



On the metabolic origin of the carbon isotope composition of CO₂ evolved from darkened light-acclimated leaves in *Ricinus communis*

Arthur Gessler^{1,2,6}, Guillaume Tcherkez^{4,5}, Oka Karyanto², Claudia Keitel¹, Juan Pedro Ferrio³, Jaleh Ghashghaie⁴, Jürgen Kreuzwieser³ and Graham D. Farquhar¹

¹Environmental Biology Group; Research School of Biological Sciences, Australian National University, GPO Box 475, Canberra, ACT 2601, Australia;

²School of Forest and Ecosystem Science, University of Melbourne, Water Street, Creswick, VIC 3363, Australia; ³Institute of Forest Botany and Tree

Physiology, Albert-Ludwigs Universität Freiburg, Georges-Köhler-Allee 53/54, 79110 Freiburg, Germany; ⁴Laboratoire d'Ecologie, Systématique et

Evolution, Département d'Ecophysiologie Végétale, CNRS-UMR 8079, Centre scientifique d'Orsay, Bâtiment 362, Université Paris-Sud XI, 91405

Orsay Cedex, France; ⁵Plateforme Métabolisme-Métabolome IFR87, Centre scientifique d'Orsay, Bâtiment 630, Université Paris-Sud XI, 91405 Orsay Cedex,

France; ⁶Present address: Core Facility Metabolomics, Centre for System Biology (ZBSA), Albert-Ludwigs Universität Freiburg, Habsburgerstr. 49, 79104

Freiburg, Germany

Summary

Author for correspondence:

Arthur Gessler

Tel: +49 76120397137

Fax: +49 7612038302

Email: arthur.gessler@sonne.uni-freiburg.de

Received: 6 August 2008

Accepted: 12 September 2008

New Phytologist (2009) **181**: 374–386

doi: 10.1111/j.1469-8137.2008.02672.x

Key words: light enhanced dark respiration (LEDR), malate, PEPc, respiration, respiratory quotient, respiratory carbon isotope fractionation.

- The ¹³C isotopic signature (δ¹³C) of CO₂ respired from plants is widely used to assess carbon fluxes and ecosystem functioning. There is, however, a lack of knowledge of the metabolic basis of the δ¹³C value of respired CO₂.
- To elucidate the physiological mechanisms driving ¹²C/¹³C fractionation during respiration, the δ¹³C of respired CO₂ from dark-acclimated leaves during the night, from darkened leaves during the light period, and from stems and roots of *Ricinus communis* was analysed. The δ¹³C of potential respiratory substrates, the respiratory quotient and the activities of phosphoenolpyruvatecarboxylase (PEPc) and key respiratory enzymes were also measured.
- It is shown here that the CO₂ evolved from darkened light-acclimated leaves during the light period is ¹³C-enriched, and that this correlates with malate accumulation in the light and rapid malate decarboxylation just after the onset of darkness. Whilst CO₂ evolved from leaves was generally ¹³C-enriched (but to a lesser extent during the night), CO₂ evolved from stems and roots was depleted compared with the putative respiratory substrates; the difference was mainly caused by intensive PEPc-catalysed CO₂ refixation in stems and roots.
- These results provide a physiological explanation for short-term variations of δ¹³C in CO₂, illustrating the effects of variations of metabolic fluxes through different biochemical pathways.

Introduction

While carbon isotope discrimination during photosynthetic CO₂ fixation is a rather well described and understood phenomenon (Farquhar *et al.*, 1982, 1989), much less is known about isotopic fractionations associated with downstream metabolic pathways (Badeck *et al.*, 2005). Such post-carboxylation carbon isotope fractionation processes, as

a consequence of equilibrium and kinetic isotope effects, not only result in differences in isotopic signatures between metabolites but also in intramolecular positional isotopic effects (Schmidt, 2003; Tcherkez & Farquhar, 2005). In addition to spatial and temporal variation in the carbon isotope composition of organic matter (e.g. Brandes *et al.*, 2006; Gessler *et al.*, 2007a, 2008), the often observed enrichment of CO₂ released from dark respiration in leaves compared with

potential organic substrates (Duranceau *et al.*, 1999; Tcherkez *et al.*, 2003; Xu *et al.*, 2004) is also likely to be a result of post-carboxylation carbon isotope effects. Duranceau *et al.* (1999) and Tcherkez *et al.* (2004) discussed this effect and suggested that it was caused by nonrandom distribution of ^{13}C in organic matter substrates (Rossmann *et al.*, 1991) along with fragmentation fractionation during respiration: the CO_2 released by the decarboxylation of pyruvate (via the enzyme pyruvate dehydrogenase (PDH)) originates from the relatively ^{13}C -enriched C-3 and C-4 atoms of a glucose molecule, whereas the CO_2 emitted from tricarboxylic acid (TCA) cycle reactions originates from the C-1, C-2, C-5 and C-6 atoms, which are relatively ^{13}C depleted (Rossmann *et al.*, 1991; Tcherkez *et al.*, 2003, 2004). Any apparent ^{13}C enrichment of CO_2 compared with the organic respiratory substrate can thus be attributable to incomplete oxidation of glucose molecules, with a higher proportion of C-3 and C-4 atoms converted to CO_2 . Tcherkez *et al.* (2003) showed that environmental conditions that changed the proportion of CO_2 molecules inherited from pyruvate (C-3 and C-4) versus acetyl-CoA (C-1, C-2, C-5 and C-6) molecules, as revealed by the respiratory quotient (RQ; CO_2 production to O_2 consumption ratio), resulted in the predicted changes in ^{13}C enrichment of respired CO_2 . However, the carbon isotope composition of evolved CO_2 in darkness is not constant under controlled conditions: the ^{13}C abundance of CO_2 evolved by rapidly darkened leaves during the light period is higher than during the night and varies with time (Hymus *et al.*, 2005; Werner *et al.*, 2007), with a plausible relationship with the cumulative amount of photosynthetically fixed CO_2 (Hymus *et al.*, 2005). Similarly, when applying tuneable diode laser measurements with high temporal resolution for the analysis of $\delta^{13}\text{C}$ in CO_2 , Barbour *et al.* (2007) observed that CO_2 released from leaves in the first 10–15 min after darkening was strongly ^{13}C -enriched as compared with both potential substrates and the steady respired- CO_2 value of dark-acclimated leaves (i.e. leaves exposed to sustained darkness).

Such a striking ^{13}C enrichment and variability of the carbon isotope composition of CO_2 respired by leaves after illumination might be attributed to either (1) shifts in the proportion of respired CO_2 derived from the decarboxylation of pyruvate by PDH (relatively ^{13}C -enriched CO_2) versus that derived from the TCA cycle (relatively ^{13}C -depleted CO_2) or (2) the effect of the so-called light-enhanced dark respiration (LEDR), which is assumed to produce ^{13}C -enriched CO_2 from phosphoenolpyruvatecarboxylase (PEPc)-derived malate (Barbour *et al.*, 2007). Under assumption (1), the carbon isotope composition of CO_2 is expected to correlate with the prevalence of pyruvate or acetyl-CoA as the CO_2 source (i.e. with the nature of the respiratory substrate). In other words, the ^{13}C abundance in CO_2 may be related to the RQ as preferential pyruvate decarboxylation would lead to higher RQ values than preferential acetyl-CoA oxidation in the TCA cycle. Under assumption (2), the carbon isotope composition of CO_2

evolved just after illumination is expected to correlate with higher respiratory rates and malate decarboxylation activity and additionally with an increase in the RQ. Until now, there has been no experimental evidence to favour one hypothesis over the other.

In contrast, heterotrophic organs have been shown to produce ^{13}C -depleted CO_2 : for example, Badeck *et al.* (2005) and recently Bathellier *et al.* (2008) reported a ^{13}C depletion of CO_2 evolved by *Phaseolus vulgaris* roots of c. 2‰ compared with root organic matter. Comparable results were found with shoots and roots of sunflower (*Helianthus annuus*), perennial ryegrass (*Lolium perenne*) and alfalfa (*Medicago sativa*) (Klump *et al.*, 2005, but see Damesin & Lelarge, 2003; Brandes *et al.*, 2006, Gessler *et al.*, 2007a; Kodama *et al.*, 2008 in trees). Several hypotheses have been proposed to explain such a pattern, among which PEPc fixation without subsequent decarboxylation of organic acids has been emphasized (Badeck *et al.*, 2005). In addition, it is plausible that, in contrast to the situation in leaves, respiratory substrates are completely broken down by respiration in heterotrophic organs, without any imbalance between PDH-derived and TCA cycle-derived CO_2 .

It is apparent, then, that the physiological origin of the carbon isotope composition of CO_2 respired by both leaves and heterotrophic organs is uncertain. However, knowledge of the carbon isotope fractionation during respiration is important for a general understanding of the controls of plant carbon metabolism (e.g. Kodama *et al.*, 2008) and of ecosystem carbon fluxes (e.g. Bowling *et al.*, 2001; Pataki *et al.*, 2003). In order to obtain a better understanding of the physiological mechanisms driving fractionation processes during respiration, we analysed the ^{13}C abundance in respired CO_2 from dark-acclimated leaves, from darkened leaves during the light period and from stems and roots, and measured the $\delta^{13}\text{C}$ of potential respiratory substrates, the respiratory quotient and the enzymatic activities of PEPc and key respiratory enzymes (pyruvate dehydrogenase, NADP-dependent isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase). We show here that the CO_2 evolved from darkened light-acclimated leaves is naturally ^{13}C -enriched and that this correlates with the malate excess produced by leaves in the light and subsequently decarboxylated in the dark.

Materials and Methods

Plant material

Ricinus communis L. plants were cultivated in a glasshouse as described by Gessler *et al.* (2007b). Seeds were germinated in vermiculite moistened with 0.5 mM CaSO_4 . After 10–12 d in the dark to induce etiolated hypocotyls, uniform seedlings were each transferred to pots filled with quartz sand. The plants were supplied daily with nutrient solution in excess that contained 4 mM nitrate as the nitrogen (N) source (Peuke *et al.*, 1994). The plants were cultivated for 35–40 d in a

glasshouse ($25 \pm 5^\circ\text{C}$) with a 16-h photoperiod provided by natural daylight plus mercury-vapour lamps (Osram HQL 400; Osram, Munich, Germany) supplying the plant with a minimum of $300\text{--}500 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Experimental design

We conducted an experiment covering a full diel course (with two measurements during the light and two during the dark period) in order to study respiration rates and the carbon isotopic composition of potential organic sources for respiration and of respired CO_2 . Respiration was measured immediately after tissues were darkened by covering them with aluminium foil (measurements took between 10 and 20 min per sample; see following section). CO_2 emitted from the middle of the stem (at a height of approx. 25 cm), from the uppermost fully expanded leaf and from the roots was collected in the morning (10:00 h), in the afternoon (15:30 h) and in the first (22:30 h) and second (03:30 h) halves of the night. Three plants were analysed at each time-point.

Phloem sap was sampled by cutting the bark with a scalpel directly below the position where stem CO_2 was analysed, according to the procedure described by Jeschke & Pate (1991). Phloem sap was transferred with a capillary into a 1.5-ml reaction tube and frozen immediately. Sampling took approx. 10 min.

After CO_2 and phloem sampling, organic matter was harvested from the same plant parts where CO_2 had been analysed and a subsample of it was immediately frozen for total organic carbon, water-soluble organic carbon, sugar, starch and lipid analyses. A subsample of the plant material from stems and roots was used for the determination of RQs (see following section). For leaf measurements a sample of another leaf darkened for approx. 2 min was used for measuring RQ.

In addition, enzyme activities (PDH, isocitric dehydrogenase (ICDH), 2-oxoglutarate dehydrogenase (2-OGDH) and PEPc) were determined at the same time-points in three additional plants growing under the same environmental conditions. The samples were taken from leaves, stems and fine roots and the tissues were not darkened before sampling in order to determine enzyme activity under ambient light conditions. This was considered to be especially important to characterize the difference in enzyme activities between light- and dark-acclimated leaves during the day and night, respectively.

In order to explore the influence of potential changes in the malate concentration on the $\delta^{13}\text{C}$ of leaf respired CO_2 during the light period we performed an additional experiment. Under the same light conditions as described above, leaf discs (fresh weight approx. 50 mg) from six *R. communis* plants were harvested in the afternoon in the light, their specific leaf area was determined and they were directly frozen in liquid nitrogen. Thereafter plants were darkened. Additional leaf discs were harvested 2, 5, 8, 11, 23, 37 and 55 min after darkening and analysed for malate concentrations.

Determination of the $\delta^{13}\text{C}$ of respired CO_2 , of CO_2 exchange and of respiratory quotients

Whole leaves, 12-cm-long intact (not excised) shoot sections and the whole root system of *R. communis* plants were placed into Plexiglas respiration chambers darkened with aluminium foil with a volume of 0.76 (leaf chamber), 0.023 (stem chamber) or 0.046 m^3 (root chamber). The chambers were sealed with an inert plastic material (Terostat VII®; Henkel Teroson GmbH, Heidelberg, Germany) and connected to a closed loop gas exchange system including a pump and a CO_2 gas analyser (modified LI-6400 with soil chamber configuration; Li-Cor Inc., Lincoln, NE, USA). The system was checked for leaks before each sampling by exhaling on all seals and connections. The air flow rate through the closed system was approx. 4 l min^{-1} . The chamber systems were additionally equipped with fans to allow a more rapid mixing of the air. At the beginning of each measurement ambient air was pumped through the system for 1 min before the closed mode was established. Then 12-ml gas samples were taken from the chambers through airtight septa using hypodermic needles and syringes and transferred into argon-flushed exetainers (Labco Ltd, High Wycombe, UK). Five samples in CO_2 concentration ranges of approx 570–1000 (leaves), 500–1500 (roots) and 500–1200 $\mu\text{mol mol}^{-1}$ (stem) were taken per measurement, which took approx. 15–20 min. The carbon isotope signature of respired CO_2 was determined with the Keeling plot approach (Keeling, 1958, 1961). Respiration rates were calculated from the linear increase in CO_2 concentration over time and based on leaf and stem area or root fresh weight.

We also determined the respiratory quotient (RQ), defined as the ratio of CO_2 production to oxygen consumption. CO_2 production and oxygen consumption were measured simultaneously in excised leaves, stem sections and roots in the dark using a gas-tight aluminium cylinder equipped with an infrared CO_2 sensor (UMS GmbH, München, Germany) and a ZrO_2 oxygen sensor. The measurements took approx. 10–15 min per sample.

Photosynthetic leaf gas exchange in the light period was measured with a portable gas exchange system (GFS3000; Heinz Walz GmbH, Effeltrich, Germany). The measurements were conducted under ambient light and temperature conditions.

Extraction of different carbon fractions

All tissue samples (leaves, stem sections and roots) were homogenized with a mortar and pestle in liquid nitrogen. For the extraction of water-soluble organic carbon 1.5 ml of deionized water was added to 0.1-g aliquots of plant material. The mixture was shaken for 1 h at 4°C . The extract was heated at 100°C for 3 min to precipitate proteins and centrifuged (12 000 g for 5 min). The supernatant was considered to be the water-soluble (exportable) carbon fraction (Brandes *et al.*, 2006). The

supernatant was then mixed with Dowex-50 (H⁺) and Dowex-1 (Cl⁻) in sequence to remove amino acids and organic acids. The eluate of the Dowex resins is representative of the soluble (neutral) sugar fraction (Brugnoli *et al.*, 1988; Xu *et al.*, 2004).

To obtain the starch fraction, the pellets from the sugar extraction were washed with ethanol (80% v/v) at 80°C to remove chlorophyll. This procedure was repeated until the washing solution was colourless. Thereafter the pellets were suspended twice with 500 ml of 6 N HCl for 1 h to solubilize starch according to Ghashghaie *et al.* (2001). The mixture was then centrifuged for 10 min at 4500 g and the pellet was discarded, and then 80% (v/v) methanol was added to the supernatant containing the HCl and the soluble starch. The sample was stored at 5°C overnight to precipitate the starch. The following morning, the extract was centrifuged at 12 000 g for 15 min and the pellet containing the starch was isolated, dried at 65°C and stored for carbon isotope analysis. According to Ghashghaie *et al.* (2001), Damesin & Lelarge (2003), Brugnoli *et al.* (1988) and Xu *et al.* (2004) no carbon isotope fractionation occurs during these extractions. The total lipid fraction was obtained from roots, stems and leaves according to Blight & Dryer (1959) with the modifications described by Ghashghaie *et al.* (2001). Three hundred mg of plant homogenates was added to 1.5 ml of a methanol-chloroform mixture (1 : 1 v/v) on ice. After 10 min of incubation under agitation, 1.5 ml of water was added to separate the phases. The hydrophilic phase was discarded whereas the heavy lipophilic phase was dried for isotope analysis.

For the analysis of malate in leaf tissues, 50 mg of the frozen leaf discs was homogenized in liquid nitrogen with a mortar and pestle. The homogenate was extracted in 1 ml of 87% (v/v) methanol at 70°C for 15 min. Fifty- μ l aliquots were vacuum-dried and thereafter dissolved in 20 μ l of methoxamine hydrochloride (20 mg ml⁻¹ pyridine) and incubated at 30°C for 90 min under continuous agitation. Then 40 μ l of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was added at 37°C for 30 min to derivatize polar functional groups. The derivatized samples were stored at room temperature for 2 h and then injected into a gas chromatography (GC)–quadrupole mass spectrometry (MS) system (GC: 7890A; MS: 5975C; Agilent Technologies, Waldbronn, Germany) operating in electron impact ionization mode. One μ l of the extract was injected in splitless mode with an injector temperature of 230°C. Separation of metabolites was performed on a fused silica capillary column (HP-5ms; Agilent Technologies) coated with a 0.25- μ m (5%-phenyl)-methylpolysiloxane stationary phase with a temperature gradient starting from 80°C and increasing by 5°C per min to 320°C. A mass to charge ratio range of 70–500 was scanned with the quadrupole mass detector at a rate of 12 scans per second. Data were deconvoluted and peak areas quantified using the AMDIS software (<http://chemdata.nist.gov/mass-spc/amdis/>) and the malate peaks were identified by comparing the measured mass spectra with the National Institute of Standards and Technology (NIST)

05 mass spectral library (<http://www.nist.gov/srd/nist1a.htm>). The malate concentration was quantified with calibration curves with external standards and based on the leaf area. The malate recovery was tested by addition of internal standards.

Determination of *in vitro* enzyme activities

The activities of the three enzymes responsible for CO₂ production during dark respiration via glycolysis and the TCA cycle plus the activity of PEPc were determined in fresh plant material. For the preparation of the enzyme extracts approx. 100 mg of leaf, stem and root material was homogenized in 1.5 ml of extraction buffer on ice. For the extraction of PDH (EC 1.2.2.2) and 2-OGDH (EC 1.2.4.2), 100 mM 3-(N-Morpholino) propanesulfonic acid (MOPS) HCl buffer (pH 7.5) with 12 mM MgCl₂ and 0.6 mM CaCl₂ was used, whereas ICDH (EC 1.1.1.42) and PEPc (EC 4.1.1.31) were extracted with 0.1 M Tris-HCl (pH 8). All extraction buffers additionally contained 15% (v/v) glycerol, 0.2% (v/v) Tween 20, 2 mM dithiothreitol (DTT) and 1 mM phenylmethylsulphonyl fluoride (PFMS).

The homogenates were centrifuged and the supernatant was mixed with polyvinylpyrrolidone (PVPP; 10% w/v) and shaken for 10 min to remove phenols. The sample was centrifuged and the clear supernatant was diluted with the same amount (1 : 1) of the extraction buffer and stored on ice until enzyme activities were analysed. Enzyme activity measurements were started by adding excess substrate for a given reaction to the enzyme assays.

All enzyme activities were measured photometrically by determining changes in NADH or NADPH concentration using absorption at 340 nm. The enzyme activity of PDH was measured according to Brown & Perham (1976) by photometrically determining the NADH production during the conversion of pyruvate to acetyl-CoA and CO₂ in the presence of coenzyme A, NAD and thiamine pyrophosphate. For the analysis of 2-OGDH activity, the increase in NADH concentration resulting from the conversion of 2-oxoglutarate and coenzyme A was monitored. The activity of ICDH was determined according to Bergmeyer (1974): isocitrate (at an end concentration in the assay of 0.44 mM) and NADP (1.0 mM end concentration) were added to the enzyme extract and subsequently the increase in NADPH concentration was monitored. PEPc activity was assessed according to Johnson *et al.* (1994) by following the disappearance of NADH at 340 nm in a malate dehydrogenase coupled reaction. Calculations of enzyme activities were based on the protein content. Total soluble protein contents were determined by the technique of Bradford (1976), with bovine serum albumin (BSA) as a standard.

Isotope measurements and isotopic calculations

The carbon isotope composition of respiratory CO₂ sampled from the static chambers was determined either on an

Isoprime isotope ratio mass spectrometer (IRMS) coupled in a continuous flow mode with a trace gas system (Micromass, Manchester, UK) or on a Delta Plus XP IRMS (ThermoFinnigan, Bremen, Germany) coupled in a continuous flow mode to a Gasbench II (ThermoFinnigan, Bremen, Germany). Repeated measurements of gas samples showed a precision of 0.12‰ (1 SD; $n = 10$) with both systems.

The carbon isotope signatures of dried bulk plant material and the different extracts were determined using an Isoprime or a Delta Plus (ThermoFinnigan, Bremen, Germany) IRMS, each coupled to an elemental analyser. The samples were combusted in tin capsules (IVA Analysentechnik, Meerbusch, Germany) and comprised *c.* 400 µg of organic carbon. The precision of the measurements as determined by repeated measurements of the standard IAEA-CO-8 (IAEA, Vienna, Austria) was 0.11‰ (1 SD; $n = 10$). Carbon isotope ratios (δ) are presented relative to the Vienna Pee Dee Belemnite standard (VPDB).

$$\delta = \frac{R_p}{R_{VPDB}} - 1 \quad \text{Eqn 1}$$

(R_p and R_{VPDB} , the isotope ratios ($^{13}\text{C}/^{12}\text{C}$) of the plant material or respired CO_2 and VPDB, respectively.)

Respiratory carbon isotope discrimination (Δ_R) was calculated as the difference between the $\delta^{13}\text{C}$ of the putative organic source and the $\delta^{13}\text{C}$ of respired CO_2 divided by 1 + the $\delta^{13}\text{C}$ of the respired CO_2 .

Statistical analyses

All statistical analyses were performed using NCSS 2004 (Number Cruncher Statistical Software, Kaysville, UT, USA). Differences between the $\delta^{13}\text{C}$ values of CO_2 and potential respiratory substrates as well as differences in enzyme activities during the diel course were determined using analysis of variance (ANOVA). In the experiment in which the change in malate concentration over time was assessed ($n = 6$), it was determined whether the data were normally distributed by applying a D'Agostino–Pearson omnibus test. Correlations between $\delta^{13}\text{C}$ and environmental/physiological parameters were calculated using the bivariate correlation procedure. The significance of correlation was calculated according to Sachs (1984).

Results

$\delta^{13}\text{C}$ of CO_2 and potential organic substrates

The $\delta^{13}\text{C}$ of respired CO_2 ($\delta^{13}\text{C}_R$) strongly varied during the diel cycle, exhibiting a maximum in leaves rapidly darkened during the light period, with an upper value of -27.4‰ (Fig. 1a). The diel pattern was inverted in stems and roots (relatively ^{13}C -enriched CO_2 during the night period) (Fig. 1b,c, respectively). The maximum difference between

day and night values was 1.1‰ in leaves, 3.3‰ in stems and 2.8‰ in fine roots.

At night, CO_2 emitted from leaves was slightly ^{13}C -enriched but did not differ significantly from the sugar and water-soluble carbon fractions. The starch pool was significantly more enriched in ^{13}C than respired CO_2 , and leaf lipids were generally ^{13}C -depleted. In the light period, leaf respired CO_2 was enriched compared with all potential organic substrates except starch.

In stem sections, the $\delta^{13}\text{C}$ values of phloem organic matter and evolved CO_2 were similar during the entire diel course. By contrast, respired CO_2 was always (albeit not always significantly) ^{13}C -depleted compared with total organic matter or its components (Fig. 1b). Such a pattern occurred also in roots, that is, respired CO_2 was ^{13}C -depleted compared with organic matter, although the difference between $\delta^{13}\text{C}_R$ and the $\delta^{13}\text{C}$ values of soluble carbon and sugars was not significant during the light period and in the second part of the night.

In order to assess the influence of the $\delta^{13}\text{C}$ of potential substrates on the diurnal and organ-specific patterns of $\delta^{13}\text{C}_R$, correlations were tested between $\delta^{13}\text{C}_R$ and the $\delta^{13}\text{C}$ values of the different organic matter pools (Table 1). When values from all tissues were combined there was no significant correlation of $\delta^{13}\text{C}_R$ with any of the variables tested. When roots were analysed separately, $\delta^{13}\text{C}_R$ showed the highest correlation with the $\delta^{13}\text{C}$ of stem phloem sap organic matter (which we assumed to be the main origin of organic carbon available for roots) and those of the root water-soluble organic matter and sugar pools. In stems, $\delta^{13}\text{C}_R$ was highly and significantly correlated with $\delta^{13}\text{C}$ values of all carbon pools, with the highest R obtained with the water-soluble organic matter fraction. In leaves, $\delta^{13}\text{C}_R$ was not significantly correlated with any of the organic matter fractions.

Respiratory fractionation and the metabolic background

Figure 2 shows the diel courses of respiration rate, RQ and respiratory fractionation with sugars as the putative organic source (Δ_R^s). RQ varied significantly neither with time nor with tissue. Daily mean values were 0.98 (leaves), 1.03 (stems) and 1.08 (roots). Respiration rates were generally higher during the light period than during the night in all the organs. An RQ of 1 indicates carbohydrates as the respiratory substrate. As a consequence we calculated carbon isotope discrimination associated with respired CO_2 with soluble sugars as the source material. The choice of soluble sugars as the source material is also supported by the fact that the $\delta^{13}\text{C}$ of sugars showed high and significant correlations with $\delta^{13}\text{C}_R$ in stems and roots (Table 1). Nevertheless, we recognize that other substrates (e.g. malate, which is associated with a theoretical RQ of 1.33; see also the Discussion section) may also contribute to respiration, and that a mixture of substrates in a certain range might still give RQ values close to 1. This should be kept in mind when

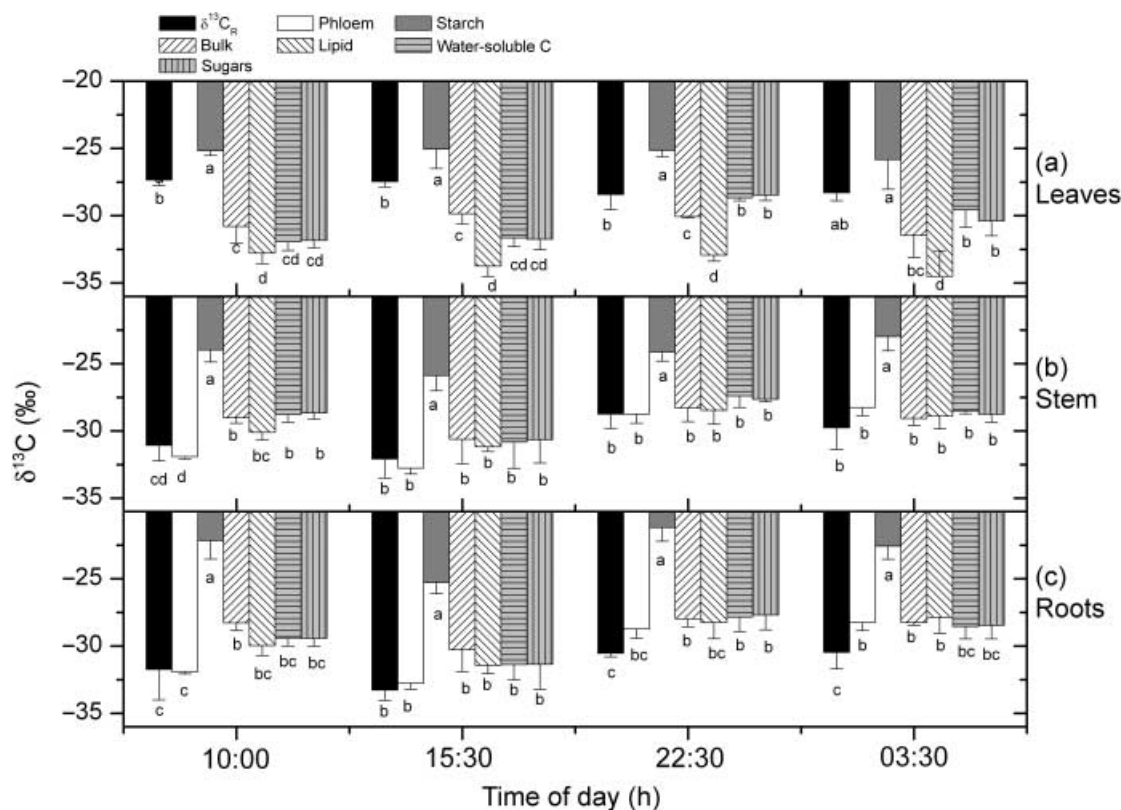


Fig. 1 ^{13}C isotopic signature ($\delta^{13}\text{C}$) of respired CO_2 ($\delta^{13}\text{C}_R$) compared with the $\delta^{13}\text{C}$ of potential respiratory sources of *Ricinus communis* leaves (a), stems (b) and roots (c) during a diel course. Different letters indicate significant differences for a particular time-point and position assessed with a one-way ANOVA. Data shown are mean values \pm SD ($n = 3$). The measurement points at 10:00 and 15:30 h were in the light, and those at 22:30 and 03:30 h in the dark period.

Table 1 Pearson's coefficients (R) for the correlation between the ^{13}C isotopic signature ($\delta^{13}\text{C}$) of respiratory CO_2 ($\delta^{13}\text{C}_R$) and the $\delta^{13}\text{C}$ of potential substrates for respiration in the respective tissues

	$\delta^{13}\text{C}$ for phloem	$\delta^{13}\text{C}$ for starch	$\delta^{13}\text{C}$ for bulk	$\delta^{13}\text{C}$ for lipids	$\delta^{13}\text{C}_{\text{ws}}$	$\delta^{13}\text{C}$ for sugar
$\delta^{13}\text{C}_R$ in						
Leaves		-0.24 <i>0.457</i>	-0.14 <i>0.666</i>	0.03 <i>0.934</i>	-0.55 <i>0.061</i>	-0.52 <i>0.082</i>
Stems	0.80 <i>0.002</i>	0.79 <i>0.002</i>	0.73 <i>0.007</i>	0.72 <i>0.008</i>	0.81 <i>0.002</i>	0.80 <i>0.002</i>
Roots	0.88 <i>< 0.001</i>	0.79 <i>0.002</i>	0.71 <i>0.009</i>	0.74 <i>0.005</i>	0.89 <i>< 0.001</i>	0.85 <i>< 0.001</i>
All tissues		-0.21 <i>0.213</i>	-0.19 <i>0.235</i>	-0.39 <i>0.170</i>	-0.02 <i>0.888</i>	-0.08 <i>0.637</i>

For tissue-specific analysis, all time-points were combined for correlation analysis in leaves, stems and roots. Additionally, all tissues and time-points were combined and correlated with combined $\delta^{13}\text{C}_R$ ('all tissues'). The $\delta^{13}\text{C}_R$ from roots was not only correlated with the $\delta^{13}\text{C}$ of starch, bulk material, water-soluble organic matter and sugars extracted from the root tissue but also with the $\delta^{13}\text{C}$ of stem phloem organic matter, as we assume that the main portion of carbon available for the roots is supplied via phloem transport. R is displayed in the first row of each cell, and the significance level P in italics in the second row. Bold values for R indicate significant correlations with $P < 0.05$; $n = 12$. $\delta^{13}\text{C}_{\text{ws}}$, $\delta^{13}\text{C}_R$ for water soluble carbon.

we refer to the ^{13}C enrichment or depletion of CO_2 , which is calculated with sugars as the putative source for respiration.

Δ_R^s was always positive (^{13}C -depleted CO_2) in roots and stems and was significantly ($P = 0.028$) higher in roots than in

stems. There were no significant diel variations in respiratory carbon isotope fractionation in both stems and roots. In leaves, Δ_R^s was always negative (^{13}C -enriched CO_2) with significant day–night differences ($P < 0.001$). In other words, leaves

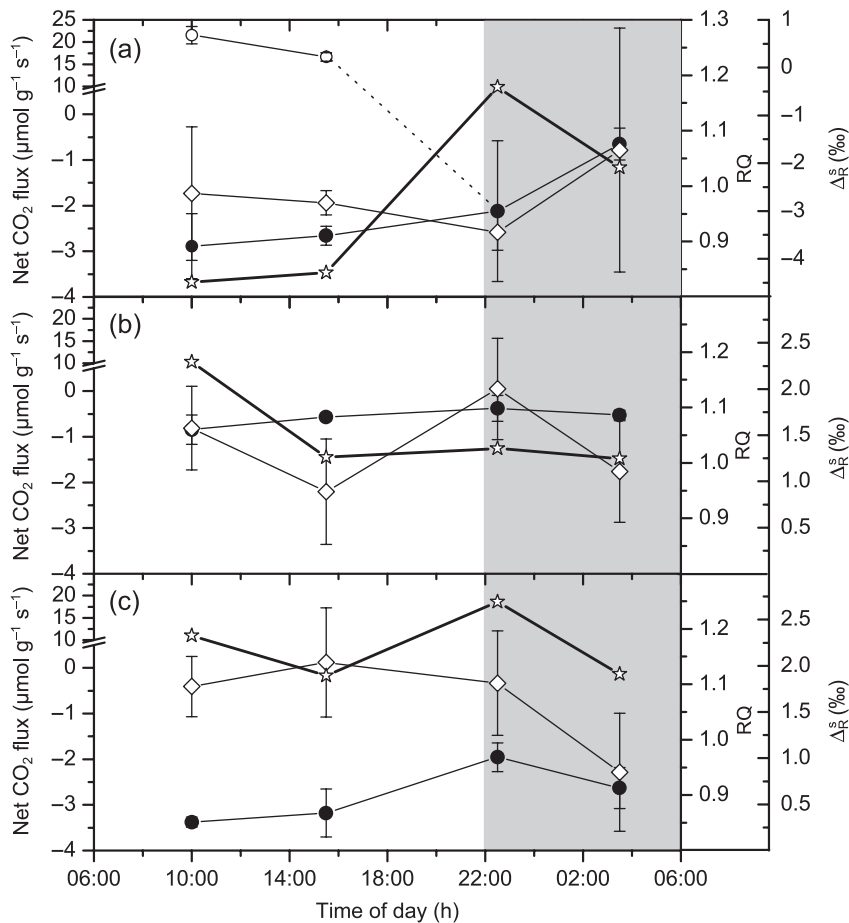


Fig. 2 Net CO₂ flux (circles), respiratory quotients (RQs; diamonds) and carbon isotope discrimination during respiration (Δ_R^s ; stars) in *Ricinus communis* leaves (a), stems (b) and roots (c). Δ_R^s was calculated relative to water-soluble sugars. For leaves, photosynthetic net assimilation rate at ambient light (open circles) is shown during daylight in addition to the respiration rates measured in the darkened gas exchange chambers (closed circles). The shaded area denotes the dark period.

analysed immediately after darkening in the light period produced significantly ¹³C-enriched CO₂ compared with the CO₂ evolved in the night, with nearly a 3‰ day–night difference. It should be noted that such a difference in Δ_R^s is larger than the difference in $\delta^{13}C_R$, simply because CO₂ and sugars exhibit opposite variations: dark-respired CO₂ is ¹³C-enriched in the light while sugars are ¹³C-enriched during the night period (Fig. 1).

Enzymatic activities involved in respiration

In order to further explore the potential metabolic causes of (1) the diel variations in respiratory carbon isotope fractionation (Δ_R^s) in leaves and (2) the difference between the value of Δ_R^s in leaves and its values in stems and roots, we assessed the diel patterns of PDH, NADP-dependent ICDH and 2-OGDH *in vitro* activities (Fig. 3).

In leaves, PDH, ICDH and 2-OGDH activities were lowest during the light period (that is, in the light, as leaves were not darkened before enzyme activity was analysed) and increased to levels of approx. 2 $\mu\text{mol mg}^{-1} \text{h}^{-1}$ at night. Day/night differences were significant for all three enzymes ($P < 0.01$).

Comparable albeit much less pronounced diel patterns in the activities of these three enzymes were also observed in stems, whereas no diel rhythm was present in roots. Leaf 2-OGDH activity, which is involved in the TCA cycle, showed the strongest inhibition by light (nearly 10-fold). ICDH activity was also inhibited by light (nearly a 5-fold decrease). PDH activity was modestly affected by light (with nearly a 2-fold inhibition). Such a trend reflects the exclusive involvement of 2-OGDH in the TCA cycle which has been shown to be strongly inhibited by light by 80–95% (Tcherkez *et al.*, 2005; Tcherkez *et al.*, 2008). ICDH inhibition by light was intermediate because the ICDH activity required for TCA cycle operation is mainly NAD-dependent. The nonmitochondrial isoforms of ICDH (cytoplasm and chloroplast) were responsible for the residual ICDH activity observed here. The PDH activity was certainly the least affected of the three as our assay combined mitochondrial and chloroplastic PDH activities, the latter not being down-regulated in the light (for a similar experimental case, see Tcherkez *et al.*, 2005).

As PEPc activity is likely to be involved in fuelling respiratory intermediates (the anaplerotic function of the enzyme), the activity was also measured and is shown in Fig. 3 (lower panel

Table 2 Pearson's coefficient (R) for the correlation between carbon isotope fractionation during respiration (Δ_R^s) and the $\text{CO}_2:\text{O}_2$ respiratory quotient (RQ), the respiration rate (r), phosphoenolpyruvate carboxylase (PEPc) activities and the absolute (PEPc – 2-OGDH) and relative (PEPc – 2-OGDH/PEPc) differences between malate-producing (PEPc) and -consuming (2-OGDH) reactions

Tissue		RQ	r	PEPc	PEPc – 2-OGDH	(PEPc – 2-OGDH/PEPc)
Leaf	R	0.52	0.61	0.87	–0.93	–0.96
	P	<i>0.47</i>	<i>0.38</i>	<i>0.12</i>	<i>0.06</i>	<i>0.04</i>
	n	12	12	4	4	4
Stems	R	0.33	–0.37	0.02	0.26	0.39
	P	<i>0.67</i>	<i>0.64</i>	<i>0.98</i>	<i>0.75</i>	<i>0.60</i>
	n	12	12	4	4	4
Roots	R	0.35	0.56	–0.12	–0.69	–0.01
	P	<i>0.65</i>	<i>0.43</i>	<i>0.89</i>	<i>0.31</i>	<i>0.99</i>
	n	12	12	4	4	4
All tissues	R	0.45	0.19	0.70 (0.72)		
	P	<i>0.14</i>	<i>0.54</i>	<i>0.01 (0.01)</i>		
	n	36	36	12 (10)		

2-OGDH, 2-oxoglutarate dehydrogenase.

R is displayed in the first row, the significance level P in italics in the second row and n in the third row. Bold values for R indicate significant correlations with $P < 0.05$. For the correlation between Δ_R^s and PEPc activity for all tissues, we additionally omitted the leaf values from the light period as Δ_R^s was assumed to be influenced by light-enhanced dark respiration (LEDR); the result of this analysis is given in brackets.

(d)). PEPc activity was constant during the diel course in the leaves, stems and roots and was not repressed in the light.

PEPc activity and malate production

As PEPc activity produces some oxaloacetate or malate molecules that are not consumed by the TCA cycle in the light (both 2-OGDH and ICDH activities are significantly repressed; see the previous section), malate probably accumulates within leaves in the light. We thus denote the difference between PEPc activity (malate synthesis) and 2-OGDH activity (malate consumption) as the 'malate synthesis excess'. From Fig. 3, it is apparent that the malate synthesis excess was very large (near $3.5 \mu\text{mol mg}^{-1} \text{h}^{-1}$) during the light period and decreased in darkness (to nearly $1.5 \mu\text{mol mg}^{-1} \text{h}^{-1}$). Correlation analyses between the respiratory fractionation Δ_R^s in leaves and the absolute (PEPc – 2-OGDH) or relative ((PEPc – 2-OGDH)/PEPc) malate synthesis excess resulted in correlation coefficients of –0.93 and –0.96, respectively (Table 2). This is in contrast with stems and roots, where no significant correlations were detected (Table 2).

Malate concentration was measured in leaves collected in the light ($t=0$) and then subsequently after darkening until 3300 s in the dark (Fig. 4). Leaf malate content rapidly decreased by > 50% within the first 25 min (1500 s) after darkening and then remained constant for the next 30 min in the dark (Fig. 4). While PEPc activity showed no diel variation it was significantly different among tissues (Fig. 3). The diel mean values were 3.9 (leaf), 5.2 (stem) and $8.89 \mu\text{mol g}^{-1} \text{h}^{-1}$ (roots).

When all tissues were combined, a significant positive correlation coefficient was obtained when relating respiratory

fractionation Δ_R^s to PEPc activity (Table 2). The correlation coefficient was even slightly higher when the leaf daytime values were omitted to remove the potential influence of malate decarboxylation on $\delta^{13}\text{C}_R$ and thus Δ_R^s at these time-points.

Discussion

The metabolic basis of respiratory fractionation in plants and, in particular, the origin of the ^{13}C enrichment of CO_2 evolved by light-acclimated leaves immediately after darkening are unclear (for a recent study, see Barbour *et al.*, 2007). As an aid to elucidating the metabolic pathways and the isotope fractionations involved in such a phenomenon, we combined rapid $^{12}\text{C}/^{13}\text{C}$ isotopic measurements of respired CO_2 with measurements of the respiratory quotient, leaf malate concentrations and *in vitro* enzyme activities.

Does the ^{13}C enrichment of leaf-respired CO_2 reflect the LEDR?

The leaf respiration rate and the ^{13}C abundance of leaf respired CO_2 were larger and Δ_R^s (respiratory fractionation, with sugars as the putative organic source for respiration) was higher in light-acclimated leaves immediately after they had been subjected to the dark (leaves darkened for 15 min in the light period) than in dark-acclimated (30 min and 5.5 h in the dark) leaves (Figs 1a, 2a). Such a pattern has previously been observed in *Ricinus* by Barbour *et al.* (2007) and was attributed to a LEDR effect, that is, the enhancement of the flux of respiratory CO_2 directly after darkening in a photosynthesis-dependent manner (Azcon-Bieto & Osmond, 1983; Atkin *et al.*, 2000). Hymus *et al.* (2005) reported for *Quercus ilex* a

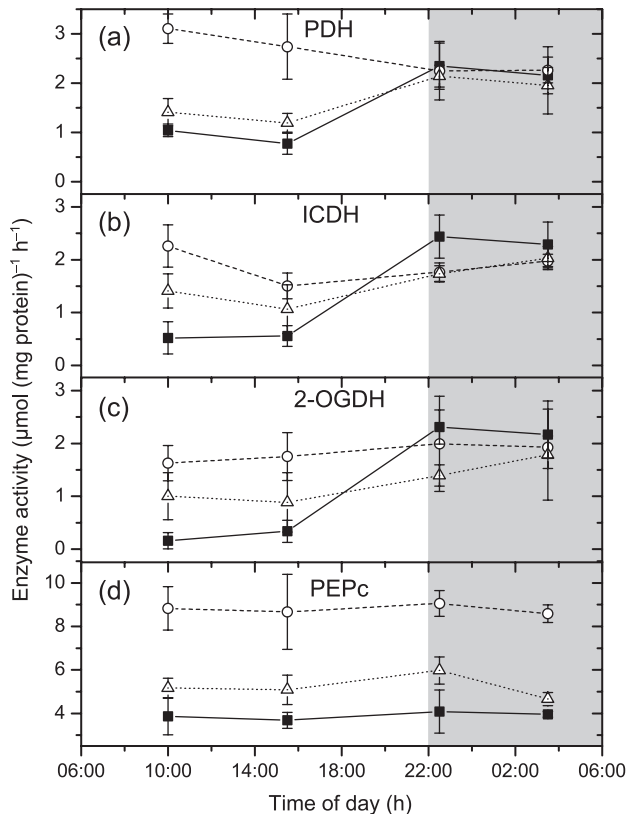


Fig. 3 Diel patterns of the activities of pyruvate dehydrogenase (PDH) (a), isocitric dehydrogenase (ICDH) (b), 2-oxoglutarate dehydrogenase (2-OGDH) (c) and phosphoenolpyruvate carboxylase (PEPc) (d) in *Ricinus communis* leaves (squares), stems (triangles) and roots (circles). The results are expressed on a protein basis for better comparability among organs. Data shown are mean values (\pm SD) from three plants. The shaded area denotes the dark period.

linear correlation between cumulative CO_2 uptake and ^{13}C enrichment of respired CO_2 resulting in a 6‰ enrichment after a CO_2 assimilation of 0.20 mol m^{-2} . After 4 h in the light (at 10:00 h), nearly $0.28 \text{ mol m}^{-2} \text{CO}_2$ was photosynthetically fixed by *R. communis* in our experiments and the ^{13}C enrichment in CO_2 (Δ_R^s) reached 4‰, a value reasonably close to that of Hymus *et al.* (2005). However, further cumulative assimilation (approx. 0.72 mol m^{-2} at 16:00 h) did not increase Δ_R^s as might have been expected from the results of previous studies with *Q. ilex* (Hymus *et al.*, 2005; Werner *et al.*, 2007). Our present results indicate a dependence of Δ_R^s upon assimilation rate rather than upon cumulative assimilation.

The respiratory substrate as indicated by the RQ was carbohydrate throughout the diel cycle (RQ = 1 with little variation; Fig. 2). Nevertheless, the isotopic composition of substrates (sugars and the water-soluble fraction) differed markedly in the light and in the dark ($> 2\%$ enriched in darkness; Fig. 1a) and was thus opposite to that of evolved CO_2 (which was ^{13}C -depleted in dark-acclimated leaves).

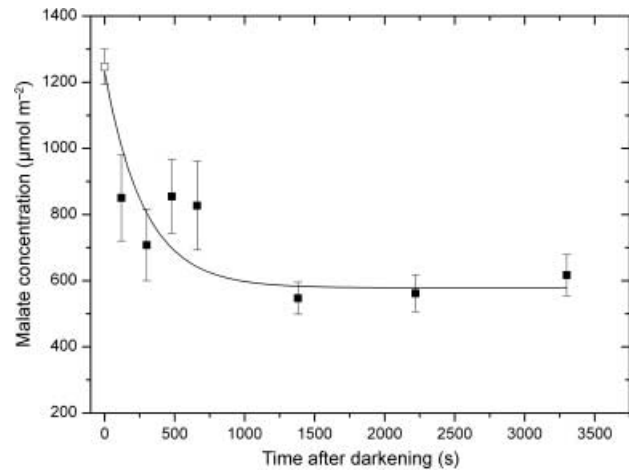


Fig. 4 Change in the malate concentration per leaf area in the leaf tissue after darkening the leaves of *Ricinus communis* plants. The white square indicates the concentration in the light at 13:00 h. Data shown are calculated from six independent replicates; the error bars denote \pm SE. The regression line follows a first-order exponential decay equation.

This clearly indicates that the post-illumination ^{13}C effect in CO_2 does not originate from a change of the $\delta^{13}\text{C}$ value of substrates (sugars) and, rather, is associated with a change of the metabolic pathways responsible for CO_2 production (that is, the nature of the reactions involved or commitment values). Accordingly, there was no correlation between the $\delta^{13}\text{C}$ for leaf CO_2 release and any of the leaf organic fractions throughout the diel course (Table 1).

The ^{13}C enrichment in leaf respired CO_2 is caused by a metabolic malate imbalance

Illuminated leaves showed a clear inhibition of several respiratory enzymes, in particular 2-OGDH and ICDH (Fig. 3). This picture is consistent with the observed inhibition of the TCA cycle activity in the light, as revealed by assays on illuminated spinach (*Spinacia oleracea*) leaf extracts (Hanning & Heldt, 1993) or for *in vivo* ^{13}C -labelling of bean (*Phaseolus vulgaris*) or detached leaves of *Xanthium strumarium* (Tcherkez *et al.*, 2005, 2008). The PDH activity was not reduced to the same extent, and this discrepancy arose from the involvement of chloroplastic PDH during the enzymatic assay (Tcherkez *et al.*, 2005), which is not down-regulated by phosphorylation in the light (Tovar-Mendez *et al.*, 2003). Similarly, the decrease of the ICDH activity is probably underestimated in the present results because of the involvement of nonmitochondrial ICDH isoforms during the assay (Hodges, 2002).

Under an assumption of mass balance of 2-oxoglutarate within the mitochondria, one may hypothesize that the TCA cycle input (metabolic flux associated with citrate synthase) is equal to the 2-OGDH flux. The resulting oxaloacetate or malate net production flux is then that of PEPc (which binds

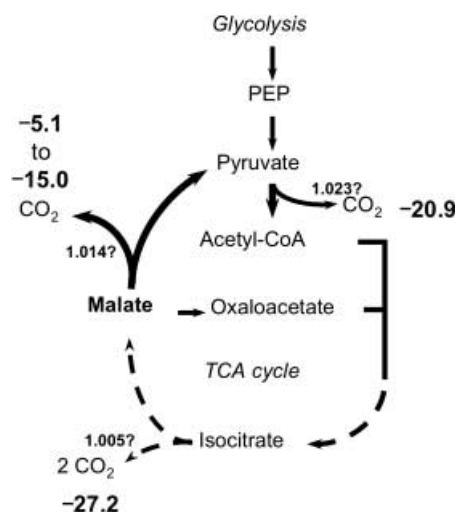


Fig. 5 Simplified metabolic scheme showing the major reactions contributing to CO₂ production during post-illumination respiration in leaves: decarboxylation of phosphoenolpyruvate carboxylase (PEPc)-derived malate (possible isotope effect of 1.014; Rishavy *et al.*, 2001), pyruvate decarboxylation (possible isotope effect of 1.023; Melzer & Schmidt, 1987) and isocitrate dehydrogenase (possible isotope effect of 1.005; Lin *et al.*, 2008). Malate is ¹³C-enriched in C-4 and so is the corresponding decarboxylated CO₂; -5.1‰ is the ¹³C isotopic signature (δ¹³C) value observed by Melzer & O’Leary (1987) and -15‰ is the calculated value for a 50% consumption of the malate content with an isotope effect of 1.014. -20.9‰ and -27.2‰ are the δ¹³C values of carbon atom positions in glucose subsequently involved in the decarboxylations (Tcherkez *et al.*, 2003).

HCO₃⁻ to phosphoenolpyruvate to produce oxaloacetate) minus oxaloacetate consumption by the TCA. Hence, malate accumulates at a rate given by the activity difference PEPc – 2-OGDH. We recognize that such a value may be underestimated, because 2-oxoglutarate molecules may come from the decarboxylation of remobilized stored citrate (Tcherkez & Hodges, 2008) and stored fumarate molecules may be converted to malate by fumarase (Tcherkez *et al.*, 2008). In addition, we recognize that *in vitro* enzyme activities as measured here might not be equal to *in vivo* activities and fluxes through given metabolic pathways. Still, there is a correlation between the estimated malate synthesis excess and Δ_R^s (Fig. 3, Table 2). In other words, the larger the disequilibrium towards malate accumulation during the previous illumination phase, the larger the ¹³C enrichment (above leaf sugars) in CO₂ subsequently decarboxylated by leaves (Fig. 5).

This scenario is consistent with the well-recognized ¹³C enrichment in PEPc-derived malate, as a consequence of the fractionation against ¹²C of PEPc fixation with respect to dissolved CO₂ (the CO₂ to HCO₃⁻ equilibrium favours ¹³C by 9‰, while PEPc fixation discriminates against ¹³C in HCO₃⁻ by 2–4‰ only; for a review, see Farquhar *et al.*, 1989). In addition, leaf aspartate (which derives from oxaloacetate through transamination) is clearly ¹³C-enriched in C-4

(the carbon atom position has a δ¹³C value of -5.1‰, that is, it is ¹³C-enriched by 22.3‰ compared with total leaf organic matter), indicating that > 50% of the aspartate pool comes from PEPc fixation in the leaf (Melzer & O’Leary, 1987). With the present data set, we may calculate what the δ¹³C of C-4 in malate should be to account for the observed ¹³C enrichment in CO₂. The malate decarboxylation rate (*d*) during the first 15–20 min after darkening (the duration of the gas exchange measurements to obtain the δ¹³C value of respired CO₂) calculated from the decrease in malate concentration in Fig. 4 was 0.54 to 0.71 μmol m⁻² s⁻¹. Assuming that only malate decarboxylation and sugar consumption contributed to CO₂ production just after darkening, and in a first step neglecting ¹²C/¹³C isotope effects, the δ¹³C value of the C-4 carbon in malate (δ_M) follows from a mass-balance equation:

$$\delta_M = \frac{\delta^{12}C_R r - \delta^{13}C_{orgsub}(r - d)}{d} \quad \text{Eqn 2}$$

(*r*, the leaf respiration rate of darkened leaves; δ¹³C_R and δ¹³C_{orgsub}, the isotope signatures of respired CO₂ and the sugar substrate for respiration, respectively.) With the *r* and δ¹³C values measured at 15:00 h, we obtain a δ_M value of -9 to -14‰, depending on the *d* value. Such a value is quite close to that calculated from the data of Melzer & O’Leary (1987) (these authors found that the δ¹³C of C-4 for aspartate was -5.1‰, that is, ¹³C-enriched by 22.3‰ compared with leaf organic matter), that is (taking into account the δ¹³C of leaf organic matter of *R. communis* in our experiment at 15:00 h), -30 + 22.3 = -7.7‰. Nevertheless, we recognize that the malic enzyme (which decarboxylates malate) may be associated with an isotope effect that favours ¹²C and may compensate for the ¹³C enrichment in malate C-4. *In vitro*, the NADP-dependent malic enzyme from chicken liver discriminates by 31‰ against ¹³C (Edens *et al.*, 1997; Hermes *et al.*, 1982) and the human NAD-dependent malic enzyme discriminates by 14‰ (Rishavy *et al.*, 2001). Under the assumption that the malic enzyme involved in post-illumination malate decarboxylation is NAD-dependent (a mitochondrial enzyme), a 14‰ fractionation may be taken into account (Fig. 5). With a 50% consumption of the initial malate content (Fig. 4) and an initial δ¹³C value of -5.1‰ in C-4 malate, the resulting CO₂ produced by malate decarboxylation (total CO₂ lost) has a δ¹³C value of approx. -15‰ (applying a classical Rayleigh mass-balance calculation; using eqn 11 from McNevin *et al.* (2006), we obtain log_e((1 + δ)/(1 - 5.1/1000)) = -14/1000 × log_e 0.5 for the δ of the malate C at the time that the fraction left is 0.5, i.e. δ = 4.6‰ (= -5.1 + 9.7‰); from mass balance, this means that the δ of the CO₂ lost must have been, on average over this period, -5.1 - 9.7 = -14.8‰). Such a value is consistent with the above minimum value of -14‰ obtained with the larger *d* value.

These data clearly support the involvement of malate decarboxylation in post-illumination ¹³CO₂ enrichment. With

values for d of 0.54 to 0.71 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and for r of 2.7 to 2.8 $\mu\text{mol m}^{-2} \text{s}^{-1}$, c . $0.62/2.75 = 22\%$ of the CO_2 emitted was from malate decarboxylation. This means that the RQ should have been 1.075 taking into account RQs for malate of 1.33 and for sugars of 1.0. In Fig. 2 the RQ in darkened leaves during the light period is approx. 1 and the standard deviation of up to 0.12 does not allow resolution of the subtle variations in RQ attributable to such changes in substrate composition.

Uncertainties still remain about whether isotope fractionations occur at levels other than the malic enzyme (Fig. 5). It has been hypothesized that the glycolytic pathway is committed in darkness, so that PDH does not discriminate between isotopes (Tcherkez & Farquhar, 2005). It is possible that TCA cycle decarboxylases discriminate against ^{13}C , depleting leaf respired CO_2 in ^{13}C (Tcherkez & Farquhar, 2005; Lin *et al.*, 2008). That said, if $^{12}\text{C}/^{13}\text{C}$ fractionations were to occur, this would compensate for the ^{13}C enrichment from malate decarboxylation; in other words, one would have to assume a much larger malate decarboxylation or a very small isotope effect associated with the malic enzyme to account for the observed ^{13}C enrichment in post-illumination evolved CO_2 . The above value of 22% calculated for the contribution of malate decarboxylation to LEDR is, however, close to the value of 30% computed by Hill & Bryce (1992) from the decrease in malate concentration in barley (*Hordeum vulgare*) mesophyll protoplasts in the first 2 min after the transition from light to dark.

PEPc-related metabolism and the ^{13}C abundance of plant organs

Our finding that evolved CO_2 is ^{13}C -enriched compared with substrates in leaves and depleted in stems and roots is in agreement with other studies that assessed respiratory fractionation in nonwoody plants. Badeck *et al.* (2005) also observed a ^{13}C enrichment (compared with the assumed organic substrate) in leaf but not in stem and root respired CO_2 in French bean (*Phaseolus vulgaris*) and Klumpp *et al.* (2005) found shoot respired CO_2 to be ^{13}C -enriched compared with root respired CO_2 in herbs and grasses. The authors of both publications assumed that PEPc activity was the main reason for the apparent depletion of root respired CO_2 , as the measured values of $\delta^{13}\text{C}$ of evolved CO_2 are not only affected by the $\delta^{13}\text{C}$ of the respiratory substrate and the possible respiratory fractionation, but also include isotope effects associated with PEPc (re-)fixation of CO_2 . The latter fractionates against ^{12}C by 5.7‰ (Farquhar, 1983), thus enriching organic matter and depleting CO_2 molecules left behind. Our results support this hypothesis as we observed a significant positive correlation between *in vitro* PEPc activity and respiratory fractionation (based on the $\delta^{13}\text{C}$ of sugars) for the whole plant (Table 2). In addition, PEPc activity was highest in roots, in agreement with the ^{13}C enrichment in organic matter or water-soluble material as compared with other organs (Fig. 1).

Our study under controlled conditions shows that enzyme activities and metabolic fluxes affect apparent respiratory carbon isotope fractionation. As a consequence, the $\delta^{13}\text{C}$ of respired CO_2 is not necessarily linked directly to photosynthetic carbon isotope discrimination because of $^{12}\text{C}/^{13}\text{C}$ fractionating steps downstream and the consumption of substrates of contrasting $\delta^{13}\text{C}$ values by respiration. On the one hand, rapid changes in the composition of the respiratory substrate and thus changes in metabolic fluxes might lead to marked short-term changes in the $\delta^{13}\text{C}$ of CO_2 , as illustrated here with leaves, which were darkened in the light periods. On the other hand, organ-specific differences in PEPc activity are likely to cause more or less continuous differences in the $\delta^{13}\text{C}$ of respired CO_2 among leaves, stems and roots, which cannot be explained by differences in the $\delta^{13}\text{C}$ of the respiratory substrate. In the future, we need to explore in more depth the physiological basis for variations in the respiratory isotope fractionation, especially under field conditions, thereby addressing organ- and species-specific differences as well as the short-term dynamics of $\delta^{13}\text{C}$ in respired CO_2 .

Acknowledgements

AG acknowledges financial support from the Deutsche Forschungsgemeinschaft (DFG) under contract numbers GE 1090/4-1 and GE 1090/5-1. Agilent Technologies is acknowledged for providing instrumental support for GC-MS measurements.

References

- Atkin OK, Millar AH, Gardstroem P, Day DA. 2000. Photosynthesis, carbohydrate metabolism and respiration in leaves of higher plants. In: Leegood RC, Sharkey TD, Von Caemmerer S, eds. *Photosynthesis and metabolism*. Dordrecht, the Netherlands: Kluwer Academic, 153–175.
- Azcon-Bieto J, Osmond CB. 1983. Relationship between photosynthesis and respiration: the effect of carbohydrate status on the rate of CO_2 production by respiration in darkened and illuminated wheat leaves. *Plant Physiology* 71: 574–581.
- Badeck FW, Tcherkez G, Nogues S, Piel C, Ghashghaie J. 2005. Post-photosynthetic fractionation of stable carbon isotopes between plant organs – a widespread phenomenon. *Rapid Communications in Mass Spectrometry* 19: 1381–1391.
- Barbour MM, McDowell NG, Tcherkez G, Bickford CP, Hanson DT. 2007. A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO_2 . *Plant, Cell & Environment* 30: 469–482.
- Bathellier C, Badeck FW, Couzi P, Harscoet S, Mauve C, Ghashghaie J. 2008. Divergence in $\delta^{13}\text{C}$ of dark respired CO_2 and bulk organic matter occurs during the transition between heterotrophy and autotrophy in *Phaseolus vulgaris* plants. *New Phytologist* 177: 406–418.
- Bergmeyer HU. 1974. *Methods of enzymatic analysis*. New York, NY, USA: Academic Press.
- Blight EG, Dryer WJ. 1959. A rapid method for total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37: 911–917.
- Bowling DR, Tans PP, Monson RK. 2001. Partitioning net ecosystem carbon exchange with isotopic fluxes of CO_2 . *Global Change Biology* 7: 127–145.

- Bradford MM. 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
- Brandes E, Kodama N, Whittaker K, Weston C, Rennenberg H, Keitel C, Adams MA, Gessler A. 2006. Short-term variation in the isotopic composition of organic matter allocated from the leaves to the stem of *Pinus sylvestris*: effects of photosynthetic and postphotosynthetic carbon isotope fractionation. *Global Change Biology* 12: 1922–1939.
- Brown JP, Perham RN. 1976. Selective inactivation of transacylase components of 2-oxo acid dehydrogenase multienzyme complexes of *Escherichia coli*. *Biochemical Journal* 155: 419–427.
- Brugnoli E, Hubick KT, vonCaemmerer S, Wong SC, Farquhar GD. 1988. Correlation between the carbon isotope discrimination in leaf starch and sugars of C₃ plants and the ratio of intercellular and atmospheric partial pressures of carbon-dioxide. *Plant Physiology* 88: 1418–1424.
- Damesin C, Lelarge C. 2003. Carbon isotope composition of current-year shoots from *Fagus sylvatica* in relation to growth, respiration and use of reserves. *Plant, Cell & Environment* 26: 207–219.
- Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. 1999. $\delta^{13}\text{C}$ of CO₂ respired in the dark in relation to $\delta^{13}\text{C}$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant, Cell & Environment* 22: 515–523.
- Edens WA, Urbauer JL, Cleland WW. 1997. Determination of the chemical mechanism of malic enzyme by isotope effects. *Biochemistry* 36: 1141–1147.
- Farquhar GD. 1983. On the nature of carbon isotope discrimination in C₄ species. *Australian Journal of Plant Physiology* 10: 205–226.
- Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 503–537.
- Farquhar GD, O'Leary MH, Berry JA. 1982. On the relationship between carbon isotope discrimination and the inter-cellular carbon-dioxide concentration in leaves. *Australian Journal of Plant Physiology* 9: 121–137.
- Gessler A, Keitel C, Kodama N, Weston C, Winters AJ, Keith H, Grice K, Leuning R, Farquhar GD. 2007a. $\delta^{13}\text{C}$ of organic matter transported from the leaves to the roots in *Eucalyptus delegatensis*: short-term variations and relation to respired CO₂. *Functional Plant Biology* 34: 692–706.
- Gessler A, Peuke AD, Keitel C, Farquhar GD. 2007b. Oxygen isotope enrichment of organic matter in *Ricinus communis* during the diel course and as affected by assimilate transport. *New Phytologist* 174: 600–613.
- Gessler A, Tcherkez G, Peuke AD, Ghashghaie J, Farquhar GD. 2008. Experimental evidence for diel variations of the carbon isotope composition in leaf, stem and phloem sap organic matter in *Ricinus communis*. *Plant, Cell & Environment* 31: 941–953.
- Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E. 2001. $\delta^{13}\text{C}$ of CO₂ respired in the dark in relation to $\delta^{13}\text{C}$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant, Cell & Environment* 24: 505–515.
- Hanning I, Heldt HW. 1993. On the function of mitochondrial metabolism during photosynthesis in spinach (*Spinacia oleracea* L.) leaves – partitioning between respiration and export of redox equivalents and precursors for nitrate assimilation products. *Plant Physiology* 103: 1147–1154.
- Hermes JD, Roeske CA, O'Leary MH, Cleland WW. 1982. Use of multiple isotope effects to determine enzyme mechanisms and intrinsic isotope effects – malic enzyme and glucose-6-phosphate-dehydrogenase. *Biochemistry* 21: 5106–5114.
- Hill SA, Bryce JH. 1992. Malate metabolism and light-enhanced dark respiration in barley mesophyll protoplasts. In: Lambers H, van der Plas LHW, eds. *Molecular, biochemical and physiological aspects of plant respiration*. The Hague, the Netherlands: SPB Academic Publishing, 221–230.
- Hodges M. 2002. Enzyme redundancy and the importance of 2-oxoglutarate in plant ammonium assimilation. *Journal of Experimental Botany* 53: 905–916.
- Hymus GJ, Maseyk K, Valentini R, Yakir D. 2005. Large daily variation in ^{13}C -enrichment of leaf-respired CO₂ in two *Quercus* forest canopies. *New Phytologist* 167: 377–384.
- Jeschke WD, Pate JS. 1991. Modeling of the partitioning, assimilation and storage of nitrate within root and shoot organs of castor bean (*Ricinus communis* L.). *Journal of Experimental Botany* 42: 1091–1103.
- Johnson JF, Allan DL, Vance CP. 1994. Phosphorus stress-induced proteoid roots show altered metabolism in *Lupinus albus*. *Plant Physiology* 104: 657–665.
- Keeling CD. 1958. The concentration and isotopic abundances of atmospheric carbon dioxide in rural areas. *Geochimica et Cosmochimica Acta* 13: 322–334.
- Keeling CD. 1961. The concentration and isotopic abundances of carbon dioxide in rural and marine air. *Geochimica et Cosmochimica Acta* 24: 277–298.
- Klump K, Schaufele R, Lotscher M, Lattanzi FA, Feneis W, Schnyder H. 2005. C-isotope composition of CO₂ respired by shoots and roots: fractionation during dark respiration? *Plant, Cell & Environment* 28: 241–250.
- Kodama N, Barnard R, Salmon Y, Weston C, Ferrio JP, Holst J, Werner R, Sauer M, Rennenberg H, Buchmann N *et al.* 2008. Temporal dynamics of the carbon isotope composition in a *Pinus sylvestris* stand – from newly assimilated organic carbon to respired CO₂. *Oecologia* 156: 737–750.
- Lin Y, Volkman J, Nicholas KM, Yamamoto T, Eguchi T, Nimmo SL, West AH, Cook PF. 2008. Chemical mechanism of homoisocitrate dehydrogenase from *Saccharomyces cerevisiae*. *Biochemistry* 47: 4169–4180.
- McNevin DB, Badger MR, Kane HJ, Farquhar GD. 2006. Measurement of (carbon) kinetic isotope effect by Rayleigh fractionation using membrane inlet mass spectrometry for CO₂-consuming reactions. *Functional Plant Biology* 33: 1115–1128.
- Melzer E, O'Leary MH. 1987. Anapleurotic CO₂ fixation by phosphoenolpyruvate carboxylase in C₃ plants. *Plant Physiology* 84: 58–60.
- Melzer E, Schmidt HL. 1987. Carbon isotope effects on the pyruvate-dehydrogenase reaction and their importance for relative ^{13}C depletion in lipids. *Journal of Biological Chemistry* 262: 8159–8164.
- Pataki DE, Ehleringer JR, Flanagan LB, Yakir D, Bowling DR, Still CJ, Buchmann N, Kaplan JO, Berry JA. 2003. The application and interpretation of keeling plots in terrestrial carbon cycle research. *Global Biogeochemical Cycles* 17: art. no. 1022.
- Peuke AD, Hartung W, Jeschke WD. 1994. The uptake and flow of C, N and ions between roots and shoots in *Ricinus communis* L. 2. grown with low or high nitrate supply. *Journal of Experimental Botany* 45: 733–740.
- Rishavy MA, Yang ZR, Tong L, Cleland WW. 2001. Determination of the mechanism of human malic enzyme with natural and alternate dinucleotides by isotope effects. *Archives of Biochemistry and Biophysics* 396: 43–48.
- Rossmann A, Butzenlechner M, Schmidt HL. 1991. Evidence for a nonstatistical carbon isotope distribution in natural glucose. *Plant Physiology* 96: 609–614.
- Sachs L. 1984. *Angewandte Statistik*. Berlin, Germany: Springer.
- Schmidt HL. 2003. Fundamentals and systematics of the nonstatistical distributions of isotopes in natural compounds. *Naturwissenschaften* 90: 537–552.
- Tcherkez G, Blligny R, Gout E, Mahe A, Hodges M, Cornic G. 2008. Respiratory metabolism of illuminated leaves depends on CO₂ and O₂ conditions. *Proceedings of the National Academy of Sciences, USA* 105: 797–802.

- Tcherkez G, Cornic G, Bligny R, Gout E, Ghashghaie J. 2005. In vivo respiratory metabolism of illuminated leaves. *Plant Physiology* 138: 1596–1606.
- Tcherkez G, Farquhar G, Badeck F, Ghashghaie J. 2004. Theoretical considerations about carbon isotope distribution in glucose of C₃ plants. *Functional Plant Biology* 31: 857–877.
- Tcherkez G, Farquhar GD. 2005. Carbon isotope effect predictions for enzymes involved in the primary carbon metabolism of plant leaves. *Functional Plant Biology* 32: 277–291.
- Tcherkez G, Hodges M. 2008. How stable isotopes may help to elucidate primary nitrogen metabolism and its interaction with (photo)respiration in C₃ leaves. *Journal of Experimental Botany* 59: 1685–1693.
- Tcherkez G, Nogues S, Bleton J, Cornic G, Badeck F, Ghashghaie J. 2003. Metabolic origin of carbon isotope composition of leaf dark-respired CO₂ in French bean. *Plant Physiology* 131: 237–244.
- Tovar-Mendez A, Miernyk JA, Randall DD. 2003. Regulation of pyruvate dehydrogenase complex activity in plant cells. *European Journal of Biochemistry* 270: 1043–1049.
- Werner C, Hasenbein N, Maia R, Beyschlag W, Maguas C. 2007. Evaluating high time-resolved changes in carbon isotope ratio of respired CO₂ by a rapid in-tube incubation technique. *Rapid Communications in Mass Spectrometry* 21: 1352–1360.
- Xu CY, Lin GH, Griffin KL, Sambrotto RN. 2004. Leaf respiratory CO₂ is ¹³C-enriched relative to leaf organic components in five species of C₃ plants. *New Phytologist* 163: 499–505.



About *New Phytologist*

- *New Phytologist* is owned by a non-profit-making **charitable trust** dedicated to the promotion of plant science, facilitating projects from symposia to open access for our Tansley reviews. Complete information is available at www.newphytologist.org.
- Regular papers, Letters, Research reviews, Rapid reports and both Modelling/Theory and Methods papers are encouraged. We are committed to rapid processing, from online submission through to publication 'as-ready' via *Early View* – our average submission to decision time is just 29 days. Online-only colour is **free**, and essential print colour costs will be met if necessary. We also provide 25 offprints as well as a PDF for each article.
- For online summaries and ToC alerts, go to the website and click on 'Journal online'. You can take out a **personal subscription** to the journal for a fraction of the institutional price. Rates start at £139 in Europe/\$259 in the USA & Canada for the online edition (click on 'Subscribe' at the website).
- If you have any questions, do get in touch with Central Office (newphytol@lancaster.ac.uk; tel +44 1524 594691) or, for a local contact in North America, the US Office (newphytol@ornl.gov; tel +1 865 576 5261).