in a freezer at −28°C before analysis.

Phloem sap extraction by centrifugation

The centrifugation method is based on the extraction of the phloem sap from phloem discs by centrifugation. The phloem discs were placed above glass-wool and centrifuged for 10 min at 14 000 *g* (MiniSpin Plus centrifuge, Eppendorf, Hamburg, Germany) in 1.5 mL Eppendorf vials (a set of 3 discs per vial). After centrifugation, the sap collected at the bottom of the vials (on average 150 μL) was stored at −28°C for further analysis and the remaining pieces of phloem discs were then dried in an oven at 65°C.

Tests (data not shown) showed that the thickness of glass-wool has no effect on the amount of collected phloem sap and that capping the vial is needed to avoid any evaporation during the centrifugation. The speed and duration of centrifugation were optimized to obtain the maximum quantity of phloem sap. This new method has the advantage of extracting the phloem sap rapidly and directly without the addition of any solvent.

DOI: 10.1002/rcm

Phloem sap content measurements

The total sugar content of phloem extracts obtained from either exudation or centrifugation was measured as follows. The solutions obtained by the exudation method were first centrifuged at 11 000 *g* for 2 min to remove the pellet of phloem tissues. A dilution in distilled water was needed for both extraction methods, i.e. 1 mL of exudates in 3 mL of distilled water for the exudation method and 20 μL of centrifuged material in 2 mL of distilled water for the centrifugation method. Then, 50 μL aliquots of the diluted solutions were injected into 2.5 mL of anthrone reagent which allows colorimetric analysis of the total sugar content (all monosaccharide, disaccharides and polysaccharides in their hydrolyzed or non-hydrolyzed forms). The preparation of the anthrone reagent was adapted from Bachelier and Gavinelli:[17] 0.5 g of anthrone, also named 9,10-dihydro-9-oxoanthracene, was dissolved in 250 mL of sulphuric acid at 95–98% without water addition. The colorimetric reaction was accelerated by heating at 80°C for 30 min and the total sugar content was then determined by measuring the absorbance at 560 nm with a spectrophotometer (Biochrom Libra S22, Biochrom, Cambridge, UK). In contrast to Bachelier and Gavinelli,[17] who measured the absorbance with filters of 580 and 620 nm, the wavelength of 560 nm was selected as the maximum absorbance of sugars in the UV-Visible spectrum. The sugar concentration was determined from calibration curves established using standard sucrose solutions with a range of known concentrations. After sugar content analysis, the remaining solutions were stored in a freezer until the carbon isotope composition ($\delta^{13}C$) analysis.

The main compounds in the phloem sap, which were extracted using both extraction methods, were individually quantified and isolated as follows. First, 50 μL of phloem extracts from the centrifugation method only were diluted in 500 μL of distilled water. The phloem extracts from both extraction methods were then filtered (filter HV, 0.45 μm type; Nihon Millipore Kogyo, Osaka, Japan) to remove any pellets. The individual compound separation and quantification were then conducted with a high-pressure liquid chromatography (HPLC) (Gilson Inc., Middleton, WI, USA) with a PL Hi-Plex Ca^{2+} column (7.7 mm diameter and 300 mm length, Polymer Laboratories, Varian Inc., Palo Alto, CA, USA) placed behind a carbohydrate precolumn (Benson Carbohydrates BC-100 Calcium, Alltech, Carquefou, France). The flow rate was maintained at 0.5 $cm^3 min^{-1}$ and the temperature of the column at 90°C. The peaks of four components, i.e. sucrose, glucose and fructose and pinitol, were observed by refractometry (refractive index detector IOTA 2, Precision Instruments, Marseille, France) and individually collected for isotope analysis. Their contents were determined from calibration curves established with standard sugars and pinitol solutions with a range of known concentrations.

Carbon isotope analysis

In order to assess the carbon isotope composition of the total phloem extracts, 100 μL or 150 μL of diluted extracts obtained by both extraction methods were transferred into tin capsules (Thermo Electron Corporation, Milan, Italy) and oven-dried at 65°C for 12 h. The individual compounds separated by HPLC were freeze-dried, suspended in distilled water and then 250 μL of each compound was transferred into tin capsules (Thermo Electron Corporation). All the tin capsules containing oven-dried samples were then put into an elemental analyzer (NA-1500; Carlo-Erba, Milan, Italy) coupled with an isotope ratio mass spectrometer (IRMS VG Optima; Micromass, Villeurbanne, France) for carbon isotope analysis. The carbon isotope composition value is expressed in $\delta^{13}C$ notation (‰ units), relative to the international standard (Pee Dee Belemnite). A laboratory standard (glutamic acid) was measured every 12 samples for correction of the drift of the IRMS instrument. According to Duranceau *et al.*,[18] no fractionation against ^{13}C occurs during the elution of phloem sap compounds. We also assumed no fractionation although our carbohydrate column was not the same.

RESULTS AND DISCUSSION

Tests on exudation method

The standard solution normally used for phloem exudation is composed of 10 mM of ethylene diaminetetraacetic acid (EDTA) and of 0.015 mM chloramphenicol (CAP) at pH 7.0.[10] EDTA is used to prevent callous formation in the sieve tubes and CAP to avoid the bacterial consumption of sugars. However, the use of deionized water without any chemical agents has already been shown to increase the precision of the measured carbon isotope composition of extracted sugars.[11] Hence, to check the effect of the chelating agent EDTA on sugar extraction, we measured the total sugar content of the exudates collected from phloem samples of mature *P. pinaster* and *P. sylvestris* trees growing in the vicinity for different concentrations of EDTA in distilled water (Fig. 1). The concentration of 0 mM corresponded to distilled water without any agents. Since the difference between the total sugar content extracted at 0 mM of EDTA

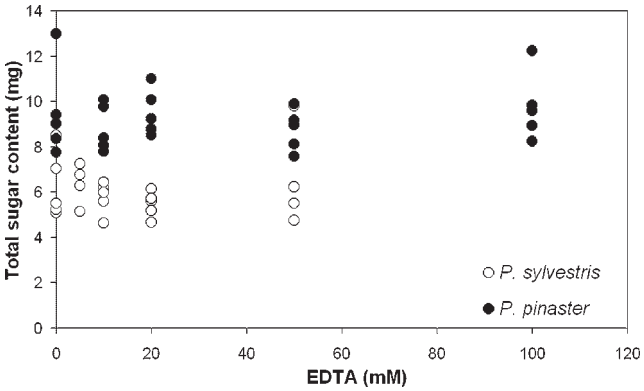

Figure 1. Total sugar content extracted from phloem discs using exudation solutions with different concentrations of EDTA for two species, *P. pinaster* (dark circles) collected on 4 May 2007 and *P. sylvestris* (white circles) collected on 15 May 2007. The total sugar content was determined by the anthrone reaction. Each data point represents total sugar content extracted from one phloem disc (five repetitions per EDTA concentration on the same pine tree). For *P. pinaster*, $p = 0.6981$ (t-test) and, for *P. sylvestris*, $p = 0.6915$ (t-test) and the Dunnett-test was not significant for either of them.

DOI: 10.1002/rcm

and the total sugar content extracted at higher concentrations was not significant (Dunnet test, Proc. GLM in SAS; SAS Institute Inc., Cary, NY, USA), we concluded that it is not necessary to add EDTA in distilled water to extract more sugars. Similarly, the activity of the antibacterial agent CAP was tested by comparing the values of the total sugar content of two different exudates collected from phloem samples of *P. pinaster* in distilled water with and without CAP addition. The difference in sugar quantity between 4 and 24 h of incubation was not significant ($p = 0.6354$, t-test), indicating that no degradation of sugars occurred in the solution without CAP. Consequently, we used distilled water without adding any carbon-containing compounds for the exudation of phloem discs during the experiments.

Finally, we evaluated the optimal duration of exudation through measurements of the total amount of sugars. Figure 2 shows the total sugar content measured on extracts after different exudation durations ranging from 30 min to 24 h at room temperature. The results showed that 4 h of incubation was sufficient since it allows the extraction of 86% of the amount extracted after 24 h. This is close to the exudation duration of 5 h previously reported by others.[10] We subsequently used an exudation time of 4 h.

Comparison of phloem sap extraction methods

A comparison between the exudation and centrifugation methods for total sugar content determination, assessed from June 2007 to December 2008, shows that the amount of compounds extracted by the centrifugation method ($48.5 \pm 1.8\,mg\,g^{-1}$ dry weight (DW)) was on average 50% less than by the exudation method ($102.1 \pm 3.3\,mg\,g^{-1}$ DW) (Fig. 3 and Table 1). Both analysis of variance (ANOVA) (Table 1) and paired t-tests (data not shown) show a significant difference between the two methods ($p < 0.001$). Consistently, the amount of the individual compounds separated by HPLC, i.e. sucrose, glucose, fructose and pinitol, extracted from phloem pieces was also higher by exudation (Fig. 4, left panel) than by centrifugation (Fig. 4, right panel) (Table 1). This is also clear from the results obtained at an hourly resolution: the mean total sugar content was $127 \pm 2.9\,mg\,g^{-1}$ DW and $43 \pm 2.5\,mg\,g^{-1}$ DW using the exudation and centrifugation methods, respectively (Fig. 5, $p < 0.0001$). On average, the centrifu-

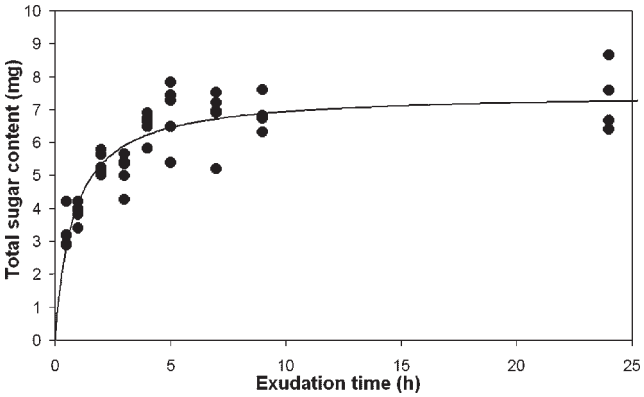

Figure 2. Total sugar content in phloem sap exudated into distilled water (without any agent) for different durations of exudation. Total sugar content was determined by the anthrone reaction. Each data point corresponds to the extract from one phloem disc sampled from one maritime pine (*Pinus pinaster*).

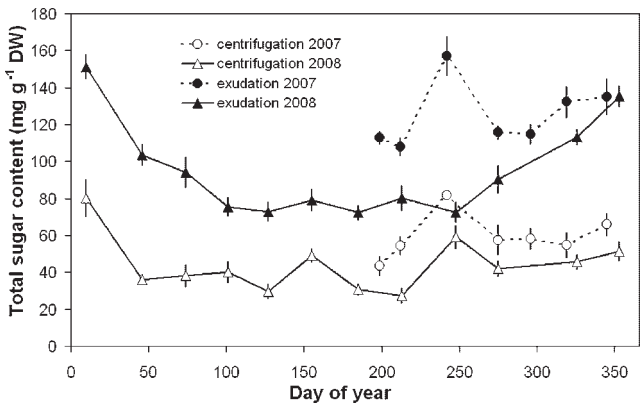

Figure 3. Seasonal changes in the total sugar content of phloem sap sampled from maritime pine trees and extracted by exudation in distilled water (dark symbols) and centrifugation (white symbols) methods in 2007 (circles and dotted lines) and in 2008 (triangles and full lines). The total sugar content was determined by the anthrone reaction. Each data point represents the mean value (±SE) of five to ten dominant trees.

Table 1. Effect of phloem sap extraction method and time of year on the sap compound contents. Phloem discs were sampled on *Pinus pinaster*. The exudation of phloem sap was conducted in distilled water. The individual compounds were analyzed using HPLC. Upper part of the table gives mean ± SE values for each extraction method for the data pooled over the studied period (n = 6 for sucrose, glucose, fructose and pinitol from June to October 2007 on two to five pine trees and n = 19 for the total sugar content measured by the anthrone reaction from June 2007 to December 2008 on 5 pine trees). Bottom part of the table gives probability values for significant effects of the method, time of year and interaction (ANOVA with repeated measurements, proc GLM, SAS software v 8.02). 'ns' means non-significant effect on the parameter

	Phloem sap compound contents ($mg\,g^{-1}$ DW)						
	Sucrose	Glucose	Fructose	Pinitol	Sugars + Pinitol	Anthrone	Anthrone - Sugars
Centrifugation	35.9 ± 2	6.4 ± 0.4	7.3 ± 0.4	3.5 ± 0.2	53.1 ± 2.7	48.5 ± 1.8	9.5 ± 2.6
Exudation	57.7 ± 3.6	12.2 ± 0.6	16.1 ± 0.6	6.0 ± 0.4	92.0 ± 4.6	102.1 ± 3.3	35.2 ± 6.3
Method	0.001	0.001	0.001	0.006	0.001	0.001	0.001
Time	ns	ns	ns	ns	ns	0.001	0.001
Method * time	0.01	ns	0.05	ns	0.01	0.001	0.01

DOI: 10.1002/rcm

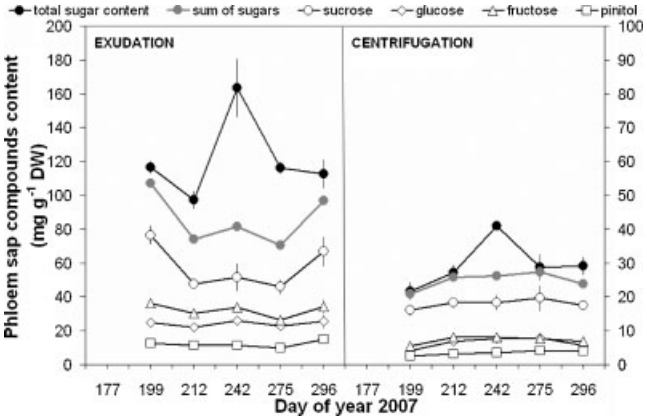

Figure 4. Monthly variations in contents of phloem sap compounds extracted from maritime pine trees obtained by exudation in distilled water (left panel) and centrifugation (right panel) methods in 2007. Total sugar content was determined by the anthrone reaction and the individual compounds by HPLC. The left axis corresponds to the mean values of total sugar (dark circles), of the sum of individual sugars (sucrose, glucose and fructose) (grey circles) and sucrose (white circles) contents. The right axis corresponds to the mean values of glucose (white diamonds), fructose (white triangles) and pinitol (white squares) contents. Each data point represents the mean value of five dominant trees. Vertical bars correspond to the standard error.

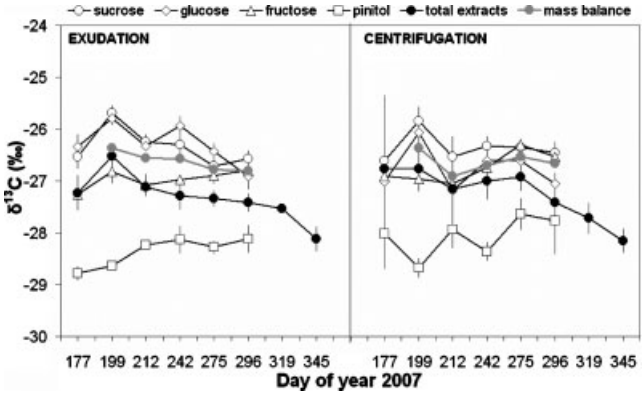

Figure 6. Monthly variations of carbon isotope composition ($\delta^{13}C$) in phloem sap compounds extracted from maritime pine trees by exudation in distilled water (left panel) and centrifugation (right panel) methods in 2007. Mean $\delta^{13}C$ of the sucrose (white circles), glucose (white diamonds), fructose (white triangles) and pinitol (white squares), which were separated by HPLC, mean $\delta^{13}C$ measured on total extracts (dark circles) and $\delta^{13}C$ values calculated from mass balance using the four constituents separated by HPLC (grey circles) are represented. The data points represent the mean $\delta^{13}C$ value of two to five dominant trees. Vertical bars correspond to the standard error.

gation extracted only 45% (fructose), 53% (glucose) and 62% (sucrose) of the amounts obtained by exudation and 47% in the case of total sugar content measured by the anthrone reaction (Table 1). The difference between the amount of sugars assayed by the anthrone reaction and the sum of individual sugars assayed by HPLC (without pinitol, because it is not reactive in the presence of anthrone reagent), was higher with the exudation method than with the centrifugation one: 35% and 20%, respectively (Table 1, last column).

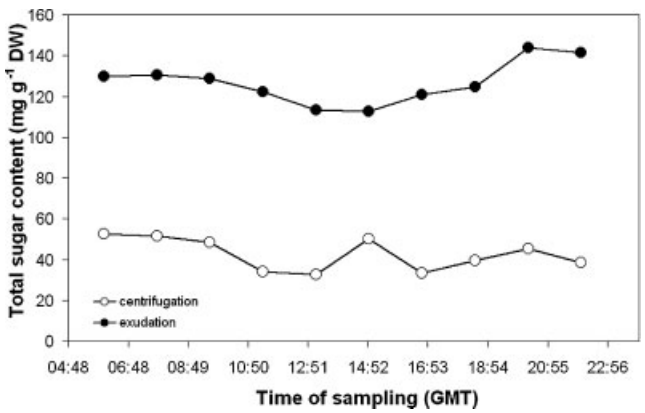

Figure 5. Hourly changes in total sugar content of phloem sap extracted by two methods: exudation in distilled water (dark symbols) and centrifugation (white symbols) on one maritime pine (*Pinus pinaster*) tree at the Bray site on 26 July 2007. Total sugar content was determined by the anthrone reaction. For the comparison of methods, $p < 0.0001$ and, for the temporal variation analysis, $p = 0.665$ (linear regression slope).

The carbon isotope composition ($\delta^{13}C$) values of the extracted compounds from the exudation and centrifugation methods from June to December in 2007 were also compared (Fig. 6). The mean carbon isotope composition of the total extracts pooled from June to December 2007 was not significantly different for either extraction method, with -27.32 ± 0.09‰ and -27.22 ± 0.12‰ for the exudation method and the centrifugation method, respectively (Table 2). Moreover, no significant difference was found between the two methods when comparing the carbon isotope composition of individual sugars, i.e. sucrose, glucose and fructose, with, respectively, mean $\delta^{13}C$ values pooled over the 6-month studied period of -26.34 ± 0.09‰, -26.28 ± 0.10‰, -26.97 ± 0.08‰ for the exudation method and -26.37 ± 0.08‰, -26.79 ± 0.23‰, -26.69 ± 0.10‰ for the centrifugation method. In the case of pinitol, the carbon isotope composition differed significantly depending on the method applied (Table 2), the pinitol extracted by centrifugation being 0.3‰ higher than that extracted by exudation. For both extraction methods, the mean carbon isotope composition of pinitol pooled over the study period was 1.4 (centrifugation) to 1.8‰ (exudation) ^{13}C-depleted compared with the mean $\delta^{13}C$ of the three sugars. The carbon isotope composition calculated from a mass balance using the values of the four compounds separated by HPLC was not significantly different when comparing the two extraction methods (Table 2, second column from the right-hand side) but the difference between the measured $\delta^{13}C$ of total extracts and calculated $\delta^{13}C$ from the mass balance was significant (Table 2, last column, $p < 0.001$). This difference, also visible in Fig. 6, was -0.56 ± 0.06‰ for the exudation

DOI: 10.1002/rcm

Table 2. Effect of phloem sap extraction method and time of year on the carbon isotope composition ($\delta^{13}C$) of phloem sap compounds. Phloem discs were sampled on *Pinus pinaster*. Upper part gives values mean ± SE values for each extraction methods for the data pooled over the studied period (n = 6 to 8 from June to December 2007 on two to five pine trees). The exudation of phloem sap was conducted in distilled water. Individual compounds were isolated by HPLC. 'Total extracts', 'mass balance' and 'extracts – mass balance' correspond to the measured $\delta^{13}C$ on the total extracts, the calculated $\delta^{13}C$ by mass balance using the individual phloem sap compounds and their difference, respectively. Bottom part of the table gives probability values for significant effects of method, time of year and interaction (ANOVA with repeated measurements and ANOVA only for the two last columns, proc GLM, SAS software v 8.02). 'ns' means non-significant effect on the parameter

	$\delta^{13}C$ (‰)						
	Sucrose	Glucose	Fructose	Pinitol	total extracts	mass balance	extracts - mass balance
Centrifugation	−26.37 ± 0.08	−26.79 ± 0.23	−26.69 ± 0.10	−28.02 ± 0.17	−27.22 ± 0.12	−26.63 ± 0.09	0.002 ± 0.16
Exudation	−26.34 ± 0.09	−26.28 ± 0.10	−26.97 ± 0.08	−28.32 ± 0.09	−27.32 ± 0.09	−26.65 ± 0.07	−0.561 ± 0.06
Method	ns	ns	ns	0.01	ns	ns	0.001
Time	0.001	ns	ns	ns	0.01	ns	ns
Method * time	ns	ns	ns	ns	ns	-	-

and not significant, 0.002 ± 0.16‰, for the centrifugation method.

The observed difference in the extraction rate between the two methods could be explained by the difference in the strength applied during extraction. The extraction of phloem sap during centrifugation consists of the detachment of compounds (mainly sugars) linked to phloem tissues by gravity whereas exudation in distilled water displaces molecules by osmosis. Extraction by exudation can be likened to the mechanism of the transfer of assimilates through phloem from source to sink organs, which occurs by osmosis and related pressure flow.[4] Indeed, less sugar can be separated from phloem tissue mechanically than through exchange with molecules of water by osmosis. Therefore, these results suggest that exudation may be more appropriate for quantitative analyses of phloem sap sugars.

The carbon isotope composition of the two types of extracts was similar. However, in the case of exudation, a significant difference was demonstrated between the carbon isotope composition measured on the total extracts and that calculated from a mass balance using values of individual compounds (Fig. 6 and Table 2). This difference can be explained by the fact that, during exudation, compounds, which are 0.6‰ more ^{13}C-depleted on average, might have been extracted in addition to sucrose, glucose, fructose and pinitol. Thus, when studying the discrimination during post-photosynthetic processes (e.g. the transfer of photoassimilates, trunk respiration[13,16] or cellulose synthesis of annual tree rings), the centrifugation method of sugar extraction seems to be more reliable. This is true, as long as the isotopic composition of sugars (i.e. mainly sucrose, glucose and fructose) is targeted but not the carbon isotope composition of the osmotically exchangeable fraction of the phloem, which appears to be constituted by additional compounds. Furthermore, the observed $\delta^{13}C$ values of phloem sap extracts of about −26‰ are consistent with previous studies. For example, Kodama *et al.*[13] showed $\delta^{13}C$ values of phloem exudate organic matter from *Pinus sylvestris* trunks of about −26‰ for few days in June 2000. Moreover, Keitel *et al.*[15] presented $\delta^{13}C$ values of phloem sap from *Fagus sylvatica* ranging from −26‰ to −30‰ between May and September. When studying the carbon isotope composition of individual sugars, our results show that sucrose and glucose were more ^{13}C-enriched than fructose, all three being more enriched than pinitol. Our results are consistent with those of Maunoury *et al.* who showed $\delta^{13}C$ values of sucrose and fructose from *Quercus petreae* of between −23.5‰ and −27‰ between April and November 2004. They also demonstrated that it was mainly sucrose that was ^{13}C-enriched rather than fructose. The ^{13}C-depletion in pinitol (by 1.4 to 1.8‰ depending on the extraction method used) could be because pinitol is not really a sugar, but in fact a cyclitol that is synthesised from glucose in response to drought stress by substituting a hydroxyl group by a methyl group.[19] Ghashghaie (unpublished data) found a similar result for *myo*-inositol, another cyclitol found in leaves, which is ^{13}C-depleted compared with sugars. Keppler *et al.*[20] found that the methyl group is strongly ^{13}C-depleted, which can explain why pinitol is more depleted than *myo*-inositol and than sugars.

Dynamics of phloem sap compounds

Seasonal variations of total sugar content measured from June 2007 to December 2008 by both methods are shown in Fig. 3. The total phloem sugar content increased significantly in winter 2007, then decreased slowly until summer 2008 and increased again until the end of December (Table 1, $p < 0.001$, ANOVA). In addition, the daily values of the total sugar content assessed by the exudation method were not significantly different over a 3-week period in June 2008, with a mean value of 74 ± 2 mg g^{-1} DW ($p = 0.395$) (Fig. 7). On 26 July 2007, no significant trend over time ($p = 0.665$) of the total sugar content for either extraction method was observed (Fig. 5). As a result, significant variations in total sugar content were observed only on a monthly time-scale. In Fig. 4 and Table 1, we also demonstrate that the individual compound contents separated by HPLC, i.e. sucrose, glucose, fructose and pinitol, did not significantly vary over a 6-month period in 2007. However, a difference between the amount of sugars assayed by the anthrone reaction and the sum of individual sugars assayed by HPLC (without pinitol, which is not reactive in the presence of anthrone reagent) was revealed. An anthrone-reactive fraction which was measured as a whole varied according to the time of year, with a

DOI: 10.1002/rcm

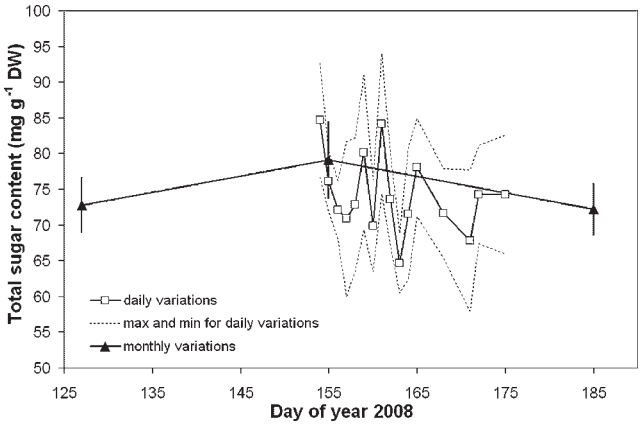

Figure 7. Daily changes in the total sugar content (white squares) in maritime pine trees obtained by the exudation method in distilled water (without any agent) over a 3-week period in June 2008. Monthly variations in total sugar content (dark triangles) are also represented over this period. Total sugar content was determined by the anthrone reaction. The data points are means of total sugar content on two different sets of five dominant trees. The error bars correspond to the standard errors and the dotted lines correspond to the minimum and maximum of total sugar content over the 3-week period ($p = 0.389$).

maximum value on day 242, corresponding to 30 August 2007 (Fig. 4). On this day, the total sugar content measured by anthrone was about 163 mg g^{-1} DW and 82 mg g^{-1} DW for the exudation and centrifugation methods, respectively, and the sugar content calculated as the sum of the three sugars was about 81 mg g^{-1} DW and 53 mg g^{-1} DW for the exudation and centrifugation methods, respectively (Fig. 4).

The variations in the carbon isotope composition of the total extracts for both methods over a 6-month period in 2007 are significant (Table 2, $p < 0.001$). The $\delta^{13}C$ of the extracts increased on day 199, corresponding to 18 July 2007, and mainly decreased from June to December 2007, reaching −28‰ at the end of that year. The carbon isotope composition of glucose, fructose and pinitol did not vary significantly over time, but the variations in the carbon isotope composition of sucrose were significant (Table 2, $p < 0.001$), peaking on day 199, as was the case for the $\delta^{13}C$ of the total extracts for both methods.

Seasonal variations were found in the amount of phloem sap compounds extracted by both methods, and in their carbon isotope compositions, but no significant variations on hourly and daily time-scales (in the case of content only). These observed seasonal fluctuations are consistent with data from Maunoury *et al.*:[16] they showed significant variations in the concentration of phloem total sugars in oak trunks, with minimum values during the period of trunk growth and maximum values once growth stopped. Therefore, these variations could be related to growth seasonality. Indeed, in summer, the total sugar content is low due to the consumption of sugars during the active growth of cambial tissues. Conversely, the lack of growth in winter allows the total sugar content to increase and the carbon isotope composition to decrease. Moreover, Maunoury *et al.*[16] showed a minimum value of the $\delta^{13}C$ of phloem sap sugars of −26.6‰ at the beginning of winter (November) and our results are consistent with this, i.e. a decrease in $\delta^{13}C$ of phloem total extracts, reaching a negative value of about −28‰. However, a study by Keitel *et al.*[15] on the seasonal dynamics of $\delta^{13}C$ of phloem sap from *Fagus sylvatica* between May and September 2000 and another one by Brandes *et al.*[21] on the $\delta^{13}C$ of phloem sap from P. sylvestris during the 2004 growing season showed that there was no decrease in $\delta^{13}C$ at the end of the sampling period.

Two events are of particular interest: the peak of carbon isotope composition of total extracts and sucrose on day 199 (18 July 2007) and the peak of total sugar content on day 242 (30 August 2007). The latter case could be a response to a brief period of water shortage and to the concurrent cessation of stem enlargement (secondary growth). As previously shown by Nguyen *et al.*,[5] in both shoots and roots of maritime pine seedlings, pinitol can accumulate in response to drought, i.e. by osmotic adjustment. However, we did not detect any significant increase in pinitol content on this day. One reason could be that this period of drought stress was too short to induce a subsequent production of pinitol. Moreover, a period of drought stress should induce stomatal closure, and changes in the carbon isotope composition of newly assimilated carbon in leaves and thus in phloem sugars transferred from the canopy to the base of tree. However, our results showed no significant variations in $\delta^{13}C$ of phloem sap in August. It may be that the $\delta^{13}C$ variations were dampened at the base of trunk of the tree compared with in the canopy. Indeed, Kodama *et al.*[13] showed that on a diurnal time-scale, the observed variations in $\delta^{13}C$ of phloem exudates organic matter at the top of *Pinus sylvestris* tree were not obvious at the base of trunk, which can be explained by a mixing of different pools of carbon during phloem sap transfer down the trunk.[22] On day 199, the total extracts and sucrose were more ^{13}C-enriched. Such a peak was also shown by Keitel *et al.*[15] in June and by Maunoury *et al.*[16] in May but the origin of this peak has not yet been elucidated.

CONCLUSIONS

The study revealed that the exudation method is more appropriate than the centrifugation method for quantitative studies of phloem sap compounds, because the quantity of the collected phloem compounds is twice that collected by centrifugation; therefore, extraction is more complete. However, the new centrifugation method appeared to be suitable for studying the carbon isotope composition of phloem sap since the compounds extracted showed no differences in their carbon stable isotope ratio. In addition, this method allows direct extraction without adding any solvent, which avoids any isotopic exchange between the compounds extracted and an external reagent. This might also prove important for the assessment of the oxygen isotope composition of phloem-transported sugars as the free carbonyl group of glucose and fructose might readily exchange oxygen with the exudation water.

The temporal dynamics of phloem sap content and carbon isotope composition have been studied on different time-scales: hourly, daily and monthly throughout a whole year.

DOI: 10.1002/rcm

Monthly variations of both content and carbon isotope composition with two particular events have been highlighted and related to growth seasonality. The phloem sap content of maritime pine showed no significant hourly or daily variations.

Acknowledgements

This study was supported by the CARBOEUROPE project (Directorate General XII, European Commission, GOCE-CT-2003-505572), by the Aquitaine region project (''DURABILITE du SYSTEME de PRODUCTION FORET-BOIS d'AQUITAINE'') and by INRA, division ''Forest, Grassland and Freshwater Ecology''. We would like to thank the Platform ''Métabolisme-Métabolome'' of the IFR 87 ''La plante et son environnement'' for the carbon isotope analysis.

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DOI: 10.1002/rcm