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The carbon isotope composition of CO_2 respired by trunks: comparison of four sampling methods

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The ^{13}C natural abundance of CO_2 respired by plants has been used in the laboratory to examine the discrimination processes that occur during respiration. Currently, field measurements are being expanded to interpret the respiration $\delta^{13}C$ signature measured at ecosystem and global levels. In this context, forests are particularly important to consider as they represent 80% of the continental biomass. The objective of this investigation was to compare four methods of sampling the CO_2 respired by trunks for the determination of its carbon isotope composition: three *in situ* methods using chambers placed on the trunk, and one destructive method using cores of woody tissues. The *in situ* methods were based either on a Keeling plot approach applied at the tissue level or on an initial flush of the chamber with nitrogen or with CO_2-free air. In parallel, we investigated the possibility of an apparent discrimination during tissue respiration by comparing the $\delta^{13}C$ signature of the respired CO_2 and that of the organic matter. The study was performed on six tree species widely distributed in temperate and mediterranean areas. The four methods were not significantly different when overall means were considered. However, considering the individual data, the Keeling plot approach and the nitrogen flush methods gave fairly homogeneous results, whereas the CO_2-free air method produced more variable results. The core method was not correlated with any of the chamber methods. Regardless of the methodology, the respired CO_2 generally was enriched in ^{13}C relative to the total organic matter. This apparent enrichment during respiration was variable, reaching as much as 3–5‰. This study showed that, on the whole, the different sampling techniques gave similar results, but one should be aware of the variability associated with each method. **Copyright © 2005 John Wiley & Sons, Ltd.**

Stable carbon isotopes (^{13}C, ^{12}C) are scaled up to the global level for the quantification of terrestrial and ocean sinks using the photosynthetic discrimination and the isotopic composition of ecosystem respiration. In this context, studies on ecosystem respiration are increasing.[1] Some of these studies have already shown variations with the age of forest ecosystems[2] and dependence on environmental conditions, such as precipitation or vapor pressure deficit.[3] To understand the respiration $\delta^{13}C$ signature of forest ecosystems, we need to quantify the isotopic composition of the CO_2 respired from its various components (leaves, trunks, branches, roots, soil). Currently, several different methods are widely used to collect respired CO_2: *in situ* sampling by accumulation in chambers (flush or Keeling plot)[4,5] and measurements on detached tissues.

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Until now, global or ecosystem models generally involved assumptions that the $\delta^{13}C$ signature of respired CO_2 was equal to the plant organic matter. However, this could not be the case. Studies of discrimination during respiration, carried out during the 1970s on leaves or whole plants, were rare and gave contradictory data.[6] More recently, for mesophyll protoplasts, Lin and Ehleringer[7] showed no discrimination during respiration when comparing the respired CO_2 and the soluble sugars. In contrast, under less artificial conditions involving bean leaves, Duranceau *et al.*[8] showed an enrichment of approximately 6‰ in respired CO_2 compared with sucrose. On beech twigs, the respired CO_2 $\delta^{13}C$ signature was lower than sucrose but generally enriched compared with that of the organic matter.[9] In a recent review, Ghashghaie *et al.*[10] presented a synthesis of the species and environmental variations of the fractionation factors involved in respiration. All these findings highlight the importance of examining *in situ* the spatial and temporal variations of the respired CO_2 $\delta^{13}C$ and the need to implement an accurate and easy method to assess it. Moreover, it is of major importance to determine if the

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different methods that are commonly used are intercomparable.

In forest ecosystems, a significant proportion of total ecosystem respiration originates from the woody tissues. Many quantitative estimates have shown that it is a large component of the annual carbon balance of forest ecosystems and therefore it partly determines the capacity of forests to stock carbon. Many studies suggest that respiration is a key process in explaining variations in ecosystem productivity.[11–13] In temperate deciduous forests, the respiration of trunks and branches represents 33–37% of the total carbon loss from ecosystem respiration,[14,15] and from 26–50% of the above-ground autotrophic respiration.[16,17]

The aim of this study was to compare the methods currently used to measure the isotopic composition of the CO_2 respired by woody tissues. Using the same chamber type, three different sampling techniques for respired CO_2 were examined: (1) accumulation according to a Keeling plot approach; (2) accumulation after flushing the chamber with CO_2-free air; and, (3) accumulation after flushing the chamber with nitrogen. The fourth technique (core method) was destructive: tissues were placed in a CO_2-free vial and allowed to respire for several hours before analysis. Our initial hypothesis was that the different methods would give similar results, with possible deviations resulting from the nitrogen flush method (because of the lack of oxygen) and from the core method (because of the injury to the tissue). In parallel, we investigated the possibility of an apparent discrimination during tissue respiration by comparing the $\delta^{13}C$ signature of the respired CO_2 with that of the organic matter. We tested these methods on trunks of various tree species widely distributed in temperate and mediterranean areas.

EXPERIMENTAL

Study sites and experimental design

This study was conducted at three sites: the ''bois de la guyonnerie'' in Bures sur Yvette (48°50′ N, 02°10′ E, elevation 65 m), 25 km southwest of Paris, France; the Barbeau Forest (48°29′ N, 2°47′ E, elevation 92 m), 50 km southeast of Paris; and near Evora, Portugal (38°32′ N, 08°00′ W, elevation 230 m). The two French sites are temperate in climate, with mean annual precipitations reaching 685 and 690 mm, respectively, and a mean annual temperature of 10.5°C. The Portuguese site has a Mediterranean climate with a mean annual precipitation of 665 mm and a mean annual temperature of 15.4°C.

To encompass a range of values and different bark types, six species were considered: two Mediterranean (*Quercus ilex* L. and *Q. suber* L.), and four temperate (*Q. petraea* Liebl., *Castanea sativa* Miller, *Pinus sylvestris* L. and *Fagus sylvatica* L.). The study was conducted between September 2003 and December 2003. The selected trees were mature, with diameters around 30 cm or greater. For each species, the CO_2 respired by trunks was collected according to four different methods: three *in situ* methods using a chamber placed on the trunk, and one destructive method using cores of woody tissues. The *in situ* methods were based either on a Keeling plot approach (KP method) applied at the trunk level, on an initial flush of the chamber with nitrogen (NF method), or on an initial flush with CO_2-free air (CFAF method). In order to minimize the effect of diurnal variations on the respiration rates between the different measurement methods on a given tree, the days selected for this field work involved only a weak diurnal temperature variation (less than 5°C). For each *Q. petraea* and *C. sativa* species, three replicated measurements were made on two trees. For all other species, a single tree was tested once.

Methods using a chamber *in situ*

Two chambers, made of transparent acrylic resin, were designed specifically to collect the CO_2 respired by trunks *in situ*. The chambers were 30 cm high and 20 cm wide. Air volume was approximately 5 L. The upper and lower edges of the chambers were curved to conform to the trunk shape. Two stainless steel air inlets/outlets passed through the chamber walls, allowing gas exchange measurements in the closed system. A screw-threaded mount with a removable cap attachment (Fig. 1) was glued into the center of the front face of each chamber. When unscrewed, it opened a 6 cm wide vent fitted with a 4×4 cm electric fan (Papst 412 F, St. Georgen, Germany) used to flush the air rapidly. When the chamber was hermetically sealed by screwing on the cap, the fan was still able to mix the air inside the chamber while measurements were taken.

A chamber was attached to a trunk at a point between 0.8 and 1.30 m in height in a place free from mosses, lichens or algae. It was sealed to the bark with a rubber sealant

Figure 1. Photograph of a custom-made chamber installed on a trunk.

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(Terosta-7, Teroson, Ludwigsburg, Germany). Two strong straps were used to fasten the chamber to the tree, firmly pressing the sealant between the bark and the edges of the chamber (Fig. 1). For some trees with a rough bark (*Pinus*, *Quercus*, *Castanea*), we had to lightly flatten the surface of the bark with a scraper to get a good seal.

Stem CO_2 efflux measurements were conducted with an EGM1 infrared analyzer (PP Systems, Hitchin, UK) operating in a closed system. To achieve this, we needed to modify the instrument. First, an air outlet was fitted on the analysis cell to reinject the sampled air into the chamber. Secondly, the operating software of the EGM1 was modified by mounting a new EEPROM (V 3.5, initially designed for soil respiration measurements) to prevent the device from triggering undesirable auto-zeros. Auto-zeros automatically inject CO_2-free air into the cell of the analyzer, and this would have disrupted the experiment (the operator still has the option of triggering auto-zeros between measurements to minimize drifts).

The seals were tested by blowing along the joints and measuring the CO_2 evolved in the chamber. For the flush methods, the air inside the chamber was flushed for 15–20 min with N_2 or CO_2-free air from portable cylinders at a flow rate of 150 L h^{-1} until the CO_2 concentration inside the chamber dropped to 20–30 ppm. Then, the system was closed and the respired CO_2 was allowed to accumulate up to 700–800 ppm (20–60 min). For the KP method, the air inside the chamber initially was flushed with ambient air, using the vent. When the CO_2 concentration inside the chamber was close to ambient (380–400 ppm), the system was closed. Air sampling was performed at five or six CO_2 concentrations, generally at 400 (just after closure), 500, 700, 1000, 1500, and 1900 ppm. Air samples were collected by drawing 30 mL of air in a gas-tight syringe (SGE, Ringwood, Australia). Each filled syringe was immediately and rapidly replaced by an empty one, to avoid introducing ambient air into the chamber. Depending on the respiration rates, each Keeling plot lasted from 45–105 min. According to the mixing model of Keeling,[18,19] a linear relationship should exist between the $\delta^{13}C$ of the CO_2 and $1/[CO_2]$ in the chamber. We used a linear extrapolation of $[CO_2]$ to obtain the $\delta^{13}C$ of the respired CO_2.[2,4]

For each tree, the same sequence was monitored: first, the CFAF method; second, the NF method; and third, the KP method. To test whether there was any sequence effect, we checked three times to see that the CFAF method gave the same results if performed after the other methods.

Detached-tissue method

During measurements with the different chamber methods, we collected cores from the trunk. Two 6 cm long cores were taken from each trunk, close to the location of the chamber. The first was kept intact, whereas the second core was split into three subsamples: the bark (including phloem and suber) and two xylem sections (superficial and deep). These two xylem parts are referred to as external and internal xylem, respectively. The intact core and the three core subsamples were immediately placed inside four 50 mL gas-tight syringes flushed with CO_2-free air. They were allowed to respire for 4–5 h at ambient temperature. The gaseous content of the syringe was then injected into the isotope ratio mass spectrometer (IRMS). The core tissues were oven-dried for 48 h at 60°C and finely ground (type MM200; Retsch).

Carbon isotope analysis

For the temperate species, the carbon isotope composition values of organic material and gas were determined, with high precision (±0.2‰), using the same IRMS (VG Optima, Fisons, Villeurbanne, France). $\delta^{13}C$ of organic matter was determined on CO_2 released from combustion of core tissue samples in an elemental analyzer (model NA-1500; Carlo Erba, Milan, Italy). For the respired CO_2, we used the system derived from Tcherkez *et al*.[20] The content of the syringes was directly injected into the elemental analyzer through a 15 mL loop (via a magnesium perchlorate column to eliminate the water vapor released by tissue). The connection between the elemental analyzer and the spectrometer was opened when the CO_2 peak eluted from the elemental analyzer.

Concerning the assessment of the isotopic signal of respired CO_2 for the Mediterranean species, the content of the syringe was transferred into septum-capped vials (16 mL) using a 50 mL gas-tight syringe equipped with a needle. The respired CO_2 $\delta^{13}C$ values were determined with an IRMS (Isoprime, Micromass, UK) in continuous-flow mode. Injections were made by an automatic sampler (Multiflow Prep-system) processing 120 samples per run in approximately 6 h, which yielded the required precision (<0.1‰). Carbon isotope ratios of the bulk organic material of cores were analyzed by Mylnefield Research Services (Dundee, UK).

The statistical analyses were performed using Biostatistica. The methods were compared using a nonparametric Friedmann test for matched samples.

RESULTS AND DISCUSSION

Comparison of the different methods

In the Keeling plots, the relationship between the inverse of the CO_2 concentration in the chamber and the carbon isotope composition of this CO_2 was well described by a linear regression (see examples in Fig. 2). The coefficients of determination (r^2) for all species were always higher than 0.95 ($n = 5–7$). These results showed that the Keeling plot approach, which is generally used at the ecosystem level to estimate the $\delta^{13}C$ of respiration,[1] may also be applied at the tissue level.

If we pool all the replications of all the species, the overall means of the carbon isotope composition of CO_2 respired by trunks obtained with the three chamber methods (CFAF, NF, KP) were not significantly different (Friedman test, 5%, $n = 7$). The overall means (standard deviation) were −23.36 (1.01), −23.69 (1.82) and −24.26‰ (1.98) for the CFAF, NF and KP methods, respectively. The maximum difference between these means is 0.9‰, which is lower than the 1‰ threshold generally considered as significant for any interpretation in biological and ecological studies.[21] We checked to ensure that the CO_2 efflux from the trunks was stable during each sampling period and that the flush with nitrogen or CO_2-free air did not lead to an increase in the CO_2 efflux (data not shown). This stability is probably explained by a high CO_2 concentration inside the trunks: concentrations of more than 10 000 ppm have been reported,[22] which implies a high

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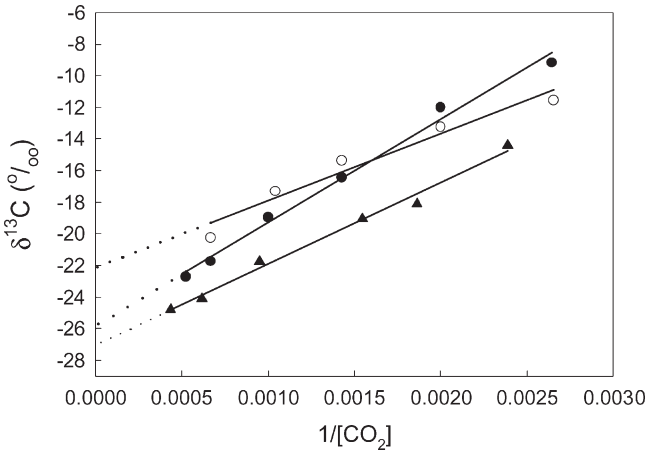

Figure 2. Examples of Keeling plots created using chambers placed on trunks of three species: *Quercus petraea* in December 2003 (open circles, $y = 4245.2x - 22.155$, $r^2 = 0.954$); *Castanea sativa* in October 2003 (filled circles, $y = 6532.6x - 25.844$, $r^2 = 0.991$); *Quercus ilex* in October 2003 (filled triangles, $y = 5153x - 27.065$, $r^2 = 0.991$).

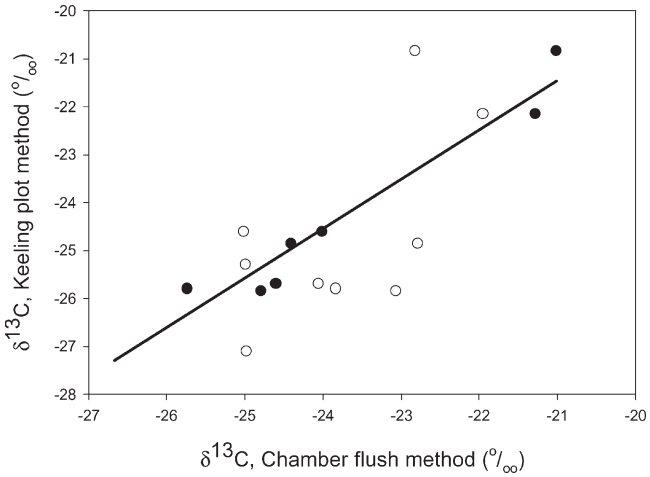

Figure 4. Comparison of the carbon isotope composition ($\delta^{13}C$) of CO_2 respired by trunks of different species. Y-axis: values obtained by the Keeling plot approach. X-axis: values obtained after an initial flush of the chamber with nitrogen (filled circles, $y = 0.8335 + 1.059x$, $r^2 = 0.94$, $P = 0.0003$), or with CO_2-free air (open circles, no significant regression).

concentration gradient regardless of the external CO_2 concentration (zero or ambient).

Considering all the replications involving the four methods (chamber and core), the overall means were not significantly different (Friedman test, 5%, $n = 5$, Fig. 3) although, on average, the core method gave the lowest values. The average differences between the core method and the KP, NF or CFAF method were −0.33, −0.88 and −1.33‰, respectively. Individual differences between tissue-based and chamber-based methods were as much as 2‰ and, in one case, −5‰ (between the core and CFAC methods). There was an acceptable correlation between results obtained from the NF and the KP methods (Fig. 4). On the other hand, the CFAF method did not correlate well with either of the other two methods and gave more variable results: an occasional difference (close to 2‰ or higher) occurred between the CFAF and KP methods; in addition, the maximal differences between the CFAF and NF methods were −1.8 and 1.9‰. The values from the core method were not correlated with any of the chamber methods ($r^2 < 0.44$). The solid fit of the data to linear regressions in the Keeling plots and the coherence of the results obtained from the NF and KP methods strongly suggest that these two methods are reliable. We cannot explain why the CFAF method, which *a priori* does not modify the O_2 concentration in the chamber, gave more variable results relative to the other two chamber methods. For the NF method, the constant CO_2 efflux during accumulation suggests that probably there is adequate O_2 inside the trunk, and that the absence of this gas in the chamber does disturb the respiration process during the experiment.

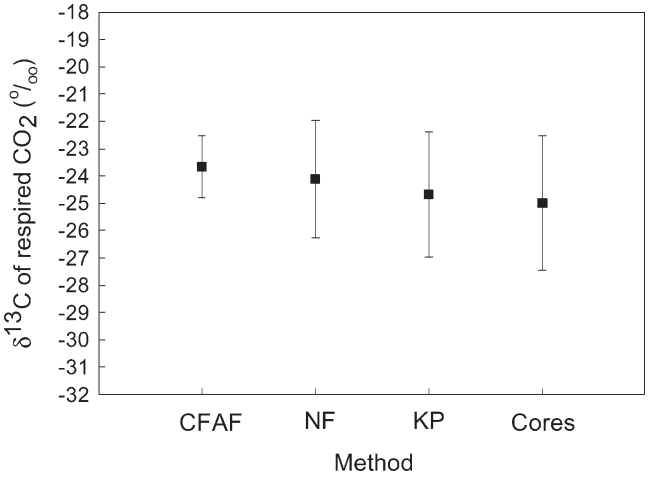

Figure 3. Comparison of the means (±SD, $n = 5$) of the carbon isotope composition of CO_2 respired by trunks determined according to four different measurement methods: three *in situ* methods using a chamber placed on a trunk, and one destructive method on collected cores. The *in situ* methods were based either on a Keeling plot approach (KP), or on an initial flush of the chamber with nitrogen (NF), or with CO_2-free air (CFAF).

Apparent discrimination during respiration

Among the chamber methods, the $\delta^{13}C$ signature of the respired CO_2 was always higher than that of the organic matter. On average, the respired CO_2 was enriched by 3.42, 2.08 and 3.92‰ compared with the organic matter values obtained by the NF, KP and CFAF methods, respectively (Table 1). The difference between the $\delta^{13}C$ of the CO_2 respired by a core and that of the organic matter of this core also was always positive, except for *C. sativa* (Table 2). Maximal differences, 3.4 and 4.3‰, were obtained in December on *Q. petraea* and *F. sylvatica*, respectively. These results suggest that there is an apparent positive discrimination during respiration, at least during the autumn and winter periods. This is consistent with the results obtained on current-year stems of *F. sylvatica* by Damesin and Lelarge.[9] To specify the discrimination during respiration, further experimentation, particularly concerning the isotopic signature of the respired substrates such as sucrose, is needed.[20]

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Table 1. Differences (in ‰) between the carbon isotope composition ($\delta^{13}C$) of CO_2 respired by trunks and that of the total organic matter of the same trunk. The respired CO_2 was collected from a chamber placed on the trunk using either a Keeling plot approach (KP), or an initial flush with nitrogen (NF), or with CO_2-free air (CFAF). The value for the organic matter was measured on a 6 cm long core collected near the chamber

Species	Date (2003)	NF (‰)	KP (‰)	CFAF (‰)
Quercus petraea	October	1.25	0.15	1.79
Q. petraea	October	1.19	1.13	3.10
Q. petraea	November	3.73	3.92	1.92
Q. petraea	December	3.29	2.42	2.62
Q. ilex	October	−0.31	−0.73	1.39
Q. suber	October	0.46	−0.09	0.22
Castanea sativa	October	3.75	2.7	5.48
C. sativa	October	3.12	2.67	4.74
Pinus sylvestris	October	3.51	2.91	2.51

The isotopic signature of the forest respiration components may exhibit large seasonal variations, as shown previously for branches[9] and for soil.[23] As a consequence, the actual values for the carbon isotope composition assessed in the present study are probably rather different from those that may be observed during the active vegetation period. The enriched values that we found (approx. −22‰) for respired CO_2 could be explained by the type of substrate used in respiration. During the leafless period, the trunk respiration, at least for deciduous species, relies on stored carbon. For *Q. petraea*, we can see that the apparent discrimination increased from October to December. The reserves, composed mainly of starch in trees, are enriched in ^{13}C compared with the total organic matter.[9,24,25] We noted that the evergreen species (*Q. ilex* and *Q. suber*) showed the lowest apparent discrimination. In contrast to the deciduous species, during winter, these evergreens probably used leaf sugars conveyed by the phloem sap, the isotopic signature of which is related to the stomatal conductance.[26]

Variations among trunk tissues

The $\delta^{13}C$ values for organic matter obtained from the bark (suber and phloem) ranged between −28.55 and −25.9‰ (see Fig. 5(a)). The values from the internal xylem are slightly more variable (−28.9 to −24.16‰). There is no general trend among tissues within species. For example, we observed an increase between bark and internal xylem for *Q. petraea*, whereas we saw a decrease for *C. sativa*. For the different species, the CO_2 respired by cores was generally enriched compared with the corresponding organic material (Figs. 5(a) and 5(b)). These results confirm at the tissue level the apparent positive discrimination during respiration. The values of

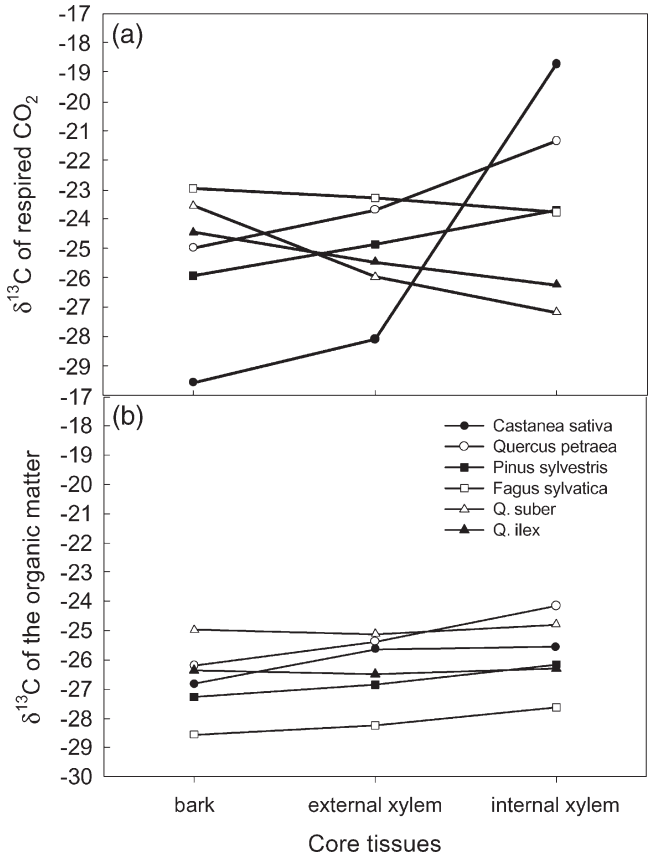

Figure 5. Carbon isotope composition ($\delta^{13}C$) of CO_2 respired by trunk tissues of different types and locations along a 6 cm long core (a) and of the corresponding total organic matter (b). To clarify the figure, only one value per species is indicated; the other values for the same species show similar differences among tissues.

Table 2. $\delta^{13}C$ values of the CO_2 respired and that of the organic matter of 6 cm long cores. The differences between the $\delta^{13}C$ values (apparent discrimination) are listed in the third column

Species	Month (2003)	$\delta^{13}C$ of respired CO_2 (‰)	$\delta^{13}C$ of organic matter (‰)	Apparent discrimination (‰)
Quercus petraea	October	−26.59	−26.93	0.34
Q. petraea	October	−24.69	−25.85	1.16
Q. petraea	November	−23.27	−24.75	1.48
Q. petraea	December	−21.18	−24.57	3.39
Q. ilex	October	−24.91	−26.37	1.46
Q. suber	October	−25.32	−25.21	−0.11
Fagus sylvatica	December	−23.38	−27.69	4.31
Castanea sativa	October	−29.04	−28.54	−0.5
C. sativa	December	−28.19	−25.68	−2.51
Pinus sylvestris	October	−24.84*	−27.52	2.68

*Data for respired CO_2 from the entire core was not available for *Pinus sylvestris*; therefore, we replaced it with the isotopic signature of the CO_2 respired by the xylem.

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the respired CO_2 were much more variable than the $\delta^{13}C$ signatures of the organic matter. The observed range was clearly larger for the internal xylem than for the other tissues. As for the organic matter, there was no general trend within species. The $\delta^{13}C$ of the CO_2 respired by *Q. petraea* increased from the external to the internal tissues. There was also an increase for *C. sativa* (though not the same trend as for the organic matter) with very high values (up to −18.2‰) obtained for the internal xylem. This species showed an increase in the $\delta^{13}C$ of the respired CO_2 from the external to the internal tissues, similar to the hardwood species. In particular, *C. sativa* had relatively narrow sapwood (3 to 5 rings) and the internal xylem sample was composed only of hardwood. The origin of the CO_2 from dead tissue is still unclear—possibly it comes from a stock of CO_2 that diffused from the living tissue and became trapped in the dead tissue. The observed enrichment could be partly the result of discrimination during diffusion, which is known to be around 3–4‰.[27]

CONCLUSIONS

This original study provides a preliminary comparison of various field sampling techniques used to evaluate the isotopic composition of CO_2 respired by trees. All four methods tested, including the destructive one, appeared acceptable. The standard Keeling plot approach can be applied at the tissue level, using a chamber. Each method was consistent with the others when mean values were considered, but differences up to 2–5‰ were occasionally observed among them. Therefore, it is not totally clear whether there is one best method. However, our comparison of different methodologies does confirm that there is generally, at least during winter, an apparent ^{13}C enrichment during trunk respiration. As a consequence, the $\delta^{13}C$ signature of the CO_2 respired by plant tissues can no longer be assumed to be equal to that of the organic matter composition. Measurements on leaves, roots and soil, as well as seasonal investigations, are required in order to be able to fully understand the ecosystem $\delta^{13}C$ signature.

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