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On the $^{13}C/^{12}C$ isotopic signal of day and night respiration at the mesocosm level†

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ABSTRACT

While there is currently intense effort to examine the ^{13}C signal of CO_2 evolved in the dark, less is known on the isotope composition of day-respired CO_2. This lack of knowledge stems from technical difficulties to measure the pure respiratory isotopic signal: day respiration is mixed up with photorespiration, and there is no obvious way to separate photosynthetic fractionation (pure c_i/c_a effect) from respiratory effect (production of CO_2 with a different $\delta^{13}C$ value from that of net-fixed CO_2) at the ecosystem level. Here, we took advantage of new simple equations, and applied them to sunflower canopies grown under low and high [CO_2]. We show that whole mesocosm-respired CO_2 is slightly ^{13}C depleted in the light at the mesocosm level (by 0.2–0.8‰), while it is slightly ^{13}C enriched in darkness (by 1.5–3.2‰). The turnover of the respiratory carbon pool after labelling appears similar in the light and in the dark, and accordingly, a hierarchical clustering analysis shows a close correlation between the ^{13}C abundance in day- and night-evolved CO_2. We conclude that the carbon source for respiration is similar in the dark and in the light, but the metabolic pathways associated with CO_2 production may change, thereby explaining the different $^{12}C/^{13}C$ respiratory fractionations in the light and in the dark.

Key-words: Day respiration; isotope; fractionation; mesocosm; sunflower.

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INTRODUCTION

Isotopic fractionation against $^{13}CO_2$ during photosynthesis (denoted as Δ) drives the isotopic signal of ecosystems, in which the carbon isotope composition of fixed CO_2 follows the well-accepted relationship involving internal CO_2 mole fraction and fractionations associated with diffusion and carboxylation (Farquhar, O'Leary & Berry 1982; Lloyd & Farquhar 1994). Nevertheless, at the ecosystem level, CO_2 evolved by plant respiration is believed to account for 30–70% of the ecosystem CO_2 exchange (Amthor 2000) so that the isotopic signal of respired CO_2 has a major influence on the $^{12}C/^{13}C$ ecosystem mass balance (Lavigne *et al.* 1997). Therefore, current efforts are devoted to elucidating the isotopic signal of CO_2 evolved by several ecosystem compartments such as trunks (Brandes *et al.* 2006; Gessler *et al.* 2007; Maunoury *et al.* 2007) and soil (Ekblad & Hogberg 2001; Bostrom, Comstedt & Ekblad 2007). At the leaf level, there is now compelling evidence that photorespiration fractionates against ^{13}C, thereby liberating ^{13}C-depleted CO_2 as compared to photosynthates (Lanigan *et al.* 2008).

In contrast, little is known on the isotopic composition of day-respired CO_2, although several authors have suggested that day respiration produces ^{13}C-depleted CO_2 (von Caemmerer 2000; Ghashghaie *et al.* 2003; Tcherkez *et al.* 2004; Lanigan *et al.* 2008). In the dark, leaf-respired CO_2 has been shown to be ^{13}C enriched, with some variations that depend on the respiratory rate, which is in turn influenced by leaf temperature or leaf respiratory substrates (Duranceau *et al.* 1999; Ghashghaie *et al.* 2001; Tcherkez *et al.* 2003). In fact, leaf-respired CO_2 is considerably ^{13}C enriched just after darkening (Mortazavi *et al.* 2005; Barbour *et al.* 2007; Werner *et al.* 2007; Gessler *et al.* 2009), and then reaches a steady value up to 6‰ enriched as compared to sucrose (Duranceau *et al.* 1999). The isotope composition of CO_2

evolved by other organs is less well documented: while roots have been repeatedly shown to produce ^{13}C-depleted CO_2 (Badeck *et al.* 2005; Klumpp *et al.* 2005; Bathellier *et al.* 2009), twigs and trunks have a more variable pattern that depends upon environmental parameters (Damesin & Lelarge 2003). However, the respiratory contribution of all non-photosynthetizing organs when integrated as isotopic fractionations at the plant level is not well defined (for a recent review, see Bowling, Pataki & Randerson 2008).

In consequence, it has often been assumed that the respiratory isotopic signal of plants, mesocosm and ecosystems is similar in the light and in the dark (e.g. Schnyder *et al.* 2003, but see Kodama *et al.* 2008 and references therein). For isotope partitioning studies, the value of ecosystem-respired CO_2 is typically measured at night, and then used to partition respiratory processes of the light period (for a specific discussion on this topic, see Zobitz *et al.* 2008). However, such an assumption does not seem consistent with published data of isotopic biochemistry of plants (see also Cernusak *et al.* 2009 for a review). Firstly, a positive relationship has been observed between the carbohydrate content and the respiration rate in many plant organs (see, e.g. Azcon Bieto & Osmond 1983; Tjoelker *et al.* 2008). In leaves, the availability of respiratory substrates has a clear impact on the $\delta^{13}C$ value of dark-evolved CO_2 (Tcherkez *et al.* 2003; Hymus *et al.* 2005; Gessler *et al.* 2009). For organs other than leaves, where circadian rhythms occur in sucrose content, there may be different $\delta^{13}C$ values in respiratory CO_2 in the light and in the dark. Secondly, sucrose molecules produced in the dark (from ^{13}C-enriched, transitory starch) and in the light (from ^{13}C-depleted, cytoplasmic triose phosphates) do not have the same isotope composition (Tcherkez *et al.* 2004; Gessler *et al.* 2008). This may drive a light/dark cycle of the $\delta^{13}C$ value of CO_2 evolved by source or sink organs depending on whether respiration is dominated by autotrophic or heterotrophic processes in those tissues. Thirdly, while some organs such as roots do not have any apparent growth circadian rhythm, others have (leaves and secondary meristems in stems) (Zweifel, Item & Hasler 2001; Walter & Schurr 2005; Deslauriers *et al.* 2007). Owing to the tight relationship between respiration and plant growth (for a review, see Amthor 2000), light/dark growth variations may have an effect on the $\delta^{13}C$ of evolved CO_2.

In the present paper, the isotopic signal of day-respired CO_2 was determined (^{13}C enrichment or depletion compared to net fixed CO_2) as compared to dark-evolved CO_2. We carried out mesocosm-level experiments with sunflower (*Helianthus annuus*) canopies, in which both the day and dark isotopic signals associated with respiration were investigated and compared with CO_2 evolved by individual organs. For this purpose, we propose two techniques to measure the isotopic impact of CO_2 evolved in the light. Furthermore, a $^{12}C/^{13}C$ labelling was conducted in order to see metabolic correlations between day-respired CO_2, dark-respired CO_2 and different plant components.

MATERIALS AND METHODS

Experimental

Plant material and growth conditions

Sunflower (*H. annuus* L. cv. Sanluca) plants were sown individually in plastic pots (5 cm diameter – 35 depth) filled with washed quartz sand. The pots were distributed at a density of 118 plants m^{-2} in two growth chambers (E15, Conviron, Winnipeg, Canada). Modified Hoagland nutrient solution (7.5 mol N m^{-3}) was supplied by an automatic irrigation system throughout the experiment. Irradiance during the 16 h photoperiod was supplied by cool white fluorescent tubes (16 × 160 W; Sylvania Germany GmbH, Erlangen, Germany) and incandescent lamps (12 × 100 W; General Electric Germany, München, Germany), and was maintained at 520 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) at the top of the canopy by adjusting the height of the lamps following plant development. Air temperature was controlled at 20/16 °C and relative humidity at 75/80% during the photo and dark periods, respectively. The CO_2 mole fraction of the air in the chamber was 200 and 1000 μmol mol^{-1} (chambers 1 and 2, respectively; for details on gas mixing and controlling, see next paragraph).

Gas exchange measurements and isotope analysis

Mesocosm level. The two growth chambers formed part of the mesocosm $^{13}CO_2/^{12}CO_2$ open gas exchange system described in detail by Schnyder *et al.* (2003). A screw compressor (S40, Boge, Bielefeld, Germany) and adsorption dryer (KEN3100, Zander, Essen, Germany) generated CO_2-free, dry air. Each chamber had an individual gas-mixing system comprising two computer-operated mass flow controllers (FC-2925V for air and FC-2900 4S for CO_2, Tylan General, San Diego, CA, USA) mixing dry air with CO_2 of known isotopic composition ($\delta^{13}C$). The CO_2 concentrations were held constant at the chamber outlets by adjusting the rate and CO_2 concentration of the air supplied to the increasing rates of photosynthesis. For each CO_2 concentration, the chambers were first supplied with CO_2 coming from a mineral source (^{13}C enriched, $\delta^{13}C$ –3.5‰) (pre-labelling period) and then originating from a fossil organic source (^{13}C depleted, $\delta^{13}C$ –44.5‰; both CO_2 from Linde AG, Munich, Germany) (labelling).

For measurements of CO_2 fluxes and associated on-line $^{12}C/^{13}C$ discrimination of the canopies, sample air was collected at the inlet and outlet (vent) of each growth chamber, and continuously pumped to a computer-controlled sample air selector (SAS) at a rate of approx. 2 L min^{-1}. During simultaneous operation of all chambers, the SAS sequentially sampled each sample air line ($n = 8$) at 2 min intervals. Sample air was split to serve the infrared gas analyser (IRGA; Li-6262; Li-Cor Inc., Lincoln, NE, USA) and isotope ratio mass spectrometer (IRMS; Delta Plus, Finnigan MAT, Bremen, Germany) in parallel. Gas lines between the SAS, IRMS and IRGA were flushed with sample air for 2 min before taking IRGA readings of absolute CO_2 and

H_2O concentration and measurement of $\delta^{13}C$ of CO_2 by IRMS. The IRMS was interfaced with the SAS *via* a steel capillary tube (i.d. 0.1 mm), a six-port, two-position valve (Valco Instruments Co. Inc., Houston, TX, USA), dryer (Nafion), gas chromatograph (25 m × 0.32 mm Poraplot Q; Chrompack, Middelburg, the Netherlands) and open split. These components all formed part of a custom-made interface (GC–GP Interface; Finnigan MAT). Sample air was pumped continuously through the steel capillary feeding the Valco valve and a 300 μL sample loop attached to it. After a 2 min flushing period, shortly before the SAS switched to the next sample air line, the content of the sample loop was swept with helium carrier gas through the interface, where water vapour was removed by the Nafion membrane, and CO_2 was separated from all other gaseous components of the air sample in the GC column. Finally, the CO_2 was introduced directly into the ion source of the IRMS *via* the open split. Samples were compared with a V-PDB-gauged, working standard reference CO_2 injected once at the start and once at the end of a measurement cycle. The overall precision of the measurement at the chamber inlets over a 24 h period was typically better than 0.15‰. A full measurement cycle, including one set of measurements (concentrations of CO_2 and H_2O, and $\delta^{13}C$ of CO_2) on the inlet and outlet of each growth chamber, was completed within 24 min.

The net CO_2 exchange flux of the canopies (N, μmol CO_2 m^{-2} s^{-1}) was obtained as the balance of CO_2 entering and leaving the chamber divided by the chamber ground area (s, m^2):

$$N = (E - O)/s$$

with E and O the fluxes of CO_2 (μmol s^{-1}) entering and leaving the chamber. The difference $E - O$ is denoted as P below ('Calculations' in Materials and methods). In the light, N is the net assimilation value at the mesocosm level, and as such, it is denoted as A (net assimilation) in the following. In the dark, N is the negative of the mesocosm-level respiration rate.

Mesocosm-scale 'on-line' $^{12}C/^{13}C$ discrimination (denoted as Δ) during photosynthesis (i.e. gas exchange in light) was obtained as given by Evans *et al.* (1986):

$$\Delta = \frac{\xi(\delta_o - \delta_e)}{1 + \delta_o - \xi(\delta_o - \delta_e)}$$

where $\xi = c_e/(c_e - c_o)$, and c_e and c_o are the CO_2 mole fractions (μmol mol^{-1}) of the air entering and leaving the chamber after correcting to standard humidity.

While N could be measured continuously from the first day of the experiment, the associated isotope analyses could be done with reasonable precision only after day 12, when rates of photosynthesis were high enough to yield significant differences in the C-isotopic composition of the CO_2 entering and leaving the chambers. The $\delta^{13}C$ of dark-respired CO_2 was measured at days 30–34, that is, when canopies were closed (plants at the three fully expanded leaves stage) and CO_2 exchange rates had reached a steady state on a day-to-day basis. At days 35 and 36, CO_2 response curves of canopies were carried out by changing the CO_2 concentration at the chamber inlet gradually from 100 to 1500 μmol mol^{-1} within 2 h. Again, measurements of net CO_2 exchange fluxes were accompanied by the simultaneous determination of Δ. The corresponding results of the A/c_a curve are shown in the Supporting Information Fig. S1. At day 37 after the start of the experiment, canopies were isotopically labelled by switching the source CO_2 from mineral (–3.5‰) to fossil organic (–44.5‰) CO_2. Gas exchange analyses continued for 5 more days with labelling CO_2 and otherwise unchanged growth conditions.

Organ level. One plant was removed from the growth chambers described above, and the attached organs were placed in a closed system for the on-line measurement of the $\delta^{13}C$ of the respired CO_2. The closed system which was described previously by Tcherkez *et al.* (2003) was directly coupled to the IRMS (Finnigan) as specified above. The procedure for accumulating respired CO_2 in the dark and measure the isotope composition was identical. All experiments were carried out on the top expanding (EL) and mature (ML) leaves, and in the adjacent stem (ST). The contribution to the mesocosm exchange of the two cotyledons was considered negligible as they had died at the time the measurements were done. The photosynthetic fractionation at the leaf level was measured following the procedure of Nogués *et al.* (2004) and Tcherkez *et al.* (2005), that is, by coupling the portable gas exchange system (TPS-2, PP systems) to the IRMS through a three-way valve.

Metabolite extraction and quantification. Plants were harvested and the different organs were frozen in liquid nitrogen, lyophilized and analysed as follows. The extraction–purification procedure for starch, sucrose, glucose and fructose was that described previously (Tcherkez *et al.* 2003). Purified metabolites were lyophilized, resuspended in 100 μL of distilled water and then transferred to tin capsules (Courtage Analyze Service, Mont-Saint-Aignan, France) and dried for isotope analysis. The isotope analysis of metabolites and total organic matter was carried out with the EA-IRMS (EA1500; Carlo-Erba, Milan, Italy, coupled to the Optima, GV Instruments (Villeurbanne, France) on the isotopic facility structure *Plateforme Métabolisme-Métabolome*, as already described (Tcherkez *et al.* 2003; Nogués *et al.* 2004).

Calculations

In the following, simple relationships are used to derive the isotopic signature of respired CO_2 in the light. Firstly, the mesocosm system was considered as a big assimilation cuvette, and equations derived from gas exchange are used to calculate an estimate of the contribution (thereafter denoted as d^*/c_a) of day mesocosm-respired CO_2 to net photosynthetic fractionation of the mesocosm. Secondly, the contribution of non-photosynthetic organs to the net photosynthetic fractionation of the mesocosm was

Table 1. Summary of the main symbols used (upper panel) and main values examined (lower panel) in the present paper

Expression or symbol used (units)	Conditions	Description
Δ (‰)	Light	Isotope fractionation associated with net photosynthesis (CO_2 exchange) of the mesocosm
Δ_A (‰)	Light	Average isotope fractionation associated with net photosynthesis (CO_2 exchange) of leaves
Δ^0 (‰)	Light	Isotope fractionation associated with mesocosm-level net photosynthetic assimilation, when net photosynthesis tends to zero
e (‰)	Light	Isotope fractionation associated with leaf day respiration
f (‰)	Light	Isotope fractionation associated with CO_2 evolution by photorespiration at the leaf level
e_{np} (‰)	Light	Isotope fractionation associated with respiration of non-photosynthetic organs in the light at the mesocosm level
e_{app} (‰)	Light	Apparent isotope fractionation associated with respiration of non-photosynthetic organs in the light after isotopic labelling at the mesocosm level
N (μmol m^{-2} s^{-1})	Light/Night	Net mesocosm CO_2 exchange
P (μmol s^{-1})	Light/Night	Net mesocosm CO_2 exchange (not scaled to surface area)
x_{day} (dl.)	Light	Proportion of recent carbon in CO_2 respired by non-photosynthetic organs in the light at the mesocosm level
x_{night} (dl.)	Night	Proportion of recent carbon in CO_2 respired by the mesocosm in darkness at the mesocosm level
d^*/c_a (‰)	Light	(Photo)respiratory isotopic contribution to the photosynthetic fractionation at the mesocosm level (Eqn 2)
$e_{np}\bar{R}q_r/P$ (‰)	Light	Respiratory isotopic contribution of non-photosynthetic organs to the photosynthetic fractionation at the mesocosm level (Eqn 3)
e_n (‰)	Night	Isotope fractionation of night respiration by the mesocosm (Eqn 7)

dl., dimensionless.

estimated by taking advantage of the net photosynthetic fractionation of leaves that was measured separately. That is, the respiratory isotopic signal of non-photosynthetic organs (thereafter denoted as $e_{np}\bar{R}q_r/P$, see the definition of symbols in Table 1 and just below) was determined by 'substracting' the effect of leaf net photosynthesis from the mesocosm-level photosynthetic fractionation. Thirdly, the respiratory isotopic signal of non-photosynthetic organs was followed after isotopic CO_2 labelling to calculate the proportion (denoted as x_{day}) of old, pre-labelling carbon to CO_2 production in the light.

The main parameters used throughout the present paper and explained below are summarized in Table 1. The expression '(photo)respiratory' CO_2 used in the following stands for the sum of photorespiratory and day respiratory evolved CO_2.

The isotopic (photo)respiratory component of Δ at the mesocosm level

The (photo)respiratory component of the net photosynthetic carbon isotope discrimination Δ (i.e. the (photo)respiratory term in Farquhar's expression giving Δ; see Eqn 1) was calculated using equations derived from Evans *et al.* (1986) as explained below. The present methods were developed to avoid the need of c_i, the internal CO_2 mole fraction, because this parameter could not be reached at the mesocosm level in the present study. The (photo)respiratory component is denoted here as d^* as follows (Farquhar *et al.* 1982):

$$\Delta = a\frac{c_a - c_i}{c_a} + a_i\frac{c_i - c_c}{c_a} + b\frac{c_c}{c_a} - \frac{d^*}{c_a} \quad (1)$$

where Δ is the net photosynthetic (observed) carbon isotope discrimination, and a, a_i and b are the fractionations associated with diffusion in air, dissolution and diffusion in water, and carboxylation, respectively. c_a, c_i and c_c are the CO_2 mole fractions in the atmosphere, in the intercellular spaces and at the carboxylation sites, respectively. d^* is equal to $e\frac{R_d}{k} + f\Gamma^*$ where e and f are the carbon isotope fractionation associated with day respiration (the rate of which is R_d) and photorespiration. Γ^* is the CO_2 compensation point in the absence of day respiration, and k is the carboxylation efficiency.

In both methods used below, it is assumed that d^* is constant with respect to c_a. This hypothesis may not be verified when c_a varies because k depends upon CO_2 mole fraction. Here, we used the region of low assimilation values in which the relative change of k is rather small, so that the assumption of a constant d^* is valid.

We also recognize that the value of d^* obtained here includes both leaf day respiration and heterotrophic respiration (from roots and stems), and so is not strictly equal to that used in the equation of Farquhar *et al.* (1982) (Eqn 1). That is, the day respiratory component eR_d/kc_a applies at the level of c_c (i.e. day-respired CO_2 is released into the intracellular CO_2 pool), while CO_2 evolved by heterotrophic organs is not released at c_c but rather into surrouding air (at c_a). Nevertheless, Eqn 1 is not altered. Let us denote as δ_h the carbon isotope composition of CO_2 evolved by non-photosynthetic organs, and ε the CO_2 amount (in μmol mol^{-1}) produced by respiration of non-photosynthetic organs. Δ_A is the net photosynthetic fractionation of photosynthetic organs (leaves). By mass balance, we have:

$$\delta_e c_e = \delta_o c_o + (c_e - c_o + \varepsilon)\left(\frac{\delta_o - \Delta_A}{1+\Delta_A}\right) - \varepsilon\delta_h$$

This re-arranges to:

$$\Delta_{obs} = \Delta_A - \frac{[\delta_o - \Delta_A - \delta_h(\Delta_A + 1)]\varepsilon}{c_e(\delta_e + 1) - c_o(\delta_o + 1)}$$

where Δ_{obs} is the observed discrimination at the mesocosm level (including both leaves and heterotrophic organs), using the equation of Evans *et al.* (1986), as indicated above. Within the numerator, the term $\delta_h\Delta_A$ may be neglected, giving $\delta_o - \Delta_A - \delta_h$, which is very close to the isotope fractionation associated with respiration of heterotrophic organs, with respect to carbon fixed by leaves. Let us denote this fractionation as e_h. The denominator is very close to $c_e - c_o$, and the ratio $\varepsilon/(c_e - c_o)$ is equal to R_h/A, where R_h denotes respiration by heterotrophic organs, and A is net mesocosm photosynthesis. This gives:

$$\Delta_{obs} = \Delta_A - \frac{e_h R_h}{A}$$

In other words, the apparent value of d^* obtained from Δ_{obs} (as explained below) here includes an additional term that represents heterotrophic respiration. That is,

$$d^* = \frac{eR_d}{k} + \frac{e_h R_h}{A} c_a + f\Gamma^*$$

The heterotrophic term $e_h R_h c_a/A$, and the leaf term eR_d/k are very similar because the carboxylation efficiency k is defined as v_c/c_c, where v_c is the carboxylation rate and c_c is internal CO_2 mole fraction. Therefore, the input of heterotrophic respiration to mesocosm CO_2 at the level of c_a does not modify the general equations described here, and the d^* value computed below simply includes heteretrophic respiration in the light. It should be noted that similarly, there would also be a contribution of heterotrophic respiration to the apparent d^* value at the leaf level because of the contribution of non-photosynthetic leaf cells such as phloem tissue, epidermis, etc. In other words, the present equations may also apply to leaf-level gas exchange experiments, even though the heterotrophic term $e_h R_h c_a/A$ is quantitatively modest in most leaves.

First method. We used the following relationships: $A = g_s(c_a - c_i) = g_m(c_i - c_c)$, where A is CO_2 net assimilation, and g_s and g_m are the stomatal and internal conductance for CO_2, respectively. Subtracting b from each side of Eqn 1, and re-arranging gives:

$$(b - \Delta)\cdot c_a = \left(\frac{b-a}{g_s} + \frac{b-a_i}{g_m}\right)\cdot A + d^* \quad (2)$$

In the following, we denote the left-hand side of Eqn 2 by $\langle\Delta\rangle$. In the present study, experiments involved A/c_a curves, that is, variation of the CO_2 level (Supporting Information Fig. S1). Importantly, the use of Eqn 2 does not require c_a to be constant as d^* is assumed c_a independent, and we used the intercept (and not the slope). When plotted against A, $\langle\Delta\rangle$ shows a non-linear relationship, simply because both conductances vary with A. Stomatal conductance typically decreases at high CO_2, and so the slope of the $\langle\Delta\rangle$-versus-A relationship increases with A. Similarly, it has been recently shown that internal conductance responds to changes in c_a (Flexas *et al.* 2007). Nevertheless, when A converges to zero, $\langle\Delta\rangle$ converges to d^*. In other words, with a set of Δ, c_a and A values, extrapolating $\langle\Delta\rangle$ at $A = 0$ gives a value of d^*. This graphical method is depicted in Fig. 1, in which the linear regression used data with A less than 17 μmol m^{-2} s^{-1} (linear region of the plot).

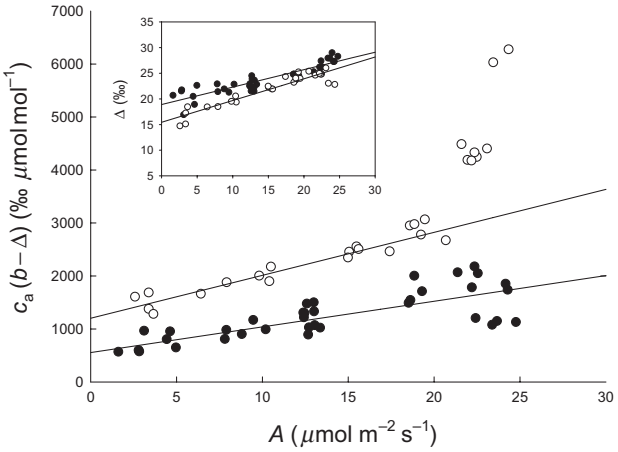

Figure 1. Plot of $\langle\Delta\rangle = c_a(b - \Delta)$ as a function of net CO_2 assimilation A at the mesocosm level when assimilation is made to vary with CO_2 mole fraction c_a (the A/c_a curve is given as a Supporting Information). The growth CO_2 conditions were 200 μmol mol^{-1} (chamber 1, closed symbols) or 1000 μmol mol^{-1} (chamber 2, open symbols). The calculations use $b = 29$‰. The extrapolated values of $\langle\Delta\rangle$ for $A = 0$ (intercepts) are 604‰ μmol mol^{-1} (chamber 1) and 1183‰ μmol mol^{-1} (chamber 2). Both regressions were significant with $P < 0.008$ ($r^2 = 0.54$ and 0.83, and $F = 11.4$ and 38.4, respectively). Inset: mesocosm-level net photosynthetic fractionation Δ as a function of A. The extrapolated values of Δ at $A = 0$ (intercepts) are 18.89‰ (chamber 1, closed symbols) and 15.44‰ (chamber 2, open symbols).

Second method. Equation 2 is still valid at the CO_2 compensation point, Γ, at which $A = 0$. This simply gives:

$$d^* = \Gamma(b - \Delta^0) \quad (3)$$

where Δ^0 is the net photosynthetic carbon isotope discrimination for $A = 0$. Clearly, such a value cannot be measured (no net CO_2 exchange) and should be extrapolated with a plot representing Δ against A. This method is depicted in the inset of Fig. 1.

The respiratory contribution of non-photosynthetic organs to mesocosm-level isotopic gas exchange in the light

The respiratory component of isotopic exchange between the mesocosm and the atmosphere was calculated using

classical mass balance equations and taking advantage of the photosynthetic carbon isotope fractionation measured at the leaf level and applied to the photosynthetizing leaves of the mesocosm. We used the following iso-flux conservation equation at the level of the mesocosm:

$$u(c_o\delta_o - c_e\delta_e) + \bar{A}q_A\overline{\delta_{fixed}} = \bar{R}q_r\overline{\delta_r} \quad (4)$$

where q_A and q_r are the total mass of leaves and non-photosynthetic organs (roots and stems), respectively; u is the air flow rate through the mesocosm chamber (in mol s^{-1}); c_o and c_e are the outlet and inlet CO_2 mole fractions (in μmol mol^{-1}) corrected to standard humidity, with the associated $\delta^{13}C$ values δ_o and δ_e, respectively. $\bar{A}$ is the average net photosynthetic rate (in μmol g^{-1} s^{-1}) of leaves. $\bar{R}$ and $\overline{\delta_r}$ are the mass average respiratory rate and the respiration average isotopic composition of CO_2 respired by non-photosynthetic organs, respectively. $\overline{\delta_{fixed}}$ is the isotope composition of net fixed carbon in leaves.

If we denote as P the net CO_2 exchange by the mesocosm (in μmol s^{-1}) in the light, we have: $P = \bar{A}q_A - \bar{R}q_r$. Equation 4 may then be rewritten using P, which is the parameter measured by the mesocosm system. This gives:

$$u(c_o\delta_o - c_e\delta_e) + P\overline{\delta_{fixed}} = \bar{R}q_r\left(\overline{\delta_r} - \overline{\delta_{fixed}}\right)$$

With the proxies $\overline{\delta_{fixed}} \approx \delta_o - \overline{\Delta_A}$ where $\overline{\Delta_A}$ is the average leaf net photosynthetic fractionation, and $e_{np} \approx \overline{\delta_{fixed}} - \delta_r$ where e_{np} is the respiratory fractionation by non-photosynthetic organs, we have:

$$-e_{np}\bar{R}q_r = u(c_o\delta_o - c_e\delta_e) + P\left(\delta_o - \overline{\Delta_A}\right) \quad (5)$$

It is not possible to separate the three variables in the left term of Eqn 5; it represents the non-photosynthetic respiratory contribution to the mesocosm-level fractionation. However, such a value is in ‰ μmol s^{-1}, which is not consistent with d^*/c_a value (see above), that is, the (photo)respiratory contribution to the mesocosm-level fractionation (in ‰). At the mesocosm level, we used here $e_{np}\bar{R}q_r/P$, which is a scaled contribution of respiratory isotopic signal, and is in ‰. This may be calculated by dividing all the terms of Eqn 5 by P. It should be emphasized that the use of Eqn 5 does not require any assumption on the carbon isotope composition of CO_2 evolved by respiration in the light. Other authors suppose a similar value in the light and in the dark. Nevertheless, this assumption is unlikely, simply because of the contribution of photosynthetizing organs to night respiration, and such organs have proven to be isotopically different (lighter) than other organs. It should be noted that the $e_{np}\bar{R}q_r/P$ value computed with Eqn 5 is relative because it depends upon the leaf photosynthetic fractionation $\overline{\Delta_A}$: any slight variation of this parameter at the mesocosm scale as compared to the leaf-level measurement causes variations in $e_{np}\bar{R}q_r/P$. In practice, we used the average of pre-labelling $\overline{\Delta_A}$ values (mature leaves). Any error in $\overline{\Delta_A}$ only induces an offset in $e_{np}\bar{R}q_r/P$ and does not impact on the percentage of new carbon (see below Eqn 6) and the covariation analysis (Fig. 4).

The contribution of recent photosynthates to respiration of non-photosynthetic organs in the light

The term $e_{np}\bar{R}q_r/P$ of Eqn 5 is expected to vary after CO_2 labelling. For example, if the labelling CO_2 is ^{13}C depleted, the CO_2 produced by respiration is expected to be strongly ^{13}C enriched relative to fixed CO_2 because of the lag phase needed to renew respiratory pools. In other words, the use of 'old', ^{13}C-enriched carbon by respiration will artificially lead to a large negative $e_{np}\bar{R}q_r/P$ term (this is illustrated in Fig. 3). That is, e_{np} is artificially decreased by the decarboxylation of old, ^{13}C-enriched carbon. In the following, we then denote it as e_{app} and use e_{np} for the intrinsic respiratory fractionation (which is independent of the ^{13}C abundance of the C source).

We may use the apparent $e_{app}\bar{R}q_r/P$ value to calculate the proportion of 'old' and recent carbon in CO_2 evolved by non-photosynthetic organs in the light. The proportion of recent carbon in CO_2 is denoted as x_{day} in the following. The isotope composition of respired CO_2 coming from 'old' carbon is approx. $\overline{\delta_r} = \delta_o - \overline{\Delta_A} - e_{np}$ where, again, e_{np} is here the 'intrinsic', $\delta^{13}C$-independent, respiratory fractionation, while that of new carbon is approx. $\delta_o' - \overline{\Delta_A} - e_{np}$ where δ_o' is the new (labelled) isotope composition of outlet air. The resulting isotope composition of respiratory CO_2 is then:

$$\overline{\delta_r}' = (1 - x_{day})\left(\delta_o - \overline{\Delta_A} - e_{np}\right) + x_{day}\left(\delta_o' - \overline{\Delta_A} - e_{np}\right)$$

that simply re-arranges to: $\overline{\delta_r}' = \overline{\delta_r} + x_{day}(\delta_o' - \delta_o)$, that is,

$$x_{day} = \frac{\overline{\delta_r}' - \overline{\delta_r}}{\delta_o' - \delta_o}$$

$\overline{\delta_r}$ in the light cannot be calculated nor measured. We take advantage here of the definitions (see the above section):

$$\left(\overline{\delta_r} - \overline{\delta_{fixed}}\right) = e_{app} \quad \text{and} \quad \left(\overline{\delta_r}' - \overline{\delta_{fixed}}'\right) = e_{app}'$$

where the symbol 'prime' indicates values obtained after labelling. As $\overline{\delta_{fixed}} \approx \delta_o - \overline{\Delta_A}$ and $\overline{\Delta_A}$ is assumed constant before and after labeling, we have:

$$\overline{\delta_r}' - \overline{\delta_r} = (\delta_o' - \delta_o) + (e_{app}' - e_{app})$$

In order to get an estimate of x_{day}, we may multiply its numerator and denominator by $\bar{R}q_r/P$, giving:

$$x_{day} = 1 + \left(\frac{e_{app}'\bar{R}q_r/P - e_{app}\bar{R}q_r/P}{\delta_o' - \delta_o} \times \frac{P}{\bar{R}q_r}\right) \quad (6)$$

x_{day} is then calculated using the $e_{app}\bar{R}q_r/P$ value calculated with Eqn 5 before and after (symbol 'prime') labelling, and the value of $\bar{R}q_r$ measured at night, when only respiration occurs (it is equal to $-P$ measured at night). Such a value of $\bar{R}q_r$ is somewhat overestimated because photosynthetic

organs respire at night; hence, the x_{day} value obtained with Eqn 6 is slightly overestimated.

The contribution of recent photosynthates to mesocosm respiration in darkness

The carbon isotope composition of CO_2 respired by the whole mesocosm (photosynthetic and non-photosynthetic organs) in darkness is denoted as δ_n. The isotope fractionation associated with night respiration of the mesocosm, with respect to CO_2 fixed in the previous light period is then:

$$e_n = \frac{\overline{\delta_{fixedm}} - \delta_n}{1 + \delta_n} \quad (7)$$

where $\overline{\delta_{fixedm}} \approx \delta_o - \Delta$ is the isotope composition of mesocosm-level net fixed carbon, and Δ is the mesocosm-level net photosynthetic fractionation. The subscript 'n' is used here to distinguish the respiratory isotope fractionation in darkness from that in the light (denoted as e; see above).

After labelling, the proportion of recent carbon in evolved CO_2 in darkness may be calculated. Such a proportion is denoted as x_{night} below, and is given by (Schnyder 1992; Schnyder *et al.* 2003; Nogués *et al.* 2004):

$$x_{night} = \frac{\delta_n^{after} - \delta_n^{before}}{\overline{\delta_{fixedm}} - e_n - \delta_n^{before}} \quad (8)$$

where the superscripts 'after' and 'before' refer to after and before labelling, respectively.

Covariation analyses

The covariation analysis was done following the isotopomic array representation of Tcherkez, Ghashghaie & Griffiths (2007). In the present study, the isotope composition of the different plant fractions (starch, sucrose, etc.) and respired CO_2 was recalculated to isotope ratios times 100. The intensity of the red or green colour represents the strength of the natural ^{13}C enrichment or depletion, respectively. Both the drawing of the array and the clustering analysis were done with the MeV 4.1 software (Saeed *et al.* 2003). The clustering of Fig. 4 is based on the cosine correlation method. The results are similar to other correlation methods, such as the Euclidean distance. To introduce mesocosm day-respired CO_2 into the correlation analysis, the estimated isotope composition ($\delta^{13}C$) of day-respired CO_2 was calculated with the values of $e_{app}\bar{R}q_r/P$ obtained with Eqn 5, as follows: $\delta^{13}C = \delta_o - \Delta - e_{app}\bar{R}q_r/P$, where δ_o is the isotope composition of mesocosm outlet air, and Δ is the mesocosm-level net photosynthetic fractionation (measured on-line). It should be noted that in Fig. 2, e_{app} is negative (favours ^{13}C) because of the breakdown of 'old', ^{13}C-enriched photosynthates. In other words, after the start of labelling, day-respired CO_2 is enriched compared to fixed CO_2.

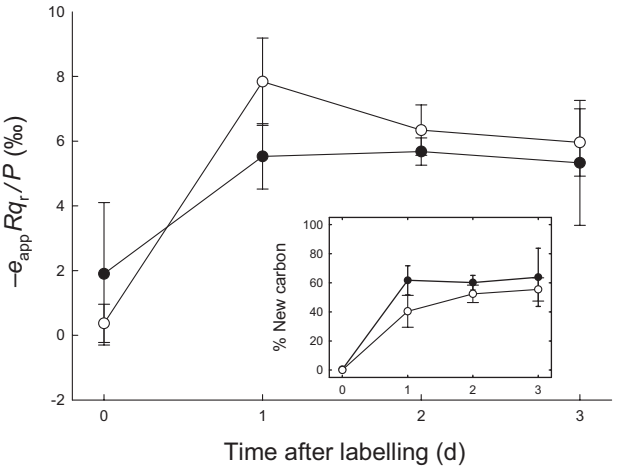

Figure 2. Time-course of the respiratory contribution of non-photosynthetic organs to the mesocosm-level photosynthetic fractionation (Eqn 5) after labelling with ^{13}C-depleted CO_2. Day 0 is the value just before labelling, day 1 is the first day of labelling and so on. For convenience reasons, the opposite value of $e\bar{R}q_r/P$ (minus sign) was plotted here because it is a negative value (pre-labelling carbon is ^{13}C enriched and so is evolved CO_2 in the light). The values of P, c_o and δ_o used here were that in the photosynthetic steady state. Inset: the corresponding proportion (in %) of recent carbon in evolved CO_2 (Eqn 6). Closed symbols: chamber 1 (200 μmol mol^{-1} CO_2); open symbols: chamber 2 (1000 μmol mol^{-1} CO_2).

RESULTS

Day-respired CO_2

The isotopic contribution of (photo)respiration to the net photosynthetic carbon isotope fractionation, usually written as $\frac{eR_d/k + f\Gamma^*}{c_a}$, is here abbreviated as d^*/c_a. At the mesocosm level, this term correponds to both leaf (photo) respired CO_2 and CO_2 evolved by other non-photosynthetic organs. It may be calculated using the intercept of the response curve of $\langle\Delta\rangle = c_a(b - \Delta)$ to net assimilation A (Eqn 2). Such response curves are shown in Fig. 1. Both chambers showed a linear relationship for low values of A. For chamber 2 (high CO_2 growth conditions), the $\langle\Delta\rangle$ value did not increase linearly at higher A values, and the slope increased at lower stomatal conductances (Eqn 2). Accordingly, the plot of Δ versus A shows indeed that the carbon isotope discrimination decreased slightly at high A values, indicating a lower c_i/c_a (Fig. 1, inset). Otherwise, stomatal aperture increased slowly with A as evidenced by the general positive trend between Δ and A (Fig. 1, inset).

The isotopic contribution of (photo)respiration at both the leaf and mesocosm scale was then calculated with the data of Fig. 1; the results are shown in Table 2. Equations 2 and 3 gave very similar results. When expressed on the same scale (at near-ambient CO_2 mole fraction, i.e. 400 μmol mol^{-1} as c_a), the isotopic contribution was larger under high than low CO_2, while both being in the range 1–3‰. The relative effect was higher when growth CO_2 mole fraction

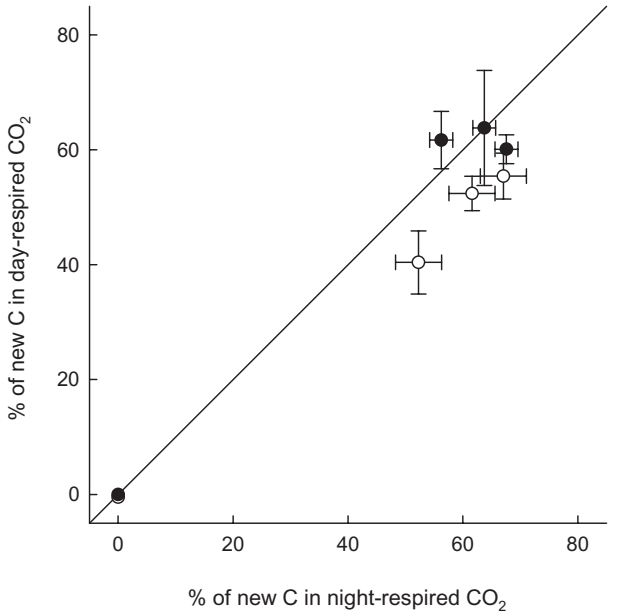

Figure 3. The relationship between the proportion (in %) of recent carbon in mesocosm night-respired CO_2 (x_{night}, Eqn 7) and that in CO_2 respired by non-photosynthetic organs in the light at the mesocosm level (x_{day}, Eqn 6), under progressive labelling with ^{13}C-depleted CO_2. Closed symbols: chamber 1 (200 μmol mol^{-1} CO_2); open symbols: chamber 2 (1000 μmol mol^{-1} CO_2). Continuous line: 1:1 line. The (0,0) point on the left-hand side corresponds to the pre-labelling conditions (day 0).

was used to compute d^*/c_a (2–3‰ versus 1‰ at 200 and 1000 μmol mol^{-1}, respectively). However, the d^*/c_a value included the photorespiration effect that was different at 200 and 1000 μmol mol^{-1} CO_2. With typical values of $f\Gamma^*$ ($f = 11$‰, Tcherkez 2006; Lanigan *et al.* 2008; $\Gamma^* \sim 40$ μmol mol^{-1}, Brooks & Farquhar 1985), the contribution of respiration $\frac{d^* - f\Gamma^*}{c_a} = \frac{eR_{day}}{kc_a}$ (Eqn 1) was very similar in both chambers, roughly around 0.5 ± 0.3‰ regardless of growth CO_2 mole fraction (Table 2).

Because c_a was larger in chamber 2, the similar $\frac{d^* - f\Gamma^*}{c_a}$ value probably came from a higher R_{day} value. In fact, respiration rates in the dark were more than 80% higher at 1000 μmol mol^{-1} CO_2 (6.3 μmol m^{-2} s^{-1} compared to 3.4 μmol m^{-2} s^{-1} at 200 μmol mol^{-1} CO_2). This arose from both a higher specific respiration rate (6.8 versus 5.4 nmol g^{-1} DW s^{-1}) and higher plant biomass (5.6 versus 3.9 g DW per plant) at 1000 compared to 200 μmol mol^{-1} CO_2. Still, both d^* values were (slightly) positive, showing that evolved CO_2 was ^{13}C depleted in the light ($e > 0$) with an average $\frac{d^* - f\Gamma^*}{c_a}$ value within the 0.6–0.8‰ range (Table 2, right column). These results are clearly sensitive to the $^{12}C/^{13}C$ fractionation associated with carboxylation (b value: see Eqns 2 & 3). While it is currently believed that $b = 29$‰, we also calculated the d^* with $b = 27$‰ (Table 2, lower): the $\frac{d^* - f\Gamma^*}{c_a}$ value was then lower, but fractionation against ^{13}C remained positive, within the 0.2–0.7‰ range.

Night-respired CO_2

The night respiration rate at the mesocosm level (N value in the dark, see Materials and methods) was 3.4 and 6.3 μmol m^{-2} s^{-1} in chambers 1 (200 μmol mol^{-1} CO_2) and 2 (1000 μmol mol^{-1} CO_2), respectively. The carbon isotope composition of CO_2 within the mesocosm or evolved by individual organs in darkness was measured, and the results are shown in Table 3, where the $\delta^{13}C$ values are also expressed as apparent fractionations with respect to net fixed CO_2 (between parentheses). At the mesocosm level, night-respired CO_2 was slightly ^{13}C enriched, so was leaf-respired CO_2, while roots produced ^{13}C-depleted CO_2, and stem respiration did not seem associated with an apparent fractionation. Such a pattern was very similar in both chambers. While there are not enough data to close the isotopic mass balance in our study, it is likely that ^{13}C-enriched, dark-respired CO_2 of the mesocosm was dominated by leaf respiration, which was the only ^{13}C-enriched signal (Table 3).

Day-respired CO_2 after labelling

The isotopic gas exchange of the mesocosm was followed during a labelling experiment that used ^{13}C-depleted CO_2 (−44.5‰) while maintaining the CO_2 mole fraction (200 and 1000 μmol mol^{-1}). The isotopic contribution of respiration of non-photosynthetic organs to the isotopic CO_2 exchange in the light (denoted as $e\bar{R}q_r/P$, Table 1) was calculated using the 'deviation' from the expected Δ (Eqn 5). The results are shown in Fig. 2 in which, for convenience, $-e\bar{R}q_r/P$ (positive value) was represented. All the values are associated with a large standard error, simply because very slight variations in P (the net mesocosm gas exchange, in μmol s^{-1}) or $\overline{\Delta_A}$ propagate into large uncertainties in $e\bar{R}q_r/P$. In other words, it is not possible to compare directly the initial value at day 0 and the isotopic contribution d^*/c_a computed above (see also Materials and methods).

That said, it is apparent that CO_2 evolved in the light was ^{13}C enriched, that is, respiration used carbon atoms that were fixed before labelling. The minimal proportion of 'new' carbon in day-respired CO_2 was calculated (Eqn 6; Fig. 2, inset). Both chambers behaved similarly with a progressive turnover of day-respired CO_2 which reached a maximum near 40% (chamber 2, at 1000 μmol mol^{-1}) and 60% (chamber 1, at 200 μmol mol^{-1}). That is, mesocosm respiration was fed by: (1) current photosynthates *via* a pool that had a half-life time of several hours (accounting for 40–60% of total respiration); and (2) stored carbon with a half-life time in the order of several days (accounting, respectively, for the remaining 40–60% of total respiration).

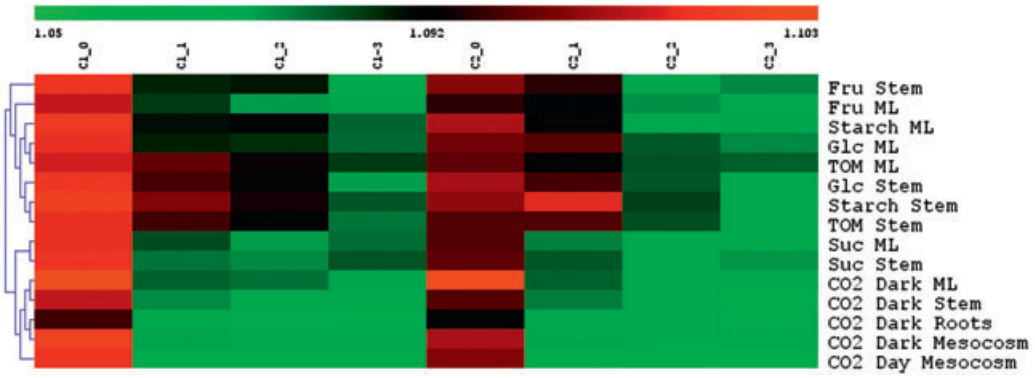

Figure 4. Isotopomic array representation and hierachical clustering (left) of isotope ratios in glucose (Glc), fructose (Fru), total organic matter (TOM), starch and sucrose (Suc), and respiratory CO_2 of mature leaves (ML), stems, roots and of the mesocosm. Data for chambers 1 (200 μmol mol^{-1} CO_2) and 2 (1000 μmol mol^{-1} CO_2) are indicated as C1 and C2, respectively, and the time after labelling (in days) is indicated as 0, 1, 2 and 3. The green and red colours mean ^{13}C depletion and 13C enrichment, respectively, as indicated by the colour scale above (in which the values are multiplied by 100 for clarity).

The comparison of day- and night-respired CO_2 by the mesocosm

The $\delta^{13}C$ value of CO_2 evolved by the mesocosm in the dark was also measured after labelling. As night-respired CO_2 is intrinsically ^{13}C enriched (see above and Table 3) while day-respired CO_2 was intrinsically ^{13}C depleted (see above and Table 2), it is more convenient to compare the proportions of 'new' carbon in respired CO_2 (Eqns 6 & 8) instead of $\delta^{13}C$ values. Such a representation is shown in Fig. 3. While being somewhat noisy, day- and night-respired CO_2 followed similar carbon sources (close to the 1:1 line). This agrees with the covariation analysis carried out with the whole set of $\delta^{13}C$ values in CO_2 and metabolites, as shown in Fig. 4. When represented as an isotopomic array, it appears that the closest relative of day-respired CO_2 (for the mesocosm) was night-respired CO_2 of both the mesocosm and individual organs (Fig. 4, bottom). Within the same cluster (Fig. 4, left), the second-order relative of respired CO_2 was stem and leaf sucrose, suggesting that, unsurprisingly, sucrose was a major source of carbon for respiration. Fructose, glucose, starch and organic matter belong to a different cluster.

Table 2. Values of the (photo)respiratory contribution d^*/c_a to net photosynthetic fractionation at the mesocosm and leaf scale

Method	Γ	$d^*/400$	d^*/c_a	$\frac{d^* - f\Gamma^*}{400}$	$\frac{d^* - f\Gamma^*}{c_a}$
With $b = 29‰$					
Chamber 1 ($c_a = 200$ μmol mol^{-1})					
Eqn 2 and Fig. 1	–	1.51	3.02	0.41	0.82 (–0.06, –0.2)
Eqn 3 and Fig. 1, inset	59	1.50	3.01	0.40	0.81 (–0.06, –0.2)
Chamber 2 ($c_a = 1000$ μmol mol^{-1})					
Eqn 2 and Fig. 1	–	3.09	1.23	1.99	0.76 (–0.01, –0.04)
Eqn 3 and Fig. 1, inset	79	2.68	1.07	1.58	0.63 (–0.01, –0.04)
With $b = 27‰$					
Chamber 1 ($c_a = 200$ μmol mol^{-1})					
Eqn 2 and Fig. 1	–	1.44	2.88	0.34	0.68 (–0.06, –0.2)
Eqn 3 and Fig. 1, inset	59	1.20	2.41	0.11	0.21 (–0.06, –0.2)
Chamber 2 ($c_a = 1000$ μmol mol^{-1})					
Eqn 2 and Fig. 1	–	2.97	1.19	1.87	0.75 (–0.01, –0.04)
Eqn 3 and Fig. 1, inset	79	2.29	0.91	1.19	0.47 (–0.01, –0.04)

In this table, c_a stands for atmospheric CO_2 mole fraction under growth conditions, that is, 200 (chamber 1) or 1000 (chamber 2) μmol mol^{-1}. Γ is the CO_2 compensation point of net CO_2 assimilation (in μmol mol^{-1}); it was obtained with A/CO_2 curves at the mesocosm level. On the right-hand side, the pure respiratory value was calculated by substracting the photorespiratory term $f\Gamma^*$, using arbitrary typical values of 40 μmol mol^{-1} for Γ^* and 11‰ for f. Between brackets: sensitivity coefficients of the calculated pure respiratory value with respect to Γ^* (in ‰ mol μmol^{-1}) and f (in ‰ ‰$^{-1}$), respectively. The sign of the values given above follows the convention of eqn 1 of Farquhar *et al.* (1982), that is, positive values mean fractionations against ^{13}C.

Table 3. Carbon isotope composition of CO_2 evolved by the mesocosm or intact individual organs

$\delta^{13}C$ of respired CO_2 in darkness (‰)				
Mesocosm	Roots	Stem	Mature leaves	Young leaves
Chamber 1 ($c_a = 200$ μmol mol^{-1}):				
–19.6 ± 2.1	–26.6 ± 2.0	–23.0 ± 0.2	–13.8 ± 1.0	–15.7 ± 1.0
[–2.7]	[+3.2]	[–0.5]	[–9.9]	[–8.0]
Chamber 2 ($c_a = 1000$ μmol mol^{-1}):				
–24.4 ± 2.1	–28.5 ± 1.0	–26.1 ± 0.6	–19.0 ± 1.2	–20.5 ± 1.0
[–0.5]	[+3.4]	[+0.9]	[–6.4]	[–4.8]

The isotope composition of inlet CO_2 during growth was –3.5‰, and so in the steady state, the $\delta^{13}C$ value of atmospheric CO_2 (δ_o) was on average +0.5‰ (chamber 1, 200 μmol mol^{-1} CO_2) and +0.7‰ (chamber 2, 1000 μmol mol^{-1} CO_2). The values are average obtained at days 32–34 when stands were closed and plants were at the three fully expanded leaves stages. Between brackets: calculated respiratory fractionation with respect to mesocosm-level net fixed CO_2 (Eqn 7). Positive values indicate fractionations against ^{13}C.

DISCUSSION

While there is currently a growing literature on the carbon isotope composition of CO_2 evolved in the dark by either leaves, roots or ecosystems, less is known on the isotope composition of day-respired CO_2 (Ghashghaie *et al.* 2003; Bowling *et al.* 2008). This lack of knowledge stems from the technical difficulties associated with the measurement of the pure, respiratory isotopic signal. Such difficulties are independent of the scale of interest (leaf to ecosystem). Disentangling the (photo)respiratory contribution to the net isotopic fractionation usually requires assumptions on either its magnitude or associated fractionation. Here, we manipulated both the atmospheric CO_2 mole fraction (two growth conditions) and the isotope composition of inlet CO_2 (labelling), and took advantage of the mesocosm gas exchange facility (Schnyder *et al.* 2003). We used new simple equations (Materials and methods; Table 1) and Δ versus A curves to gain information of the carbon isotope composition of respiratory CO_2 evolved in the light, without prerequisite assumptions.

Is day-respired CO_2 ^{13}C depleted or ^{13}C enriched?

When plotted against A, the $c_a(b - \Delta)$ intercept directly gave the d^* value. Scaled to atmospheric CO_2 mole fraction of interest (d^*/c_a), this gave the (photo)respiratory component of mesocosm photosynthetic fractionation (Eqn 2; Fig. 1). Our results show that day-(photo)respired CO_2 was ^{13}C depleted as compared to net fixed CO_2, and this difference depended on growth CO_2 conditions (Table 2). However, the contribution of photorespiration was dissimilar under different CO_2 conditions, and the estimated pure respiratory signal was close to +0.7‰ in both chambers (Table 2, with $b = 29$‰), that is, day-respired CO_2 was slightly ^{13}C depleted. We, nevertheless, recognize that such a value depended upon the chosen values of f (photorespiratory fractionation) and Γ^*, that is, they were assumed to be independent of growth CO_2 mole fraction. Both assumption are nevertheless reasonable (Thomas *et al.* 1993; Lanigan *et al.* 2008), and our sensitivity analysis shows that variations in Γ^* and f variations have little effect on the pure respiratory signal (Table 2). It may be argued that day-respired CO_2 is ^{13}C enriched at the leaf level, as shown by isotopic analyses of CO_2 evolved from darkened leaves during the light period (see, e.g. Hymus *et al.* 2005). It is believed that such a ^{13}C enrichment comes from the metabolism associated with light-enhanced dark respiration (Barbour *et al.* 2007; Gessler *et al.* 2009). In addition, our value (depletion by 0.7‰) agrees with the data from previous investigations: in *Senecio*, Ghashghaie *et al.* (2003) found an intercept (Δ versus A/c_a curve) of around 1.5‰, which corresponds to a pure respiratory component of +0.31‰, with their values of $f = 11$‰ and $\Gamma^* = 39$ μmol mol^{-1}. This is also in agreement with the theoretical study of Tcherkez *et al.* (2004), in which the commitment of ^{13}C-depleted triose phosphates to mitochondrial respiration in the light led to ^{13}C-depleted CO_2. That said, measurements under 2% O_2 (non-photorespiratory conditions) gave an intercept of about 1‰, and such a value indicates a much larger respiratory component (Ghashghaie *et al.* 2003) than the value of 0.31‰ quoted above. In fact, there is now a body of evidence that day respiratory metabolism is affected by the photorespiration rate (Tcherkez *et al.* 2008 and for a review see Noguchi and Yoshida 2007) and likely therefore, so is the isotopic contribution of evolved CO_2 to net photosynthetic fractionation.

The similar values of 0.3–0.8‰ associated with the respiratory contribution to photosynthetic fractionation at both the leaf and mesocosm level show that presumably, CO_2 evolved by leaves and non-photosynthetic organs was isotopically similar. With typical mesocosm values of $R_{day} = 3$ μmol m^{-2} s^{-1} and $k = 0.1$ mol m^{-2} s^{-1}, the respiratory fractionation (usually denoted as e) is then about 5 ± 1‰ at the leaf level under a CO_2 mole fraction of 400 μmol mol^{-1}. This value agrees with the theoretical estimation of Tcherkez *et al.* (2004) (see also the discussion below).

Such a pattern is in clear contrast with night-respired CO_2 which is ^{13}C enriched by several per mil in leaves (Ghashghaie *et al.* 2003 and references therein, and Table 3) and *ca*. 0.6‰ (Schnyder *et al.* 2003) to 3‰ (Table 3) in the

mesocosm. At the forest level, night-time respired CO_2 appears slightly ^{13}C enriched compared to photosynthates with, however, considerable variations (Bowling, Baldocchi & Monson 1999; Cai *et al.* 2008). Under our conditions, the ^{13}C enrichment of mesocosm dark-evolved CO_2 came from leaves that were indeed the sole organs to produce ^{13}C-enriched CO_2. As such, leaves probably dominated the CO_2 production in the night in the present study. This scenario agrees with another study on sunflower (Klumpp *et al.* 2005) in which night-time-respired CO_2 was ^{13}C enriched in shoots and ^{13}C depleted in roots, suggesting that the slight ^{13}C enrichment of night-respired CO_2 at the stand scale originated from shoots.

The day/night oscillation of the isotopic composition of CO_2 respired by the mesocosm may come from: (1) the decrease of the contribution of leaves because of the inhibition of leaf respiration by light (Atkin *et al.* 2000); (2) the decarboxylation of ^{13}C-enriched malate in the dark in leaves (Gessler *et al.* 2009); (3) the change of respiratory carbon source (^{13}C-enriched starch at night and ^{13}C-depleted sucrose in the light) (Gessler *et al.* 2008); and (4) a change of metabolic pathways or commitments so that $^{12}C/^{13}C$ fractionations of respiratory enzymes vary. While assumptions (1) and (2) are likely and supported by experimental results, assumptions (3) and (4) are not demonstrated yet and are addressed below.

Are day- and night-respired CO_2 correlated?

We used here isotopic labelling to make the respiratory isotopic signal to vary: the inlet CO_2 source to the mesocosm was changed from –3.5‰ to –44.5‰. The day respiratory contribution to the photosynthetic fractionation was then calculated with Eqn 3. It should be emphasized that such a value included the production of respired CO_2 by non-photosynthetic organs, as we use the *net* photosynthetic fractionation of leaves in Eqn 3. Unsurprisingly, the respiratory component was associated with ^{13}C-enriched CO_2 (Fig. 2), clearly showing the contribution of ^{13}C-enriched, old carbon sources to day respiration. The calculated proportion of 'new' carbon was indeed 40–60% (Fig. 2, inset). Similar values have already been obtained at both the leaf (Nogués *et al.* 2004), plant (Lehmeier *et al.* 2008), mesocosm (Schnyder *et al.* 2003) and ecosystem scale (Gamnitzer, Schäufele & Schnyder 2009) in the dark. Our data show that: (1) day- and night-respired CO_2 had a very similar turnover pattern (Fig. 3); and (2) the covariation analysis indicated that the closest relative of day-respired CO_2 was night-respired CO_2 (Fig. 4). We therefore conclude that both day and night respiratory substrate pools were likely to be fed by the same carbon source. The latter may comprise several components: the kinetics of CO_2 turnover indeed suggest that at least two carbon sources provide substrates to respiratory metabolism. It has been argued that one of them arises from carbohydrates because of the respiratory quotient of 1 (Nogués *et al.* 2004). Our data support this assumption, because the closest relative to evolved CO_2 was stem (i.e. mainly phloem) and leaf sucrose (Fig. 4).

While both day- and dark-respired CO_2 comprised always a lower proportion of 'new' carbon under high CO_2 (chamber 2) than under low CO_2 (chamber 1) (Fig. 3), the absolute decarboxylation rate of 'new'carbon was similar under both conditions: it was $60\% \times 3.4$ μmol m^{-2} s^{-1} = 2.04 μmol m^{-2} s^{-1} at low CO_2, and $40\% \times 6.3$ μmol m^{-2} s^{-1} = 2.52 μmol m^{-2} s^{-1} at high CO_2. Therefore, the rate at which recently fixed carbon fed respiration did not vary with CO_2 conditions, while the rate of remobilization increased as CO_2 (and carbon availability) increased. Consistent with this are observations that when pools of respiratory intermediates (such as citrate) and storage molecules (such as starch) increase under high CO_2 conditions at fixed N supply, the specific N content (Bernacchi *et al.* 2007) and amino acid pools were identical or even smaller (Geiger *et al.* 1999; Li *et al.* 2008). Such a metabolic effect tends to impede the turnover of respiratory metabolites, as evidenced here (Fig. 2).

Why is day-respired CO_2 ^{13}C depleted?

While assumption (2) above (decarboxylation of ^{13}C-enriched material in the dark) has received strong support in the literature, it is likely that both day- and night-evolved CO_2 originate from similar substrates (see just above). In fact, the involvement of the decarboxylation of ^{13}C-enriched malate in leaves has been shown to last less than half an hour (Barbour *et al.* 2007). Therefore, different metabolic processes probably explain why the natural $^{13}CO_2$ abundance is dissimilar in the light and in the dark. One of them is the inhibition of leaf respiration that is accompanied by the decrease of both the pyruvate dehydrogenase and TCA cycle activity (Randall *et al.* 1990; Hanning and Heldt 1993; Tcherkez *et al.* 2005, 2008, and for a review, see Hurry *et al.* 2005). These two metabolic steps fractionate against ^{13}C (Melzer and Schmidt 1987; Tcherkez & Farquhar 2005), and the associated isotope effects are expected to increase as the metabolic commitment decreases (O'Leary 1980), thereby depleting evolved CO_2 in ^{13}C. By contrast, the $\delta^{13}C$ value of CO_2 evolved by roots under continuous darkness has been shown to be independent of the carbon source availability (Bathellier *et al.* 2009). This suggests that the day/night transition does not induce major changes in the root-respired isotopic signal, unless the $\delta^{13}C$ of root-imported sucrose (carbon input) varies. Because leaf respiration seemed to be a major component of mesocosm respiration in our study, it is therefore likely that the inhibition of leaf respiration by light contributed to the ^{13}C depletion of mesocosm CO_2.

We nevertheless recognize that day/night cycles are accompanied by circadian variations of the ^{13}C abundance in sucrose (Tcherkez *et al.* 2004; Gessler *et al.* 2008), with ^{13}C-enriched values at night and ^{13}C-depleted values in the light. Such variations are caused by the $^{12}C/^{13}C$ isotope effect of aldolases (Gleixner & Schmidt 1997), that catalyse the production of fructose-1,6-bisphosphate from triose phosphates, thereby depleting day sucrose and enriching transitory starch (and night sucrose). Such a circadian

variation in sucrose that feeds non-photosynthetic organs is likely to contribute to further deplete mesocosm day-respired CO_2 in ^{13}C, because non-photosynthetic organs were taken into account in our estimate of day respiratory fractionation.

Perspectives

Our results indicate that the respiratory isotopic signal is very dynamic and follows day/night variations, which correlate predominantly with leaf-level ^{13}C signals, and this appears to be unaffected by growth CO_2 conditions. In that sense, the mesocosm can be considered as a big leaf under our experimental conditions. We, nevertheless, gave little attention to other parameters that may change this pattern (such as temperature, nitrogen availability, etc.) through an effect on the root/shoot ratio, the growth rate and the $\delta^{13}C$ value of evolved CO_2. In fact, the isotope composition of night-time-respired CO_2 is sensitive to temperature, vapour pressure deficit or light level (Cai *et al.* 2008). In addition, the results can certainly not be extrapolated to natural forest ecosystems because trees comprise woody organs (branches and trunks) that have particular isotopic signals. In natural conditions, CO_2 respired by heterotrophic organs of trees is ordinarily ^{13}C enriched (Brandes *et al.* 2006; Gessler *et al.* 2007; Maunoury *et al.* 2007) with noticeable diel variations (Kodama *et al.* 2008). Such a pattern is in clear contrast to herbaceous plants in which heterotrophic organs produce ^{13}C-depleted CO_2 (see references above and the present study). Therefore, further studies are needed to determine whether day-respired CO_2 is similarly ^{13}C depleted in natural ecosystems.

On-line carbon isotope discrimination measured at the leaf level is often used to gain information on, for example, mesophyll conductance, and quite frequently, the respiratory contribution to the net photosynthetic carbon isotope fractionation is thought to be negligible (Warren 2006; Flexas *et al.* 2007). Our results suggest that this very contribution may not be negligible at the mesocosm level (Table 2) particularly when the $\delta^{13}C$ value of CO_2 used for isotopic measurements strongly differs from that of growth CO_2.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Typical A/c_a curves at the mesocosm level. The growth CO_2 conditions were 200 μmol mol^{-1} (chamber 1, closed symbols) or 1000 μmol mol^{-1} (chamber 2, open symbols).

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