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Impact of carbohydrate supply on stem growth, wood and respired CO_2 $\delta^{13}C$: assessment by experimental girdling

FLORENCE MAUNOURY-DANGER,[1,2,3,4,5] CHANTAL FRESNEAU,[1,2,3] THOMAS EGLIN,[1,2,3] DANIEL BERVEILLER,[1,2,3] CHRISTOPHE FRANÇOIS,[1,2,3] CAROLINE LELARGE-TROUVERIE[6] and CLAIRE DAMESIN[1,2,3]

[1] *Laboratoire Ecologie, Systématique et Evolution, UMR 8079, Université Paris-Sud XI, 91405 Orsay CEDEX, France*

[2] *CNRS, 91405 Orsay CEDEX, France*

[3] *AgroParisTech, 75231 Paris, France*

[4] *Laboratoire des Interactions Ecotoxicologie Biodiversité Ecosystèmes (LIEBE), Université Paul Verlaine—Metz, CNRS UMR 7146, Campus Bridoux, Avenue du Général Delestraint, 57070 Metz, France*

[5] *Corresponding author (maunoury@univ-metz.fr)*

[6] *Plateforme Métabolisme-Métabolome IFR87, Batiment 630, Institut de Biotechnologie des Plantes, Université Paris-Sud 11, 91405 Orsay CEDEX, France*

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Summary The present study examines the impact of the C source (reserves vs current assimilates) on tree C isotope signals and stem growth, using experimental girdling to stop the supply of C from leaves to stem. Two-year-old sessile oaks (*Quercus petraea*) were girdled at three different phenological periods during the leafy period: during early wood growth (Girdling Period 1), during late wood growth (Girdling Period 2) and just after growth cessation (Girdling Period 3). The measured variables included stem respiration rates, stem radial increment, $\delta^{13}C$ of respired CO_2 and contents of starch and water-soluble fraction in stems (below the girdle) and leaves. Girdling stopped growth, even early in the growing season, leading to a decrease in stem CO_2 efflux (CO_{2R}). Shift in substrate use from recently fixed carbohydrate to reserves (i.e., starch) induced ^{13}C enrichment of CO_2 respired by stem. However, change in substrate type was insufficient to explain alone all the observed CO_{2R} $\delta^{13}C$ variations, especially at the period corresponding to large growth rate of control trees. The below-girdle mass balance suggested that, during girdling periods, stem C was invested in metabolic pathways other than respiration and stem growth. After Girdling Period 1, the girdle healed and the effects of girdling on stem respiration were reversed. Stem growth restarted and total radial increment was similar to the control one, indicating that growth can be delayed when a stress event occurs early in the growth period. Concerning tree ring, seasonal shift in substrate use from reserves (i.e., starch) to recently fixed carbohydrate is sufficient to explain the observed ^{13}C depletion of tree ring during the early wood growth. However, the inter-tree intra-ring $\delta^{13}C$ variability needs to be resolved in order to improve the interpretation of intra-seasonal ring signals in terms of climatic or ecophysiological information. This study highlighted, via carbohydrate availability effects, the importance of the characterization of stem metabolic pathways for a complete understanding of the $\delta^{13}C$ signals.

Keywords: carbon isotope composition, leaf assimilates, reserve, Quercus petraea, stem respiration, tree ring.

Introduction

Photosynthesis discriminates against ^{13}C and plant organic matter is ^{13}C depleted by, on average, 20‰ compared with atmospheric CO_2 in C_3 plants (Farquhar et al. 1989). Carbon isotope discrimination during photosynthesis is now well understood and has been described by Farquhar et al. (1982, 1989). Changes in plant isotopic signals [e.g., total organic matter (TOM), tree ring or respired CO_2] are, therefore, mainly explained by variations in these discrimination steps. However, a ^{13}C enrichment is generally observed in non-photosynthetic tissues compared with leaves (e.g., Terwilliger and Huang 1996, Brugnoli and Farquhar 2000, Badeck et al. 2005), suggesting that discrimination during photosynthesis is not the only process that explains isotopic signals ($\delta^{13}C$) at the scale of different plant tissues. Various hypotheses have been proposed to explain the ^{13}C enrichment of non-photosynthetic tissues (for reviews, see Badeck et al. 2005 and Cernusak et al. 2009). Apart from biochemical composition (Eglin et al. 2008, Cernusak et al. 2009), possible non-photosynthetic discriminations concern carbon transfer, fixation and catabolism. Possible discrimination during transport of sugars via phloem was discussed in previous studies (Damesin and Lelarge 2003, Brandes et al. 2006, Gessler et al.

2004, 2009*b*). PEP carboxylase, whose activity is higher in non-photosynthetic tissues (particularly in twigs) than in leaves (Berveiller and Damesin 2008, Gessler et al. 2009*a*), is known to discriminate against ^{12}C during carbon fixation (Cernusak et al. 2009). The respiration process is likely to be also an important mechanism responsible for non-photosynthetic tissue $\delta^{13}C$. Actually, in leaves, respiration releases ^{13}C-enriched CO_2 leading to ^{13}C depletion of leaf tissue (Duranceau et al. 1999, Ghashghaie et al. 2003, Tcherkez et al. 2003, Gessler et al. 2009*a*). However, this would not be the case for non-photosynthetic tissues since CO_2 respired by roots has shown either no enrichment (Klump et al. 2005) or even ^{13}C depletion (Badeck et al. 2005, Bathellier et al. 2008) compared with the respiratory substrate pool. This difference between $\delta^{13}C$ of leaf- and root-respired CO_2 could partly explain the difference observed in the organic matter of the two organs (Bathellier et al. 2009). Inconsistent with these results for root of herbaceous species, woody organs (trunk and twigs) generally produce ^{13}C-enriched CO_2 relative to TOM or respiratory substrates (Damesin and Lelarge 2003, Gessler et al. 2007, Maunoury et al. 2007), suggesting that the influence of discrimination during respiration does not explain the ^{13}C enrichment between leaf and these non-photosynthetic organs. Moreover, the impact of respiration on total-biomass $\delta^{13}C$ is likely to vary during the year according to substrate availability and respiratory rate. The $\delta^{13}C$ of respired CO_2 could be altered by changes in the relative amounts of C diverted to either the decarboxylation of pyruvate during glycolysis (^{13}C enriched because of non-homogeneous C isotope distribution in the glucose molecule) or decarboxylation in the Krebs cycle (^{13}C depleted) (Tcherkez et al. 2003, 2004). More studies are still needed to determine the potential contribution of these processes in the determination of whole-tissue $\delta^{13}C$ (Cernusak et al. 2009).

In trees, carbohydrate storage (i.e., mainly starch in oaks, Barbaroux and Bréda 2002) and remobilization may also strongly influence the carbon isotope signals of sugars exported from leaves (Brandes et al. 2006, Gessler et al. 2008), tree organic matter (Damesin and Lelarge 2003, Eglin et al. 2009) and respired CO_2 (Eglin et al. 2009). Starch is known to be generally ^{13}C enriched in comparison with photosynthetic sugars (Brugnoli et al. 1988, Tcherkez et al. 2004). When glucose derived from starch hydrolysis is used as a substrate for respiration or for tree growth, respired CO_2 or TOM should be ^{13}C enriched compared with respired CO_2 or TOM built from new leaf assimilates. For example, part of the annual radial stem growth of ring-porous trees (including a major part of the early wood) occurs before leaves are mature and able to supply C to other tree parts (Dougherty et al. 1979, Hinckley and Lassoie 1981, Lachaud and Mansouri 1993, Breda and Granier 1996, Barbaroux and Bréda 2002). Early wood is, therefore, assumed to be built using stored starch from the previous year (Helle and Schleser 2004, Weigl et al. 2008). Thus, due to ^{13}C enrichment in starch compared with soluble sugars in woody parts (Damesin and Lelarge 2003), reserve use should significantly impact early wood $\delta^{13}C$. Moreover, several studies have demonstrated that, during biotic or abiotic stresses, trees can rely on reserves as a complementary C source (Daudet et al. 2005, Smith and Stitt 2007). When a lack of current assimilates occurs at the tree level, reserve use may have an important role in controlling the carbon isotope composition of tree ring organic matter and respired carbon dioxide. Previous studies assumed that ^{13}C enrichment observed in TOM (Damesin and Lelarge 2003) or in CO_2 respired by the stem (Maunoury et al. 2007), when leaves were not functional, was due to reserve use.

The aim of this study was to characterize the impact of a change in the C source (reserves vs recent assimilates) used for stem functioning on the $\delta^{13}C$ of stem organic matter and respired CO_2. We hypothesized that, when a lack of assimilates occurs at the stem scale, the use of reserves leads to the ^{13}C enrichment of both TOM (i.e., current ring) and respired CO_2. To test this hypothesis, we carried out a girdling experiment on 2-year-old oaks (*Quercus petraea*) that consisted in removing the bark and phloem all around the stem, thus causing phloem sap flow to cease. In this way, the stem was forced to use its own C reserves to maintain its metabolic activity after the application of girdling.

As the impact of reserve use and respiration process on $\delta^{13}C$ signals is likely to vary throughout the year, trees were girdled, following Jordan and Habib (1996), at three different periods:

(i) just after budburst, when leaves were not mature and radial growth had just begun;
(ii) during stem growth, when leaves were totally mature and represented the main C source for the other tree organs;
(iii) just after the cessation of stem radial growth, when the stem was no longer a C sink.

Stem radial growth and respiration rate were measured throughout the year. In parallel, carbohydrate contents and carbon isotope composition ($\delta^{13}C$) of carbohydrates and of respired CO_2 were examined for both stems (below the girdle) and leaves. Leaf measurements were not studied per se but provided information about assimilate source tissue. At the end of the leafy period, intra-annual ring $\delta^{13}C$ variations were determined. Furthermore, a C mass balance was built for the part of the stem located below the girdle, in order to provide information about carbohydrate use (quantity and $\delta^{13}C$) during the girdling period.

Materials and methods

Experimental design

Experiments were conducted on 2-year-old sessile oaks (*Q. petraea* (Matt.) Liebl.) from March to December 2006. Trees were planted during December 2005 in an open field at the University of Paris XI (+48°42′7.15″, +2°10′13.92″,

Figure 1. Girdle (A) at the application date and (B) after several weeks (5 weeks for Girdling Period 1, 8 weeks for Girdling Period 2) when stem tissues had regenerated.

25 km southeast of Paris, France, at an elevation of 65 m) in 90-L pots (53 cm height, 49 cm diameter) containing a sand–compost mixture (1:1). A randomized design with trees at 1-m spacing was established. In 2006, budburst occurred from 10 April to 10 May. The average annual temperature and annual rainfall were 11.43 °C and 848.76 mm, respectively, in 2006. All of the trees were watered once per week (up to field capacity) and sprayed twice in the year (23 June and 13 July) with pesticides targeting aphids, mealybugs and oidium (Decis®: deltamethrin, Vertimec®: abamectin, Systane®: myclobutanil).

Trees were girdled at three periods of ring growth (Girdling Period 1, during early wood growth: 9 May; Girdling Period 2, during late wood growth: 30 June; and Girdling Period 3, just after growth stopped: 1 September) by removing 2 cm of phloem all around the stem at 30 cm above the root collar (Figure 1A). Several weeks after the first two girdling dates (5 weeks for Girdling Period 1 and 8 weeks for Girdling Period 2), stem tissues had regenerated (Figure 1B) and the phloem sap flow restarted. Tissue healing was visually determined. Such tissue regeneration was not observed for Girdling Period 3. It is important to distinguish the 'girdling date' from the 'girdling period'. The former indicates the precise date of girdling application and the latter is the entire period during which phloem tissues were missing. Girdle healing ended the 'girdling period'.

Respiration rate and CO_2 $\delta^{13}C$ of stem and leaves were monitored on three trees from both control and girdled groups. For destructive measurements (carbohydrate analyses and ring collection), four sampling campaigns (Figure 2) were performed (in June, August, October and December). For the three first campaigns, at the end of each girdling period (June, August and October), three control trees and three trees from the current girdled group were sacrificed. For Girdling Periods 1 and 2, these sampling dates corresponded to the end of girdling periods. For Girdling Period 3, there was no phloem healing and, therefore, no 'end of the girdling period', so the sampling date was arbitrarily fixed at 7 weeks after the girdling date (October).

In order to compare girdled trees to the control trees during dormancy and to sample current-year rings, three control trees and three trees from each girdled group were sacrificed during a fourth sampling campaign (December).

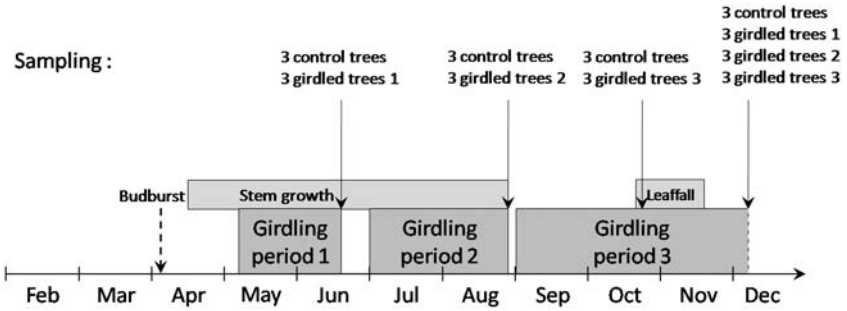

Figure 2. Experimental design and sampling date timetable for destructive analysis (stem biochemical analyses and ring collection). Girdling periods (i.e., periods during which phloem tissues were missing) are indicated in grey. Non-destructive measurements (respiration rate and $\delta^{13}C$ of leaves and stem) were performed on three control and girdled trees throughout the year. For each girdling, three other control and girdled trees were monitored from the sampling date until December.

Stem growth and leaf mass per area (LMA) measurements, which were less time consuming, were performed on the same groups but on more than three trees from each group, ranging from three to seven trees per group.

LMA and stem diameter

To measure LMA, one leaf from each tree was collected once per week. Leaf area was measured using an area meter (Delta-T Devices, UK). Leaf dry weight was determined after dehydration for 48 h in a 60 °C oven. For each of the 41 trees, stem diameter (in millimetres) was measured at 20 cm above the collar with an electronic calliper, in two radial directions, twice per week. For girdled trees, stem measurements were made below the girdle.

Stem CO_2 efflux rate and CO_2 sampling

For stem CO_2 efflux measurements, the system described by Damesin et al. (2005) and Maunoury et al. (2007) was adapted to young stems. Once per week, the CO_2 efflux of the stems (of three control trees and three trees of the current girdling group) was measured using home-made Plexiglas chambers (10 cm height and 4.5 cm diameter, 0.2 L). We denoted this efflux as 'stem respiration', keeping in mind that the CO_2 released in the chamber could also come from CO_2 transported by the xylem, as previously suggested (Teskey and McGuire 2002). Stem respiration was measured at ambient temperature using a closed dynamic system. Chambers were fixed on the stem, at 20 cm above the collar and below the girdle, with malleable rubber sealant (Terostat-7; Teroson, Ludwisburg, Germany) and elastic bands. The chambers were connected to an infra-red gas analyser (IRGA, EGM4; PPSystems, Hitchin, UK) by two independent flexible tubes 40 cm in length (Excelon BEV-A LINE IV, a polycarbonate tube with inert liner; Thermoplastic Processes, Stirling, NJ). The CO_2 increase was checked for linearity over a period of 2 min by taking an intermediate value of CO_2 inside the chamber at the end of the first minute. Three repetitions were performed every time. From these measurements, respiration rates were calculated and expressed per unit volume of stem (at this age, all parts of the stem have living cells and are capable of contributing to stem respiration). The chamber was removed after each series of measurements and reinstalled at the same place for each campaign. Twice a month, after respiration measurements, the outlet and inlet pipes were linked from the chamber to a flask and the entire system was purged with nitrogen for 10 min until the CO_2 concentration inside the chamber dropped to 10–20 μmol mol^{-1}. A pump was added into this closed system, which allowed the CO_2 respired by the stem to accumulate homogeneously in the chamber and flask. The N_2-filled flask accumulated 700–800 μmol mol^{-1} of CO_2 diffusing from the stem. At the stem level, the N_2 flush method has been previously demonstrated to yield the same results as the Keeling plot method (Damesin et al. 2005).

Simultaneously, leaf-respired CO_2 (of the same six trees) was sampled and analysed with a method close to the 'in-tube incubation method' described by Werner et al. (2007). Just after collection, entire leaves were placed inside 50-ml valved syringes (SGE, Australia) previously flushed with pure nitrogen (N_2) for at least 1 min after inserting the leaf. The leaves were left to respire in the dark, by placing the syringes in a black box, at ambient temperature. An incubation time of 10 min was required to collect sufficiently respired CO_2. Tests of incubations using CO_2 free air were performed, and no significant differences in $\delta^{13}C$ were observed compared with incubations using N_2 ($n = 44$; $P = 0.72$).

On the same day, gas samples contained in flasks (stem-respired CO_2) and syringes (leaf-respired CO_2) were analysed in the laboratory with a mass spectrometer (see the section 'Isotopic analysis' below) to determine the isotopic signal of CO_2 respired ($\delta^{13}C_R$) by the leaves and stem.

Wood sampling and biochemical extraction

At the end of each girdling period, tissues (leaves and stem wood) of three trees of the current girdled group and five trees of the control group were sampled (at the level of respiration measurements for stems) and directly placed at −20 °C before being lyophilized and then finely ground in a ball mill (Type MM200; Retsch, Haan, Germany). The remaining three trees of each girdled group were sampled on the last sampling date in December (in order to compare with the results obtained at the end of each girdling period).

Carbohydrate contents and their carbon isotope signal were determined in the leaves and stem wood (considering all the rings). Procedures from Duranceau et al. (1999) were used for starch and water-soluble fraction extraction. From 50 mg of powdered tissue, the water-soluble fraction (containing soluble sugars) was extracted at 4 °C, with 1 ml of fresh water in a 2-ml Eppendorf microfuge tube. The soluble fraction was heated to 100 °C for 3 min to denature all proteins of the extract. After centrifugation (5 min, 5 °C, 12,000 *g*), the supernatant was frozen. The extract was then filtered through a 0.45-μm filter (HV; Nihon Millipore Kogyo K.K., Japan) and transferred to a tin capsule (Courtage Analyse Service, Mont Saint-Aignan, France). Each capsule containing liquid samples was dried (for 48 h at 60 °C), then weighed in order to obtain the soluble fraction quantity and analysed to determine $\delta^{13}C$.

Starch was extracted from the pellet by HCl solubilization (in 2 ml of 6 M HCl). Tubes were incubated at 5 °C for 1 h. After centrifugation (20 min, 5 °C, 12,000 *g*), the supernatant was retained in 50-ml tubes and the residue was again suspended in HCl. Pure methanol was added to the supernatant (containing HCl-solubilized starch) and incubated at 5 °C overnight to precipitate the starch. The starch powder, obtained after centrifugation and lyophilization of the new pellet, was weighed prior to transfer to a tin capsule for carbon isotopic analysis.

$\delta^{13}C$ of intra-ring total organic matter

In December, current-year rings of three girdled trees and three control trees were isolated, lyophilized and fixed with Tissue-Tek embedding medium (Sakura Finetek, Tokyo, Japan) on a mobile support, allowing sample adjustment with the blade. Rings were cut into 60-µm-thick sections using a cryostat (Leica CM3050 S; Leica Microsystems, Bensheim, Germany). Sections were cleaned (with seven rinses of water), dried (50 °C for 48 h) and weighed before being transferred to tin capsules for carbon isotopic analysis. Ring borders and the boundary between early and late wood were determined with a binocular magnifier. The length of the growth period corresponding to each section was estimated using stem increment data.

Isotopic analysis

Gas samples were injected into the elemental analyser (Model NA-1500; Carlo Erba, Milan, Italy) through a 15-ml loop, which is a system derived by Tcherkez et al. (2003). The various gases were separated (mainly N_2/O_2 and CO_2) and the connection valve between the elemental analyser and the isotope ratio mass spectrometer (IRMS; VG Optima, Fison, Villeurbanne, France) was opened when the CO_2 peak emerged from the gas chromatograph column of the elemental analyser.

The solid samples of water-soluble fraction, starch and organic matter were analysed with the same elemental analyser and IRMS. Gases resulting from combustion were separated, and the CO_2 peak was sent to the IRMS. Isotope composition was calculated from the sample isotopic ratio $^{13}C/^{12}C$ (R_s) measured by the IRMS and is expressed as the conventional delta (in ‰) according to the relationship:

$$\delta^{13}C = \frac{(R_s - R_{VPDB})}{R_{VPDB}} \quad 10^3$$

where VPDB (Vienna Pee Dee Belemnite) is the international standard. According to the quality assessment of the analysis platform, a laboratory working standard (i.e., −28.06‰ glutamic acid for organic matter samples and air with 500 µmol mol^{-1} of −53.1‰ CO_2 for air samples) was measured after each group of 12 samples in order to correct for any offset of the IRMS. The precision for isotopic measurements was ±0.2‰, based on repeated measurements of the laboratory working standards.

Isotopic mass balance

At the end of each girdling period, a mass balance was established for the stem located below the girdle. The stem portion inside the stem chamber was considered. Reserve supply was assumed to be local. Measurements of the starch and soluble fractions of the controls (Table 1) were used as the pre-girdle values and the measurements at the end of the girdling period (i.e., girdled-tree values; Table 1) were considered as the post-girdle values. This provided an estimate of the quantity of C consumed during the girdling period (i.e., missing substrate). The mean $\delta^{13}C$ of this missing substrate was estimated from an isotopic mass balance by multiplying the previously calculated decreases in substrate (starch and soluble fraction) by their respective isotopic compositions (i.e., girdled-tree values; Table 2).

With these calculations, two assumptions were tested:

(1) Considering that the girdled trees are not growing (see the 'Results' section), no substrate should be disappearing into new biomass and substrate should be completely consumed by stem respiration. Therefore, the quantity of missing substrate should be in agreement with the measured respiration rates.
(2) Considering that all the missing substrate was used for stem respiration, one might expect the $\delta^{13}C$ of respired CO_2 to match that of the missing substrate.

Statistical analysis

At the end of each girdling period, *t*-tests (R 2.9.1; R Development Core Team, 2005) were performed to compare treatment vs control values of carbohydrate contents and $\delta^{13}C$, LMA, stem diameter, respiration rate and respired CO_2 $\delta^{13}C$. In December, ANOVA and post hoc tests (Tukey HSD) were used to compare values between the three

Table 1. Change in water-soluble fraction (SF) and starch (St) contents in leaf and stem after each girdling period: 1 (June), 2 (August) and 3 (October) and after all girdling periods (December). Values are expressed as percent of dry weight (n = 3–5) ± standard error. The asterisk indicates results significantly different from the control value ($P \leq 0.05$). nd, not determined.

		June		Aug		Oct		Dec
		% SF	% St	% SF	% St	% SF	% St	% St
Leaf	Girdling period 1	24.9 ± 2.4	2.6 ± 2.0	nd	nd	nd	nd	nd
	Girdling period 2	nd	nd	21.1 ± 2.3	6.9 ± 1.8*	nd	nd	nd
	Girdling period 3	nd	nd	nd	nd	24.9 ± 8.0	1.7 ± 1.1	nd
	Control	21.9 ± 1.8	3.6 ± 0.2	22.6 ± 0.1	2.5 ± 0.9	24.3 ± 1.9	2.2 ± 0.7	nd
Stem	Girdling period 1	5.9 ± 1.1*	1.9 ± 0.1*	nd	nd	nd	nd	6.0 ± 1.3
	Girdling period 2	nd	nd	6.1	3.6 ± 0.6*	nd	nd	4.8 ± 1.6
	Girdling period 3	nd	nd	nd	nd	5.0 ± 0.6*	5.2 ± 0.7*	2.7 ± 0.5
	Control	9.7 ± 2.2	6.1 ± 0.9	9.6 ± 1.1	6.6 ± 0.5	9.7 ± 2.0	7.1 ± 0.3	4.0 ± 0.5

Table 2. $\delta^{13}C$ changes in water-soluble fraction (SF), starch (St) and TOM in leaf and stem measured after the three girdling periods: 1 (June), 2 (August) and 3 (October); a fourth measurement was taken at the end of the year (December). Values represent the mean ($n = 3–5$) ± standard error. The bold letters indicate the results of statistical analyses for trunk or leaf. Each date was independently analysed. The significance level was set at 5%. nd, not determined.

		June			Aug			Oct			Dec	
		$\delta^{13}C$ SF	$\delta^{13}C$ St	$\delta^{13}C$ TOM	$\delta^{13}C$ SF	$\delta^{13}C$ St	$\delta^{13}C$ TOM	$\delta^{13}C$ SF	$\delta^{13}C$ St	$\delta^{13}C$ TOM	$\delta^{13}C$ St	$\delta^{13}C$ TOM
Leaf	Girdling period 1	−26.6 ± 0.3**b**	−24.9 ± 1.3**a**	−27.0 ± 0.3**b**	nd	nd	nd	nd	nd	nd	nd	nd
	Girdling period 2	nd	nd	nd	−27.1 ± 0.1**c**	−22.6 ± 0.4**a**	−27.0 ± 0.4**c**	nd	nd	nd	nd	nd
	Girdling period 3	nd	nd	nd	nd	nd	nd	−26.6 ± 0.1**bc**	−25.3 ± 0.7**ab**	−27.4 ± 0.1**cd**	nd	nd
	Control	−26.7 ± 0.4**b**	−23.9 ± 0.3**a**	−26.8 ± 0.4**b**	−26.6 ± 0.4**c**	−23.9 ± 0.8**b**	−26.6 ± 0.6**c**	−28.0 ± 0.3**cd**	−25.1 ± 0.3**a**	−28.2 ± 0.2**d**	nd	nd
Stem	Girdling period 1	−22.3 ± 0.8**a**	−22.0 ± 0.4**a**	−22.4 ± 0.7**a**	nd	nd	nd	nd	nd	nd	−22.7 ± 0.6**a**	−24.2 ± 0.5**a**
	Girdling period 2	nd	nd	nd	−23.9 ± 0.1**bc**	−23.3 ± 0.1**ab**	−24.0 ± 0.3**bc**	nd	nd	nd	−22.7 ± 0.1**a**	−24.2 ± 0.4**a**
	Girdling period 3	nd	nd	nd	nd	nd	nd	−24.1 ± 0.4**ab**	−23.2 ± 0.5**a**	−24.8 ± 0.4**b**	−23.3 ± 0.4**a**	−24.7 ± 0.4**a**
	Control	−23.6 ± 0.2**b**	−22.6 ± 0.2**a**	−23.9 ± 0.2**b**	−24.0 ± 0.5**bc**	−22.9 ± 0.3**a**	−24.6 ± 0.2**c**	−24.5 ± 0.3**b**	−23.3 ± 0.3**a**	−25.0 ± 0.1**b**	−22.9 ± 0.4**a**	−24.7 ± 0.4**a**

'long-term girdled groups' and the control group. The significance level was set at 5%.

Results

LMA and stem growth

For control trees, LMA (Figure 3A) decreased in very young leaves from 82.2–50.1 gDM m^{-2} (end of April) and increased during leaf maturation to 103.6 gDM m^{-2} (at the beginning of July). Girdling treatments significantly increased LMA for Girdling Period 1 ($P \leq 0.079$, at the end of Girdling Period 1) and for Girdling Period 3 ($P \leq 0.003$, at the end of Girdling Period 3) compared with controls (Figure 3A). After each girdling period, the LMA of girdled trees returned close to control values and were not significantly different from control values at the last LMA sampling (Day 312, $P \leq 0.927$ for Girdling Period 1, $P \leq 0.897$ for Girdling Period 2 and $P \leq 0.222$ for Girdling Period 3). Girdling Period 1 occurred just after the budburst period and did not alter the leaf growth period. Indeed, whatever the girdling period, there was no significant difference in leaf area between girdled trees and control trees ($P \leq 1$, $P \leq 1$ and $P \leq 0.88$ for Girdling Periods 1, 2 and 3; data not shown).

The annual stem diameter increment was, on average, 5.5 ± 0.2 mm for control trees (Figure 4A). After the girdling date, diameter growth totally stopped during Girdling Periods 1 and 2 (Figure 4A) and no change was noticed after Girdling Period 3 ($P \leq 0.314$, 7 weeks after the girdling date) because stem diameter also stopped increasing in controls. As previously explained (see the 'Materials and methods' section), the girdled section healed and the phloem sap flow restarted after Girdling Periods 1 and 2. For Girdling Period 1, stem growth restarted 3 days after girdle healing and reached the control total increment ($P \leq 0.549$ at the end of the growth season). In contrast, stem growth did not restart after Girdling Period 2 and the total stem increment was reduced, on average, by 15.3% relative to control trees ($P \leq 0.058$ at the end of the growth season).

Stem respiration rate

The stem respiration rate of control trees increased from 44 to 1957 μmol CO_2 m^{-3} s^{-1} between April and July (Figure 4B). It then progressively decreased after the growth period and reached 16 μmol CO_2 m^{-3} s^{-1} in December. With girdling treatments, the stem respiration rate was reduced compared with controls (Figure 4B) and was −78.8% (i.e., −1542.6 μmol CO_2 m^{-3} s^{-1}; $P \leq 0.001$) and −31.7% (i.e., −92.7 μmol CO_2 m^{-3} s^{-1}; $P \leq 0.001$) lower than controls at the end of Girdling Periods 1 and 2, respectively. After girdle healing (Girdling Periods 1 and 2), the respiration rate increased and became higher than the controls by 78.9% (i.e., +761.7 μmol CO_2 m^{-3} s^{-1}; $P \leq 0.001$) for Girdling Period 1 and by

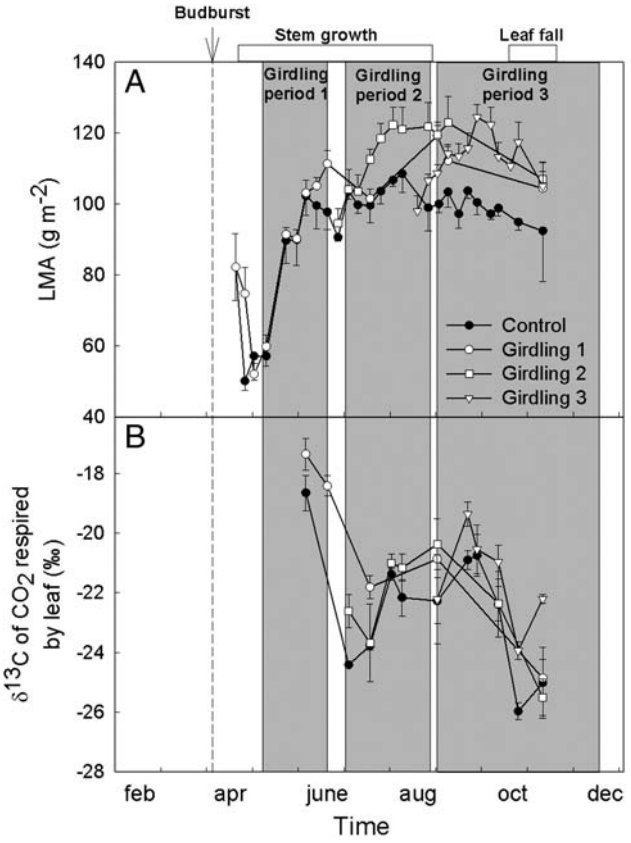

Figure 3. Seasonal change in (A) LMA ($3 < n < 7$) and (B) in $\delta^{13}C$ of CO_2 respired by leaves ($n = 3$) for Girdled Trees 1 (open circles), 2 (open squares) and 3 (open triangles) in comparison with controls (filled circles). Girdling periods are indicated in grey. Error bars represent standard errors of the mean.

305.8% (i.e., +195.8 μmol CO_2 m^{-3} s^{-1}; $P \leq 0.035$) for Girdling Period 2, 1 month after girdle healing (Figure 4B). After Girdling Date 3, the stem respiration rate of girdled trees remained close to that of the controls.

Carbohydrate content and $\delta^{13}C$

For leaves, after Girdling Periods 1 and 3, carbohydrate contents did not differ significantly from controls (Table 1). However, after Girdling Period 2 (in August), starch content was significantly higher in girdled-tree leaves than in control leaves ($P \leq 0.010$). Except after Girdling Period 3, leaf starch was significantly ^{13}C enriched compared with soluble fraction (by, on average, 2.7‰; Table 2). Except for starch $\delta^{13}C$ in Girdled Trees 2, the $\delta^{13}C$ of carbohydrates and TOM were not significantly different between girdled and control leaves.

At the end of each girdling period, starch and soluble fraction contents of girdled stems (Table 1) were significantly lower than that of control stems (except for soluble fraction content at the end of Girdling Period 2). In December, the starch content of girdled stems was not significantly different from that in control stems. Each studied fraction had a higher $\delta^{13}C$ in stems than in leaves (Table 2). For control

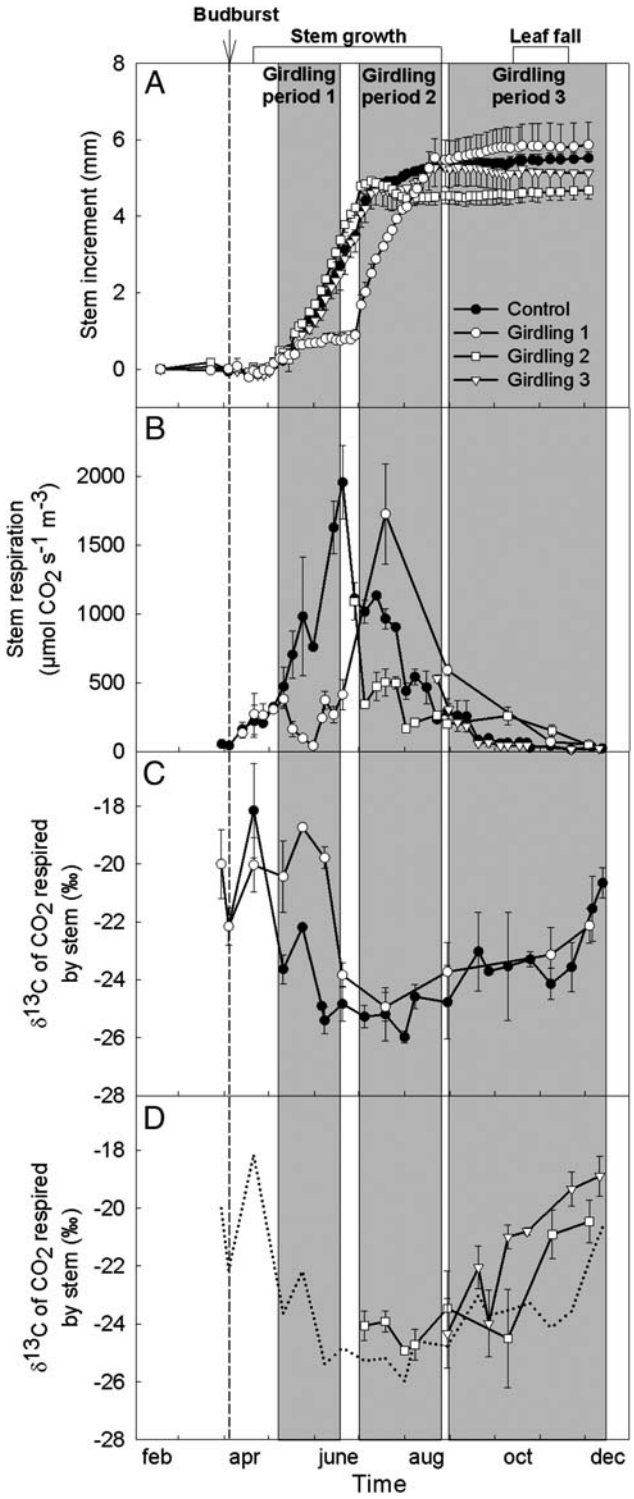

Figure 4. Seasonal change (A) in stem diameter increment ($3 < n < 7$), (B) in stem respiration rate (in micromoles of CO_2 per cubic metre of living tissue per second) and in $\delta^{13}C$ of CO_2 respired by stem (C) of Controls and Girdled Trees 1 and (D) of Girdled Trees 2 and 3 during Girdling Periods 1 (open circles), 2 (open squares) and 3 (open triangles) in comparison with controls (filled circles and dotted line; $n = 3$). Girdling periods are indicated in grey. Error bars represent standard errors of the mean.

Table 3. Results of C and isotopic mass balances built for the stem below the girdles. Carbon mass balance includes calculated missing substrate (i.e., quantity of C consumed during girdling period), measured C loss by respiration during the same period and percentage of the missing substrate used for maintenance respiration. Isotopic mass balance presents the calculated $\delta^{13}C$ of the missing substrate to be compared with the measured $\delta^{13}C$ of respired CO_2.

	C mass balance			Isotopic mass balance	
Girdling	Missing substrate, kg C m^{-3} (calculated)	Respired C, kg C m^{-3} (measured)	C lost by respiration, %	Missing substrate $\delta^{13}C$, ‰ (calculated)	Respired CO_2 $\delta^{13}C$, ‰ (measured)
1	27.6	11.1	40.1	−22.1	−20.7
2	30.7	19.5	63.6	−23.6	−24.4
3	30.2	6.2	20.4	−23.8	−22.4

groups in June, August and October, the soluble fraction was significantly ^{13}C depleted compared with starch. In contrast, there was no significant difference between starch and soluble fractions $\delta^{13}C$ at the end of each girdling period and for all groups in December.

$\delta^{13}C$ of respired CO_2 (CO_{2R} $\delta^{13}C$)

For the control, the $\delta^{13}C$ of CO_2 respired by leaves varied over the year (Figure 3B) and showed values becoming enriched from July (−24.4‰) to September (−20.8‰) and then progressively depleted until December. Leaf CO_{2R} $\delta^{13}C$ was significantly ^{13}C enriched over the Girdling Periods 1 ($P \leq 0.006$ at the end of Girdling Period 1) and 3 ($P \leq 0.001$ at the end of Girdling Period 3), by, on average, 1.3 and 1.6‰, respectively, in comparison with the controls at the same periods (Figure 3B).

For the control, stem CO_{2R} $\delta^{13}C$ varied over the year (Figure 4C) in the same range as leaf CO_{2R} $\delta^{13}C$, between −18.2‰ (in April) and −26.0‰ (in August). A relative maximum occurred in mid-April, at the beginning of the growth period. After this time, stem CO_{2R} $\delta^{13}C$ decreased and remained low throughout the growth period (until the end of August); it then progressively increased, reaching a second relative maximum (−20.7‰) in early winter (at the beginning of December). Stem CO_{2R} $\delta^{13}C$ was significantly ^{13}C enriched in comparison with the controls (Figure 4C and D), for Girdling Period 1 ($P \leq 0.001$) and Girdling Period 3 ($P \leq 0.049$) by, on average, 3.3 and 2.0‰, respectively. After these girdling periods, stem CO_{2R} $\delta^{13}C$ was not different from the controls. After Girdling Date 2, no significant difference in respired CO_2 $\delta^{13}C$ was observed between girdled trees and control trees.

Isotopic mass balance

Results are summarized in Table 3 and show that maintenance respiration used 40.1, 63.6 and 20.4% of all C consumed by the stem located below the girdle, inside the measurement chamber, during Girdling Periods 1, 2 and 3, respectively. Considering that the girdled trees were not growing, this suggested that a large part of the substrate pool (59.9, 36.4 and 79.6%) was invested in metabolic pathways other than respiration and stem growth. Moreover, the calculated $\delta^{13}C$ for the missing substrate did not match the measured ones for stem-respired CO_2, suggesting that this calculated $\delta^{13}C$ corresponded to a mix between respiratory substrates and substrates invested in other metabolic pathways and/or that discrimination during respiration led to a ^{13}C enrichment of released CO_2, which was not negligible at the stem scale.

Tree ring $\delta^{13}C$

The tree ring $\delta^{13}C$ intra-seasonal patterns of both control and girdled trees (Figure 5B and C) exhibited large variations (up to 2.5‰ for Control Tree 3). For all studied tree rings, TOM $\delta^{13}C$ was maximal at the beginning of growth and showed a large decrease thereafter. The maximal value was extremely variable between trees (from −22.11‰ for Girdled Tree 1 to −25.78‰ for Control Tree 1). For Control and Girdled Trees 2, the decrease in tree ring $\delta^{13}C$ mainly occurred during the early growth season (on average, −1.26‰ in 26 days). Considering all of the six trees, ring $\delta^{13}C$ dynamics exhibited higher inter-tree variability in late wood than in early wood.

For Girdled Tree 1, the tree ring $\delta^{13}C$ decrease was delayed to July, when growth had restarted, since girdling stopped radial trunk growth (Figure 5A). The tree ring $\delta^{13}C$ of Girdled Tree 1, therefore, did not vary during the girdling period. After Girdling Period 1, tree ring $\delta^{13}C$ was still near the initial maximal value (Figure 5B); it then decreased rapidly to a minimum (−2.81‰ in 39 days).

Discussion

Effect of substrate on $\delta^{13}C$ of respired CO_2 and tree ring organic matter

Decrease of soluble fraction and starch contents in stem during the girdling periods supports the idea that girdling stopped the sugar flux from the leaves to the parts below the girdle leading to starch use by stems. The observed ^{13}C enrichment of CO_2 respired by stem during Girdling Periods 1 and 3 was expected and is consistent with literature results about starch: (i) starch is enriched in ^{13}C compared with the TOM or soluble sugars (O'Leary 1981, Brugnoli et al. 1988, Damesin and Lelarge 2003, Xu et al. 2004, Tcherkez et al. 2004); (ii) no discrimination occurs during starch remobilization (Bathellier et al. 2008, Maunoury-Danger et al. 2009). The changes in substrate type (reserves vs newly formed

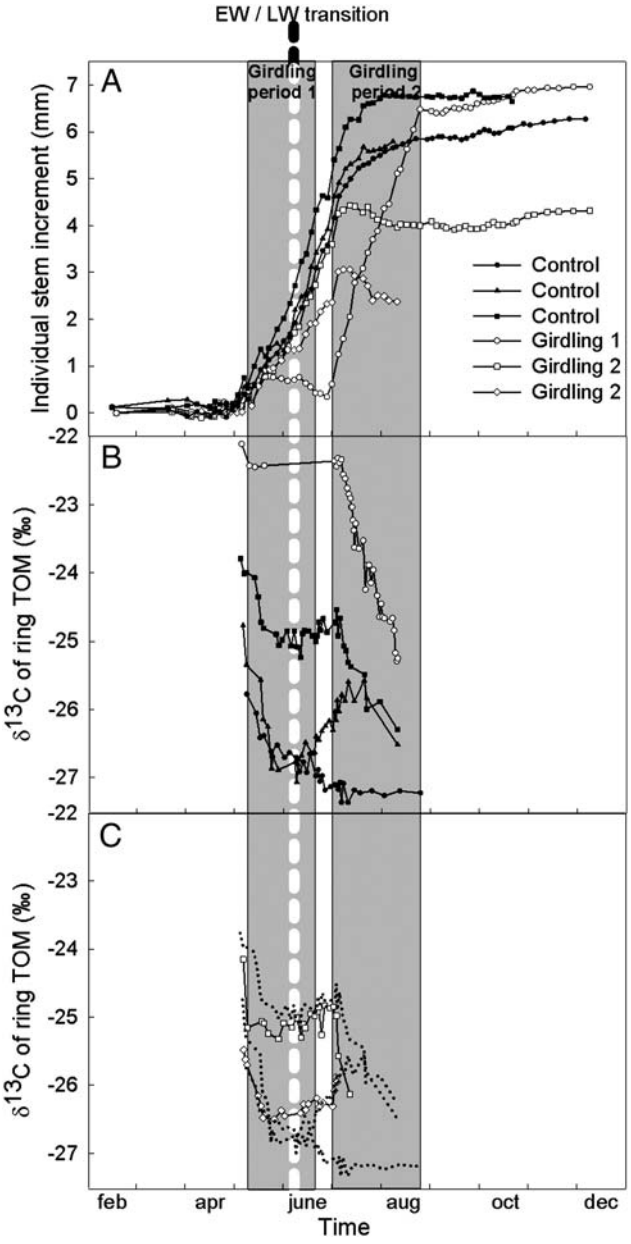

Figure 5. Seasonal change (A) in individual stem diameter increment and in $\delta^{13}C$ of individual ring TOM for (B) Control and Girdled Trees 1 and (C) Control (dotted lines) and Girdled Trees 2. Girdling periods are indicated in grey. EW, early wood; LW, late wood.

photosynthates) throughout the year allowed us to explain both the seasonal $\delta^{13}C$ patterns of stem-respired CO_2 (CO_{2R}), which were similar to patterns previously observed for adult sessile oaks (see Maunoury et al. 2007), and the beginning of an intra-ring $\delta^{13}C$ pattern. The progressive decrease in ring $\delta^{13}C$, simultaneously observed with the $\delta^{13}C$ decrease in stem CO_{2R} during stem growth, could, therefore, be explained (at least partly for stem CO_{2R} and completely for ring TOM) by the substrate shift from ^{13}C-enriched starch reserves to more depleted, recently assimilated sugars. Actually, the decrease in tree ring organic matter $\delta^{13}C$ ranged between 1 and 2‰ (see Figure 5B and C) and was consistent with the $\delta^{13}C$ difference between starch and soluble fractions in stems (~1.1‰; see Table 2).

During autumn, just prior to leaf senescence and after the growth period, leaves progressively lose their capacity for photosynthetic carbon assimilation and export to other tree organs. It can be assumed that, during this period, substrates available for stem function shift from photosynthetic sugars to starch, coinciding with the progressive increase in $\delta^{13}C$ of stem CO_{2R}, as was observed for control and all girdled groups. The increase in $\delta^{13}C$ of CO_2 respired by stems from Girdling Period 1 and control stems was delayed relative to Girdling Period 2 and even more delayed relative to Girdling Period 3 (Figure 4C and D). Because Girdling Periods 2 and 3 were not reversed (or was reversed late in the case of Girdling Period 2), it can be assumed that reserve filling (before leaf fall) was reduced and that the stem labile carbohydrate pool (i.e., soluble fraction) emptied out. For their respiration, stems from Girdling Periods 2 and 3, therefore, relied on their reserves earlier than Control and Girdled Trees 1 which is consistent with the lower—but not significant—December starch content for Girdled Stems 2 and 3 compared with Girdled Stems 1 and controls. These results highlight the role of substrate type (recent assimilates vs starch) influencing the $\delta^{13}C$ patterns of ring TOM and stem CO_{2R}.

As a consequence, in ring-porous species, early wood $\delta^{13}C$ is linked to starch $\delta^{13}C$ (i.e., to environmental conditions during reserve filling of the previous year), whereas late wood $\delta^{13}C$ is linked to newly assimilated sugars $\delta^{13}C$ and reflects recent photosynthetic discrimination associated with the environmental conditions of the current year (Farquhar et al. 1982, 1989). Therefore, one might expect that, the less starch that is used for ring growth, the better is the climatic or physiological information quality given by the ring $\delta^{13}C$ for the current year. In fact, several studies showed that ring parameters (ring width or ring $\delta^{13}C$) were more strongly correlated with current environmental parameters (like soil water content) for late wood than for early wood (Breda and Granier 1996, Raffali-Delerce et al. 2004, Weigl et al. 2008). Moreover, during late wood formation, inter-tree variability strongly increased, suggesting that photosynthetic discriminatory responses to the environment differed between trees (e.g., through stomatal conductance or stomatal density), as previously observed among six genotypes of *Quercus robur* (Roussel et al. 2009). This large inter-tree variability in late wood $\delta^{13}C$ was also observed in adult oaks (Michelot, personal communication). This result highlights the necessity of using a large number of samples of rings for climatic or ecophysiological intra-annual studies. Further studies are now needed to understand this inter-tree variability.

Implication of other post-photosynthetic processes in $\delta^{13}C$ of CO_{2R}

Changes in $\delta^{13}C$ of starch and soluble fraction in stems were ~1.1‰ (see Table 2), while seasonal changes in stem CO_{2R} $\delta^{13}C$ reached 7‰ (see Figure 4C). Additionally, the lack of increase in CO_{2R} $\delta^{13}C$ for Girdling Period 2 and the similar-

ity of $\delta^{13}C$ values of stem soluble fraction (assumed to be substrate for respiration) between control and girdled stems failed to support the hypothesis that the change of substrates was the only factor responsible for the ^{13}C enrichment of CO_2 by Girdled Stems 1 and 3. Other post-photosynthetic processes must, therefore, influence the CO_{2R} $\delta^{13}C$.

Alternative mechanisms, such as a change in metabolic pathways, could influence the CO_{2R} $\delta^{13}C$. CO_2 released by the decarboxylation of pyruvate during glycolysis is relatively enriched in ^{13}C; meanwhile CO_2 released in decarboxylation in the Krebs cycle is depleted in ^{13}C (Tcherkez et al. 2003) because of the non-random distribution of the glucose molecule (Tcherkez et al. 2004). However, after girdling, one could expect that most of the carbon should be used to support maintenance respiration and thus be directed through the Krebs cycle. Therefore, when respiration was reduced, the released CO_2 should have been ^{13}C depleted. The mass balance showed that C consumed during the girdling period was only partly used by maintenance respiration and, thus, only partly directed through the Krebs cycle. These results suggested that, even if girdled trees were not growing, 36.4 and 79.6% of the carbon was deviated before the Krebs cycle and invested in other metabolic pathways. The pyruvate dehydrogenase pathway, releasing relatively ^{13}C-enriched CO_2, was, therefore, involved.

After the girdling date, one might expect that the tree directed more C to secondary metabolism to produce compounds as a wounding response. Many studies showed changes in bark and xylem biochemical composition, particularly in phenolic and tannin contents, after bark wounding (Brignolas et al. 1995, Klepzig et al. 1995, Krekling et al. 2000). Such a wound also results in the accumulation of phenolic inclusions in ray parenchyma cells and activation of phloem parenchyma cells containing phenolics (Franceschi et al. 1998, 2000, Krekling et al. 2000, 2004). The aromatic amino acids phenylalanine and tyrosine are biosynthetic precursors of the major part of phenolic compounds and are ^{13}C depleted during their biosynthesis (Macko et al. 1987), leading to relatively ^{13}C-depleted phenolic compounds (Benner et al. 1987). The calculated $\delta^{13}C$ of the missing substrate suggested that C was invested in depleted compounds (relative to respiratory substrates) during girdling periods (1 and 3). Biosynthesis of phenol compounds may, therefore, be a good candidate as a C sink during the girdling period. Further studies are, however, needed in order to quantify C investment in phenolic compound production during girdling periods and to confirm its impact on the ^{13}C enrichment of the respired CO_2 by stem. These studies should explore whether C investment in the wounding response depends on the period of girdling application and/or if C was used by other metabolic pathways (e.g., in root functioning).

C accumulation in parts above the girdle

During girdling periods, phloem sap flow ceased and leaf sugars were not exported to stem and roots, resulting in a C accumulation in parts above the girdle, as previously observed (Iglesias et al. 2002, Daudet et al. 2005, Peuke et al. 2005, Wang et al. 2006, Maier et al. 2010). However, after each girdling period, leaf soluble fraction content did not differ significantly from the controls, suggesting that a sugar excess in leaves was used by an increase in respiration (Johnsen et al. 2007, Maier et al. 2010) or stored either in other carbon forms (different from soluble sugars) or in woody parts above the girdle.

In the case of Girdling Period 2, the significantly higher starch content in girdled-tree leaves observed after Girdling Period 2 (Table 1) suggests that the excess sugar was converted into starch. Moreover, the carbohydrate accumulation appeared to cause a source–sink imbalance in the leaves, leading to a reduction in photosynthesis (reduced by 94.7% compared with the control, 20 days after Girdling Date 2; data not shown) as previously observed (Iglesias et al. 2002, Johnsen et al. 2007, Urban and Alphonsout 2007). Two likely explanations previously proposed for this negative feedback on photosynthesis are either an excess of starch grains leading to physical damage to thylakoids (Schaffer et al. 1986) or inhibition of photosynthetic genes regulated by carbohydrate content (Paul and Foyer 2001).

During Girdling Periods 1 and 3, C excess should be allocated in other ways than starch storage, as suggested by the similar leaf starch content of control and girdled-tree leaves. In these cases, significant LMA increase could be attributable to an increase of C investment in the growing biomass (particularly during Girdling Period 1) or to C accumulation into other compounds implicated in leaf nitrogen remobilization, occurring in the same period as Girdling Period 3, during leaf senescence (Niinemets et al. 2004).

Impact of C accumulation on leaf $\delta^{13}C$ signals

For control trees, the $\delta^{13}C$ pattern of respired CO_2 $\delta^{13}C$ was different between leaves and stems (see Figures 3B and 4C). Contrary to the stems (Maunoury et al. 2007), many studies provided evidence for a large diurnal pattern in leaf CO_{2R} $\delta^{13}C$ (Hymus et al. 2005, Prater et al. 2005, Priault et al. 2009). Even if leaf measurements were all achieved during the morning, one might expect that our data set cannot be well interpreted at the seasonal scale because of the excessively large diurnal variability.

However, these leaf values suggested that C accumulation in the above-girdle parts led to the increase in leaf CO_{2R} $\delta^{13}C$ for Girdling Periods 1 and 3 (see Table 2 and Figure 2B) without any change in photosynthesis discrimination (no change in leaf carbohydrate $\delta^{13}C$). Increase in leaf CO_{2R} $\delta^{13}C$ could be due to the C accumulation in leaves, which could be compensated by the increase in respiration (Johnsen et al. 2007, Maier et al. 2010), leading to a stronger discrimination during respiration.

Stem growth period plasticity

Whatever the period during the growing season (during early wood or late wood formation), girdling led to a total growth

inhibition that is consistent with the simultaneous decrease in the stem respiration rate during each girdling period, as previously observed (Johnsen et al. 2007, Maier et al. 2010), and leading to a residual respiration corresponding to maintenance respiration. These results showed that, when a cessation of the C supply from leaves (or cessation of phloem flow) occurred at the stem level, reserves might be used as an alternative C source for stem maintenance respiration, but not for stem growth, as previously indicated (Maier et al. 2010). The link between leaves and stem is, therefore, needed to allow stem growth. Actually, cambium activity and induction of vascular tissue is known to be regulated by auxin (e.g., Aloni 2004), which is transported by phloem in various tissues (e.g., Goldsmith et al. 1974, Morris and Thomas 1978, for a review, see also Friml and Palme 2002). The stop of auxin flow by girdling might explain why starch was used to support growth prior to budburst and not after the girdling date.

Furthermore, girdling was reversed (after Girdling Periods 1 and 2), leading to growth restarting, if girdling was applied early enough in the season (Girdling Period 1). Girdled Stems 1 reached the same total increment than control trees, even if their growth period was decreased by 5 weeks, compared with controls. The higher growth rate observed for Girdled Trees 1 in comparison with control trees suggested that summer growth conditions were more favourable than spring ones. We can assume that, later in the season (Girdling Period 2), leaves are unable to supply sufficient C to compensate the stem growth cessation or that hormonal signals inducing growth (such as auxin) are decreased.

This work suggests that a lack of assimilate due to stress events (e.g., last frosts or insect defoliation) may have little impact on reserve accumulation (see stem starch contents of Girdled Trees and Control Trees 1 in December; Table 1) and no impact on ring width if it occurs early enough in the growth season, assuming that damaged leaves can be replaced. Delaying its growth, the tree was able to return to the previous state, as though no stress had happened. The earlier the disturbance (girdling) occurred, the higher the tree 'resilience' (following the ecosystem notion, see Holling 1996) was. New questions are thus raised to understand whether this 'resilience' could be interpreted as an adaptation to a stress event and to determine mechanisms and conditions of this 'resilience'.

Conclusion

This study demonstrated that leaf carbohydrate supply vs reserve use could be an important factor controlling stem growth and $\delta^{13}C$ of both ring and stem CO_{2R}. The use of ^{13}C-enriched reserves vs ^{13}C-depleted new assimilates lead to higher $\delta^{13}C$ stem signals. Large responses observed during the earlier girdling period (Girdling Period 1) indicate that this impact is major when the high growth rate period is implicated. Nevertheless, change in substrate type is insufficient to explain all the observed CO_{2R} $\delta^{13}C$ variations, and other processes like changes in C investment through various metabolic pathways must also influence variations in stem CO_{2R} $\delta^{13}C$. Besides, the inter-tree intra-ring $\delta^{13}C$ variability, especially during late wood formation, highlights the need for further studies on post-photosynthetic processes at the individual and/or clonal population scales, in order to improve the interpretation of intra-seasonal ring signals in terms of climatic or ecophysiological information.

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