13C/12C isotope labelling to study leaf carbon respiration and allocation in twigs of field-grown beech trees

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In situ 13C/12C isotopic labelling was conducted in field-grown beech (Fagus sylvatica) twigs to study carbon respiration and allocation. This was achieved with a portable gas-exchange open system coupled to an external chamber. This method allowed us to subject leafy twigs to CO2 with a constant carbon isotope composition (δ13C of −51.2%) in an open system in the field. The labelling was done during the whole light period at two different dates (in June 2002 and October 2003). The δ13C values of respiratory metabolites and CO2 that is subsequently respired during the night were measured. It was found that night-respired CO2 is not completely labelled (only ca. 58% and 27% of new carbon is found in respired CO2 immediately after the labelling in June 2002 and October 2003, respectively) and the labelling level progressively disappeared during the next day. It is concluded that the carbon respired by beech leaves after illumination was supplied by a mixture of carbon sources in which current carbohydrates were not the only contributors. In addition, as has been found in herbaceous plants, isotopic data before labelling showed that carbon isotope discrimination favoring the 13C isotope occurred during the night respiration of beech leaves. Copyright © 2005 John Wiley & Sons, Ltd.

Determining the carbon balance of the terrestrial ecosystem and understanding how it depends on the environmental conditions are currently major objectives of the scientific community (e.g. AMERIFLUX and CARBOEUROFLUX projects). In this context, forests are particularly important as they represent 80% of the terrestrial biomass. As a consequence, currently considerable effort has been made to understand forest growth and pathways of carbon fluxes in forest ecosystems. Carbon input in the system via tree photosynthesis has been well characterized and simulated using the biochemical Farquhar’s model on leaf assimilation. The carbon (C) output (i.e. respiration) has also been characterized. The processes have been described at the ecosystem level and also on an annual scale. In contrast, allocation of assimilated C (i.e. its distribution in the different tree tissues) has received less attention. The process has thus been studied in simulations. Knowledge of the distribution of C in individual trees is considered essential to a proper understanding of forest growth.

Labelling techniques using 14C or 13C have been used for many years to study the allocation of carbohydrates produced by photosynthesis between different parts of the plant, especially on herbaceous species grown in controlled-environmental conditions or in natural abundance conditions. Lacointe and coworkers have tested the branch autonomy theory inside tree crowns (i.e. the direct effect of light on photosynthesis is not compensated for by carbohydrate exchanges between branches of different light status) using a 13C/14C double-labelling experiment on 3-year-old walnut trees. They found that in September (in the northern hemisphere) branch autonomy was nearly total (i.e. there was no exchange between branches); however, in winter, significant amounts of C were imported by branches (representing up to 10% of total branch reserves). Nevertheless, data on the precise partitioning of photo-assimilated C between distinct parts on adult trees in the field are very scarce in the literature.

Direct measurements of the 14C or 13C labelling in respired CO2 in trees are surprisingly limited, and, as far as we know, such measurements have never been carried out on adult trees in the field. It has been recently shown on herbaceous species grown in controlled conditions that most of the C released by dark respiration in leaves after a period of light does not come from C recently assimilated by photosynthesis. Because of the complexity of tree structure and of possible interactions between autotrophic (mainly leaves) and heterotrophic (i.e. trunk, roots, etc.)
tissues, it is possible that such a result is not valid in ligneous plants. In particular, C stored in branches and trunk (as reserves) may act as a sink and a source of C depending on the season and growth conditions, thus blurring the picture seen in herbaceous plants. For example, Barbaroux and Bréda showed on beech trees that accumulation of some reserves occurred simultaneously with growth during the vegetative period. Damesin and Lelarge showed that a portion of stored C (naturally enriched in 13C) is used in the initial phase of leaf and stem construction and during winter respiration.

In this study, a 13C/12C isotope labelling technique was used to study the allocation of recently assimilated C by photosynthesis at short term (over 2 days) and further night respiration after the photoperiod in adult trees of Fagus sylvatica. An experimental set-up using a portable gas-exchange open system LI-6400 (LICOR Inc., Lincoln, NB, USA) was adapted for in situ carbon labelling. We have taken advantage of the difference in the C isotope composition (δ13C) between atmospheric CO2 (−10.5‰) and commercially available 13C-enriched CO2 (−51.2‰), and consequently the 13C abundance in the CO2 used for the labelling is of the same order of magnitude as that found in nature. This allows one to pick up the contribution of stored C vs. current photosynthates to CO2 production by respiration. It is noteworthy that this would not have been possible if heavily labelled C had been used (i.e. several percent in 13C would have blurred the contribution of non-labelled C). The objective of the study was to examine the contribution of leaf-assimilated C to respiratory losses (i.e. CO2), leaf metabolite (soluble sugars and starch) production and carbohydrate export from stems. The labelling was achieved on current-year twigs at two contrasted periods of the tree life (at the end of leaf growth in June and at the end of leaf lifespan in October).

**EXPERIMENTAL**

**Plant material and experimental site**

Three (isolated) 20-year-old beech (F. sylvatica) trees grown in the campus of the University of Paris XI at Orsay, France (48°42′N, 02°10′E) were used in this study. A complete description of the plant material and the experimental site has been published. Mean annual precipitation, and minimum and maximum temperatures of the site are 685 mm, 7°C and 16°C. Photosynthetically active photon flux density (PPFD) and air temperature (Tair) were measured during the two dates: 18–19 June 2002 and 17–18 October 2003 (Fig. 1). The δ13C of the air CO2 surrounding the trees was −10.5 ± 0.4‰ (average of the two measuring periods). Sun-exposed leaves in twigs and current-year produced stems (Sn, i.e. S2002 or S2003, respectively) at the bottom of the crown were used for the isotopic labelling. The labelling was also followed in the stem (of the same branch) grown during previous years.

**Gas-exchange and C-labelling procedures**

For both measuring periods, the same sequence was monitored on one (attached) twig of the bottom of the crown (including 6 sun-exposed leaves and the current year stem): (i) sampling of respired CO2 at predawn; (ii) labelling during the light time of the day (i.e., ca. 12 and 8 h labelling periods for June 2002 and October 2003, respectively); and (iii) sampling of respired CO2 immediately after the labelling and three following times the day after (i.e. at the end of the first night, in the middle of the second day, and at the end of the second day). At the end of the experiment (i.e. beginning of the second night), the labelled leaves and the corresponding current-year stem, the 1-year-old and 2-year-old stems and the nearest current year ramification present on the 1-year-old stem (see Figs. 4 and 5 for schemes)

![Figure 1. Photosynthetically active photon flux density (PPFD, circles) and air temperature (Tair, squares) during the two measuring periods (18–19 June 2002, closed symbols, and 17–18 October 2003, open symbols) in the campus of the University of Paris Sud at Orsay, France.](image-url)
were sampled for isotopic analysis. Non-labelled twigs of the same trees were also sampled as control.

**Closed system for dark respiration**

Before the labelling, attached leaves (typically six) of a twig were placed in a respiration chamber for measurements of dark-respired CO$_2$ (unlabelled). The respiration chamber was first ventilated with CO$_2$-free air and then the CO$_2$ respired was allowed to accumulate in the chamber.$^{10}$ The gas contained by this chamber was introduced into a 50-mL syringe (Magnum syringe; Alltech France, Carquefou, France). The sample gas in the syringe was then introduced into the 15-mL gas sample loop of an elemental analyser (EA, NA-1500; Carlo-Erba Instruments, Milan, Italy),$^{19}$ and the isotope analysis was conducted in the continuous flow mode. Briefly, the gas contained by the loop was introduced into the EA with helium for gas chromatography through a six-way valve. The connection valve between the EA and the isotope ratio mass spectrometer (IRMS, VG Optima, France) was opened just for the isotope analysis.

The chamber was made of aluminum (20 cm $\times$ 12 cm $\times$ 6 cm), with a clear plastic lid, and accommodates the six leaves of the twig (total leaf surface ca. 0.01 m$^2$). Two fans were enclosed in the chamber and gave a boundary layer conductance to water of ca. 6.7 mol m$^{-2}$ s$^{-1}$. The temperature of the chamber was controlled at ca. 20 $^\circ$C with circulating water from a cooling water bath to the jacket of the leaf chamber, and the leaf temperature was measured with a copper-constantan thermocouple plugged to the thermocouple sensor connector of the LI-6400 leaf-clip chamber. The ingoing air flow was 1 L min$^{-1}$ and was monitored by the LI-6400. The molar fractions of CO$_2$, the humidity and the light were measured with the infrared gas analyser (IRGA) and the light sensor of the LI-6400. The CO$_2$ used for labelling was obtained from a bottle (Air Liquide, Grigny, France) with a $\delta^{13}$C value of $-51.2 \pm 0.1%$. The $\delta^{13}$C value of the ingoing air was also regularly checked by taking a sample into a glass balloon (see below).

When the photosynthetic measurements were made, the outgoing air of the chamber was no longer directed to the IRGA but instead to 150-mL glass balloons (Scott Glass, Mainz, Germany). Balloons were taken to the lab, and the sample gas was introduced into the sample loop of the EA using a pump. The on-line photosynthetic discrimination ($\Delta_{obs}$) was calculated using the following relationship,$^{21}$

$$\Delta_{obs} = \frac{\delta_{o} - \delta_{d}}{1 - \delta_{d} + \xi(\delta_{d} - \delta_{o})},$$

where $\delta_{d}$ and $\delta_{o}$ are the $\delta^{13}$C values of outlet and inlet air, and $\xi = c_{o}/(c_{d} - c_{o})$, where $c_{o}$ and $c_{d}$ are the inlet and outlet CO$_2$ molar fractions.

After the labelling, leaves were removed from the gas-exchange chamber and were placed back into the respiration chamber for measurements of dark-respired CO$_2$ (labelled) as described above. At the end of the measurements (i.e. at the beginning of the second night), leaves and stems were immediately frozen in liquid nitrogen. They were then lyophilized, and powdered for metabolite analysis.

**Carbohydrate extractions and quantification**

The starch and sucrose, glucose and fructose extraction procedures were as previously described.$^{17,19}$ Briefly, leaf and stem powder was suspended with 1 mL of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany). After centrifugation, starch was extracted from the pellet by HCl solubilization. Soluble proteins of the supernatant were heat denatured and precipitated and soluble sugars and organic acids of the protein-less extract were separated by high-performance liquid chromatography (HPLC). After lyophilization, purified metabolites were suspended in distilled water, transferred to tin capsules (Courtagne Analyze Service, Mont Saint-Aignan, France) and dried for isotope analysis. Isotope analysis of metabolites was conducted using the same EA and IRMS as were used for gas measurements.

**RESULTS**

The environmental conditions during the two measuring dates (i.e. 18–19 June 2002 and 17–18 October 2003) were typical of the temperate climate under oceanic influence of the Paris region (Fig. 1). June 2002 was warm (maximum PPFD and $T_{air}$ values were 1900 $\mu$mol m$^{-2}$ s$^{-1}$ and 32 $^\circ$C, respectively) and October 2003 was relatively cold (maximum PPFD and $T_{air}$ values were 1200 $\mu$mol m$^{-2}$ s$^{-1}$ and 11 $^\circ$C, respectively).

**Labelling in dark-respired CO$_2$**

The $\delta^{13}$C of respired CO$_2$ in the leafy twig of beech trees was ca. $-18.5%$ before the labelling (measured in the closed system) at the end of the night. Then the leafy twig was placed in the open system for isotopic labelling with CO$_2$ depleted in $^{13}$C ($\delta^{13}$C of $-51.2%$) during a sunny day in June 2002 (Fig. 2) or October 2003 (Fig. 3). During the experiment conducted in June 2002, beech leaves assimilated ca. 300 mmol C per m$^2$ during the day ($A$ was ca. 6.5 $\mu$mol m$^{-2}$ s$^{-1}$, Fig. 2). Low light intensities produced lower assimilation rates during October 2003 (Fig. 3) than during June 2002 (Fig. 2). It should be also noted that, although $T_{air}$ was different during the two measuring periods and variable during the day, leaf temperature was maintained at 20 $^\circ$C in order to maintain...
similar stomatal and photo-respiratory conditions on the leaves during the labelling.

Leaf respiration increased after sunset (to ca. $-1.5 \, \mu\text{mol m}^{-2} \text{s}^{-1}$) and progressively decreased during the night (to ca. $-0.5 \, \mu\text{mol m}^{-2} \text{s}^{-1}$). During the whole night of June 2002, leaves respired ca. 100 mmol C per m$^2$; this is one-third of the C assimilated during the day. This dark-respired CO$_2$ was labelled, i.e., $\delta^{13}\text{C}$ of the evolved CO$_2$ was $-44.5\%$ at the beginning of the night and $-48.3\%$ at the end of the night (Fig. 2). During June 2002, the (photosynthetic) C isotope discrimination ($\Delta^{13}\text{C}$) was ca. $15\%$ in the morning as stomata were still opening (this corresponds to a $p_i/p_a$ value ca. 0.5,

Figure 2. Changes in the photosynthetic rate ($\bullet$, $\mu\text{mol m}^{-2} \text{s}^{-1}$), $\Delta^{13}\text{C}$ ($\bullet$, $\%$), dark-respiration rates ($\square$, $\mu\text{mol m}^{-2} \text{s}^{-1}$) and $\delta^{13}\text{C}$ of respired CO$_2$ ($\blacksquare$, $\%$) in leafy twigs of beech trees during June 2002 for two consecutive days. The top bar indicates the night and day periods, respectively (during the day the $\delta^{13}\text{C}$ of CO$_2$ given to the leaves is also shown). Data are the means of two replicates (standard deviations are shown when larger than the symbols).

Figure 3. Changes in the photosynthetic rate ($\bullet$, $\mu\text{mol m}^{-2} \text{s}^{-1}$), $\Delta^{13}\text{C}$ ($\bullet$, $\%$), dark-respiration rates ($\square$, $\mu\text{mol m}^{-2} \text{s}^{-1}$) and $\delta^{13}\text{C}$ of respired CO$_2$ ($\blacksquare$, $\%$) in leafy twigs of beech trees during October 2003 for two consecutive days. The top bar indicates the night and day periods, respectively (during the day the $\delta^{13}\text{C}$ of CO$_2$ given to the leaves is also shown). Data are the means of two replicates (standard deviations are shown when larger than the symbols).
data not shown) and reaches 20% during the day (i.e. $P_o/P_a$ value ca. 0.7). As the average $\Delta^{13}C$ of the leaves was ca. 17%, and the $\delta^{13}C$ of dark-respired CO$_2$ by leaves may have been $^{13}C$-enriched by $c_{dark}$ ~4–6%$^{22}$ (compared with the respiratory substrates), the $\delta^{13}C$ of the respiratory substrates (mainly carbohydrates) should have approached ~64% (i.e. ~51 – $\Delta^{13}C + c_{dark}$) if the substrates used to sustain respiration had been fully labelled (with a CO$_2$ of $\delta^{13}C$ of ~51%), but this was not the case (Fig. 2). Furthermore, as expected, the $\delta^{13}C$ value of respired CO$_2$ progressively increased to ~27.2% at the beginning of the second night; during the second day, leaves assimilated a similar amount of non-labelled CO$_2$, i.e. with a $\delta^{13}C$ value of ~10.5%.

Shorter light periods and a lower light intensity in October 2003 led to a lower labelling in the current-year stem in comparison to June 2002 (they are ca. 5% $^{13}C$-depleted) and October 2003 (ca. 4% $^{13}C$-depleted, Figs. 4 and 5) compared with the non-labelled (control) leafy twigs (control values are in the figure legends). However, metabolites are less labelled in October than in June, simply because of a lower assimilation and smaller photosynthetic isotope discrimination (Figs. 2 and 3). By contrast, the nearest (lateral) current-year ramification (Sn) in Figs. 4 and 5 are not labelled, having isotope abundances similar to the control. We recognize that several parameters such as their temperature were not controlled and, consequently, the photo-respiratory flux was not also. This may induce a different photosynthetic discrimination that could compensate for the transfer of labelled C from the labelled branch. However, metabolites of the lateral current-year ramification are not depleted in $^{13}C$ after the labelling and we assume here that it reflects no labelling, i.e. no transfer of $^{13}C$-depleted products from the labelled branch.

The leaf total organic matter (TOM) was slightly labelled (by ca. 2%$^{13}C$) in the leaves (in the chamber, i.e. 2002). Further, metabolites were also labelled in the current-year stem in spring (Sn, i.e. S2002, Fig. 4), but this is not the case for the

![Figure 4](image-url)  
**Figure 4.** Schematic representation of the $\delta^{13}C$ of sucrose, glucose, fructose, starch and TOM (from left to right) of leaves and stems during June 2002 at the end of the measurement period (i.e. at the beginning of the second night) and so the isotope composition of the organics reflects both the labelling (with a $\delta^{13}C$ value of ~51.2% during the first day) and its dilution by subsequent assimilation (during the second day leaves assimilated a similar amount of non-labelled CO$_2$, i.e. with a $\delta^{13}C$ value of ~10.5%).

At the end of the measurement period, all the metabolites (sucrose, glucose, fructose and starch) of the leaves in the labelling chamber were still labelled both in June 2002 (they are ca. 5% $^{13}C$-depleted) and October 2003 (ca. 4% $^{13}C$-depleted, Figs. 4 and 5) compared with the non-labelled (control) leafy twigs (control values are in the figure legends). However, metabolites are less labelled in October than in June, simply because of a lower assimilation and smaller photosynthetic isotope discrimination (Figs. 2 and 3). By contrast, the nearest (lateral) current-year ramification (Sn) in Figs. 4 and 5 are not labelled, having isotope abundances similar to the control. We recognize that several parameters such as their temperature were not controlled and, consequently, the photo-respiratory flux was not also. This may induce a different photosynthetic discrimination that could compensate for the transfer of labelled C from the labelled branch. However, metabolites of the lateral current-year ramification are not depleted in $^{13}C$ after the labelling and we assume here that it reflects no labelling, i.e. no transfer of $^{13}C$-depleted products from the labelled branch.

The leaf total organic matter (TOM) was slightly labelled (by ca. 2%$^{13}C$) in the leaves (in the chamber, i.e. 2002). Further, metabolites were also labelled in the current-year stem in spring (Sn, i.e. S2002, Fig. 4), but this is not the case for the
current-year stem 2003 (Sn) in autumn (Fig. 5). Metabolites in Sn-1 (i.e. S2001, Fig. 4) were slightly labelled. It is interesting to note that sucrose and starch are the most abundant carbohydrates in leaves (ca. 7.1% and 4.7% of TOM, respectively) during June 2002 (Fig. 4). Leaves had their maximum sucrose values in summer when their glucose and fructose levels were the lowest. Fructose and starch are the most abundant carbohydrates in the stem (Fig. 4). During October 2003, starch decreases in the leaves and further increases in the stem (Fig. 5).

DISCUSSION

The relationships between leaf photosynthesis and respiration in trees

After ca. 300 mmol C per m² (June 2002) or ca. 100 mmol C per m² (October 2003) had been assimilated by leaves of field-grown beech trees during the day, the night-respired CO₂ was labelled, indicating that a pool of respiratory C was fed by photosynthesis and used by respiration in the dark. This pool may be carbohydrates in the cytosol, although transitory starch may be also rapidly mobilized (i.e., after 75 min) in the dark after a light period. Another possibility is the remobilization of sucrose stored in the vacuole contributing to the respiration feeding. Further experiments are needed to specify the contribution of each carbohydrate pool to feed the respiratory pool. Nevertheless, it can be noted that the δ¹³C value of CO₂ produced the second day is closer to the δ¹³C value of starch (in both June and October) than it is to the Suc or Glc values, suggesting that starch might be the main contributor to respiration feeding in darkness.

All the C used by leaf respiration does not come from recent assimilates. It is interesting to note that there was only ca. 58% of new C in respired CO₂ in June 2002. This value decreases to 27% in October 2003 (for calculations of the percent of new carbon in respired CO₂, see Nogués et al.¹¹). This implies that non-labelled C atoms (e.g. from ‘old’ starch) were still feeding night respiration after the labelling. This effect has been shown on the herbaceous species Phaseolus vulgaris grown in controlled conditions. As previously suggested, night respiration in leaves is fed by a mixture of ‘new’ and ‘old’ carbon, the contribution of the latter being as high as 50% after 16 h of illumination. Clearly, this also applies to CO₂ production in the dark by beech leaves grown in field conditions. Interestingly, in June (Fig. 2), the δ¹³C of the C in the respiratory CO₂ was the lowest at the end of the first night suggesting that a first respiratory pool was filled up first by some newly photosynthesized C, and that another respiratory pool was then fed slowly from the first one. In October (Fig. 3) there was an indication of such a process during the first hour of the night where there is a decline in the δ¹³C of the respiratory CO₂. The comparatively high δ¹³C value at the end of the night presumably results from the low
rate of photosynthesis during the previous day with the consequence of a low labelling in the first respiratory pool.

Fractionation during dark-respiration in tree leaves
On one hand, photosynthetic CO₂ assimilation discriminates against ¹³CO₂ so the fixed carbon is ca. 20% depleted in ¹³C compared with atmospheric CO₂.²⁷ On the other hand, it has been recently shown on herbageous greenhouse-grown plants that discrimination occurs during night respiration leading to the production of ¹³C-enriched CO₂ (between 4 and 6% compared with sucrose²⁵). Before the labelling, the ¹³C of respired CO₂ in attached leafy twigs of beech trees was ¹³C-enriched (ca. –18.5‰, Figs. 2 and 3) compared with the respiratory metabolites and TOM of the leaves (ca. –24 and –27‰, respectively, control values in the legends of Figs. 4 and 5). Such apparent discrimination during dark respiration in tree leaves grown in field conditions has been also recently shown by other workers.²⁸ They demonstrated that there is considerable short-term variation in the leaf-respired CO₂ in forest canopy (although leaves were removed from the trees), and that dial changes in leaf carbohydrate status could be used to predict changes in the leaf-respired CO₂ empirically. The ¹³C value of CO₂ respired by twigs was (at least for the periods considered) even higher than have been measured on current-year stems in the same species (in Damesin and Lelarge¹⁷ leaves were not measured and maximal stem ¹³C values were –22.1‰). It has also recently been shown that leaf respiratory CO₂ is ¹³C-enriched (relative to TOM) in five species of C₃ trees grown under controlled environmental conditions.²⁹ So ¹³C enrichment in dark-respired CO₂ seems to be a general feature in Angiosperms. Tcherkez and coworkers¹⁹ proposed that this isotopic discrimination during respiration can be explained by (i) the C source used for respiration, (ii) the iso gas exchange/IRMS coupling. We thank Max Hill for technical assistance with the HPLC procedure. This work was supported by the European Community’s Human Potential Program under contract HPRN-CT-1999-00059, NETCARB.

CONCLUSIONS
The ¹³C/¹²C isotopic labelling technique allowed us to subject leafy twigs of beech trees to CO₂ with a constant C isotope composition (i.e. δ¹³C of –51.2‰) in an open system in the field, in order to study leaf carbon respiration and allocation. The ¹³C values of respiratory metabolites and CO₂ that is subsequently respired during the night were measured. It is found that night-respired CO₂ is not completely labelled (only ca. 58% and 27% of new carbon is found in respired CO₂ immediately after the labelling in June 2002 and October 2003, respectively) and the labelling level progressively disappeared during the next day. Consequently, the C respired after illumination by beech leaves was supplied by a mixture of C sources in which current carbohydrates were not the only contributors. Furthermore, isotopic data before labelling showed that a C isotope discrimination favoring the ¹³C isotope occurred during night respiration of beech leaves.

Acknowledgements
We are grateful to Marc Berry for setting up the gas-exchange/IRMS coupling. We thank Max Hill for technical assistance with the HPLC procedure. This work was supported by the European Community’s Human Potential Program under contract HPRN-CT-1999-00059, NETCARB.

REFERENCES

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