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A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO_2

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ABSTRACT

We describ an open leaf gas exchange system coupled to a tunable diode laser (TDL) spectroscopy system enabling measurement of the leaf respiratory CO_2 flux and its associated carbon isotope composition ($\delta^{13}C_{Rl}$) every 3 min. The precision of $\delta^{13}C_{Rl}$ measurement is comparable to that of traditional mass spectrometry techniques. $\delta^{13}C_{Rl}$ from castor bean (*Ricinus communis* L.) leaves tended to be positively related to the ratio of CO_2 produced to O_2 consumed [respiratory quotient (*RQ*)] after 24–48 h of prolonged darkness, in support of existing models. Further, the apparent fractionation between respiratory substrates and respired CO_2 within 1–8 h after the start of the dark period was similar to previous observations. In subsequent experiments, *R. communis* plants were grown under variable water availability to provide a range in $\delta^{13}C$ of recently fixed carbohydrate. In leaves exposed to high light levels prior to the start of the dark period, CO_2 respired by leaves was up to 11‰ more enriched than phloem sap sugars within the first 10–15 min after plants had been moved from the light into the dark. The ^{13}C enrichment in respired CO_2 then decreased rapidly to within 3–7‰ of phloem sap after 30–60 min in the dark. This strong enrichment was not observed if light levels were low prior to the start of the dark period. Measurements of *RQ* confirmed that carbohydrates were the likely respiratory substrate for plants ($RQ > 0.8$) within the first 60 min after illumination. The strong ^{13}C enrichment that followed a high light-to-dark transition coincided with high respiration rates, suggesting that so-called light-enhanced dark respiration (LEDR) is fed by ^{13}C-enriched metabolites.

Key-words: light-enhanced dark respiration; tunable diode laser.

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INTRODUCTION

The stable carbon isotope composition of leaf-respired CO_2 ($\delta^{13}C_{Rl}$) has enormous potential to allow partitioning of ecosystem respiration into various components (Tu & Dawson 2005), to provide information on key physiological processes (Ghashghaie *et al.* 2003) and to trace carbon fluxes through plants and ecosystems (e.g. Barbour *et al.* 2005). However, difficulties in measuring and understanding variation in $\delta^{13}C_{Rl}$ have limited its application. Recent experimental work has demonstrated significant variability in $\delta^{13}C_{Rl}$ (Tcherkez *et al.* 2003; Ocheltree & Marshall 2004; Xu *et al.* 2004; Hymus *et al.* 2005), and theoretical work (Ghashghaie *et al.* 2003; Tcherkez *et al.* 2005) has provided a framework with which to understand some of this variability.

CO_2 respired by isolated leaf protoplasts displays no apparent fractionation (denoted as e_d in this paper to distinguish respiration in the dark from mitochondrial respiration in the light) compared to substrates (Lin & Ehleringer 1997). However, intact leaves commonly respire ^{13}C-enriched CO_2 compared to likely respiratory substrates. For example, *Phaseolus vulgaris* leaves respired CO_2 in the dark that was 6‰ more enriched than sucrose (Duranceau *et al.* 1999), while in *Nicotiana sylvestris* and *Helianthus annuus*, values of e_d were 4 and 3‰, respectively (Ghashghaie *et al.* 2001). Further, $\delta^{13}C_{Rl}$ varies with time spent in the dark (Tcherkez *et al.* 2003; Nogués *et al.* 2004; Tu & Dawson 2005), with light level during growth (Ocheltree & Marshall 2004) and over a diurnal cycle in field-grown *Quercus* leaves (Hymus *et al.* 2005).

When carbohydrates are the respiratory substrate, ^{13}C-enriched respiratory CO_2 is thought to result from the non-statistical (i.e. non-random) carbon isotope distribution within carbohydrate molecules (Ghashghaie *et al.* 2001). Carbon atoms in positions C-3 and C-4 of glucose are 2 and 6‰ more enriched than the average for the whole molecule (Roßmann, Butzenlechner & Schmidt 1991). These atoms are released as CO_2 during glycolysis by pyruvate dehydrogenase (PDH) and are thought to predominate in respired CO_2, so that the latter is enriched in ^{13}C (Tcherkez *et al.*

2003). Tcherkez *et al.* (2004) have termed this effect 'fragmentation fractionation'. The overall result is an enrichment in CO_2 released and a depletion of ^{13}C in acetyl-CoA and subsequent fatty acids. Further, PDH itself may fractionate against ^{13}C during acetyl-CoA formation (DeNiro & Epstein 1977; Melzer & Schmidt 1987).

This picture is complicated by the dynamics of respiratory metabolism of leaf cells. When carbohydrates are limiting (i.e. leaf starch or sucrose pools are fully consumed), substrates other than carbohydrates may be used for respiration. In particular, Tcherkez *et al.* (2003) showed that if the citric acid cycle coupled to β-oxidation of fatty acids dominates over PDH in producing CO_2, then the CO_2 respired will be depleted in ^{13}C. This pattern explains the positive relationship between the ratio of CO_2 produced to O_2 consumed [i.e. the respiratory quotient (*RQ*)] and $\delta^{13}C_{Rl}$, with the most ^{13}C-enriched CO_2 when PDH activity dominated and *RQ* was close to 1, and more ^{13}C-depleted CO_2 when *RQ* dropped below 0.6 as time in the dark increased (Tcherkez *et al.* 2003). *RQ* indicates the type of substrate used in respiration because more reduced substrates, like fatty acids, require more oxygen per CO_2 molecule produced than less reduced substrates like carbohydrates.

Although previous work describes and explains the slow kinetics (half an hour to several days in continuous darkness) of the carbon isotope composition of leaf-respired CO_2 in darkness, the rapid kinetics just after illumination is unknown. However, it is recognized that leaves experience an important modification of the respiratory regime during the transition from high light to dark: after the photorespiratory burst, the respiration rate often displays a peak within 10–20 min, termed light-enhanced dark respiration (LEDR) (Atkin *et al.* 2000). Organic acids, which are thought to accumulate under high light conditions (Cornic 1973) and may provide the respiratory substrates for LEDR, often differ in $\delta^{13}C$ compared to carbohydrates (Ghashghaie *et al.* 2001), so measurements of $\delta^{13}C$ of the CO_2 evolved in darkness during LEDR may help to determine its metabolic origin.

A new instrument for measurement of the stable isotope composition of atmospheric CO_2 provides the potential of high-frequency measurements of leaf-respired $\delta^{13}C$. Tunable diode laser (TDL) spectroscopy is a relatively new technique for online CO_2 measurements at high frequency, and has proven valuable for ecosystem scale measurements (Bowling *et al.* 2003; Griffis *et al.* 2004). We adopted this technique for quantification of leaf respiration by plumbing it to a portable gas exchange system designed for leaf-level measurements, and tested the coupled system in this study using castor bean (*Ricinus communis* L., Euphorbiaceae).

We have three objectives in this paper. Firstly, we demonstrated the application of TDL absorption spectroscopy coupled to a portable gas exchange system for leaf-level measurements of $\delta^{13}C_{Rl}$, and compared values and measurement precision with published studies using traditional mass spectrometric techniques. The first experiment involved exposing leaves to a long-term period of darkness (52 h) for comparison with previously published experiments. Secondly, we quantified short-term temporal variability in $\delta^{13}C_{Rl}$ during the high respiration rates associated with LEDR at the start of the dark period. This experiment was conducted in order to test the hypothesis that measuring $\delta^{13}C_{Rl}$ during LEDR enhances understanding of the substrate for respiration during the LEDR peak. Thirdly, we quantified short-term variability in photosynthetic carbon isotope fractionation ($\Delta^{13}C_A$) and $\delta^{13}C_{Rl}$ during a switch from low light to dark in the absence of LEDR in order to determine the degree to which $\delta^{13}C$ of very recently fixed carbon is reflected in $\delta^{13}C_{Rl}$. The experiments were conducted on castor bean plants grown under two levels of water availability to provide a range in $\delta^{13}C$ of respiratory substrates.

MATERIALS AND METHODS

Growth conditions

Castor bean (*R. communis* L.) plants were grown in 7 L pots with potting mix and a slow-release fertilizer in a glasshouse at Los Alamos National Laboratory, NM, USA, during April and May 2005 (for the first and second experiments) and during July and August 2006 (for the third experiment). Average half-hourly photosynthetically active radiation (PAR) was measured with a quantum sensor (LI190SA; Li-Cor, Inc., Lincoln, NE, USA), and average half-hourly air temperature and relative humidity were measured (CS500L; Campbell Scientific, Inc., Logan UT, USA) and recorded with a datalogger (CR23; Campbell Scientific, Inc.). A shade cloth stretched across the roof of the glasshouse reduced the direct light within the glasshouse by about half, giving a maximum midday irradiance of 1050 μmol m^{-2} s^{-1} PAR.

For the first and second experiments, average daytime air temperature within the glasshouse varied between 24.1 and 31.2 °C, and relative humidity between 19 and 35%. Average daytime air temperature over the experimental period was 27.1 ± 0.8 °C, and relative humidity 25 ± 2%. Average night temperature varied between 18.2 and 22.1 °C and relative humidity between 14 and 40%. Average night temperature over the experimental period was 20.6 ± 0.4 °C, and relative humidity 27 ± 2%. The plants were well watered every second day, except five plants for which water was withheld for 7 d. Droughted plants were measured on the sixth and seventh day after watering ceased, and were observed to be slightly wilted on the morning of the seventh day.

For the third experiment, average daytime air temperature over the experimental period was 28.3 ± 2.4 °C, and relative humidity 42 ± 9%. Average night temperature over the experimental period was 21.5 ± 2.5 °C, and relative humidity 56 ± 13%. The plants were well watered every second day, except three plants for which water was withheld for 7 d.

Experimental description

Three different experiments were conducted. The first experiment (hereafter referred to as the 'long-term respiration' experiment) aimed at demonstrating the measurement technique and comparing the technique and measurements to previously experiments. Hence, changes in respiratory CO_2 production (R_c), respiratory O_2 consumption (R_o) and the carbon isotope composition of leaf-respired CO_2 ($\delta^{13}C_{Rl}$) were measured (at the 3 min temporal resolution available with the TDL) over an extended period of continuous darkness after 24 h of illumination. The goal of the 24 h illumination period was to maximize carbohydrate storage within the leaves, and the extended dark period to deplete these carbon reserves and so induce changes in respiratory substrates. Plants grown under high light and well-watered conditions were moved into a controlled-environment growth cabinet (E15; Conviron, Winnipeg, Manitoba, Canada) set at 30 °C and 70% relative humidity, and maintained under constant light (400 μmol m^{-2} s^{-1} at the level of the uppermost leaves) for 24 h. The cabinet lights were then turned off, and the plants kept in the dark for 52 h to assess the variation in $\delta^{13}C_{Rl}$ over the extended dark period. The plants remained in the dark growth cabinet while not being measured, but were moved under the cardboard box during R_c measurement. The results were compared to an experiment of similar design described by Tcherkez *et al.* (2003).

The second experiment (hereafter referred to as the 'short-term respiration' experiment) aimed to quantify short-term variability in $\delta^{13}C_{Rl}$ during the first 40 min of a dark period, after leaves had been exposed to differing levels of cumulative irradiance to provide varying levels of LEDR. To do this, changes in R_c, R_o and $\delta^{13}C_{Rl}$ were measured immediately after the plants were placed in the dark. The plants were moved into the dark at different times of the day, so that the leaves were exposed to light periods of different intensity and length over the 2 d of measurement. Five well-watered and five droughted plants were measured. The cumulative intercepted irradiance on the day of measurement (in mol m^{-2}) was calculated for each leaf using quantum sensors at the height of the uppermost leaves. Just prior to R_c measurements, the plants were placed in the dark under a large cardboard box. The air temperature within the darkened cardboard box was the same as ambient glasshouse air temperature, and the plants spent between 25 and 35 min in the dark.

The third experiment (hereafter referred to as the 'short-term photosynthesis–respiration' experiment) aimed to quantify short-term variability in photosynthetic discrimination and in $\delta^{13}C_{Rl}$ during a switch from light to dark. Light levels prior to the start of the dark period were relatively low to avoid possible complications in interpretation of $\delta^{13}C_{Rl}$ because of LEDR. Changes in the net CO_2 exchange (photosynthesis and respiration) and either photosynthetic ^{13}C discrimination ($\Delta^{13}C_A$, when expressed relative to $\delta^{13}C$ of substrate CO_2, or $\delta^{13}C_A$ when expressed relative to the standard, as described further) or $\delta^{13}C_{Rl}$ were measured for 30 min in the light and then for 60 min after each plant had been moved into the dark. Three well-watered and three droughted plants were measured, with the soil water content of each pot evaluated gravimetrically. Watering regimes and the degree of water stress were qualitatively similar between Plants 3 and 5 in the short-term respiration experiment and those in the short-term photosynthesis–respiration experiment.

Gas exchange measurements

Measurements of leaf photosynthesis or respiration rate (i.e. CO_2 production rate, R_c) were made on the youngest fully expanded leaf (leaves 3–6, depending on the growth environment) using a portable photosynthesis system (LI6400; Li-Cor, Inc.) fitted with one of two custom-built leaf chambers. The dark respiration chamber, used for both the long- and short-term respiration experiments, was milled from stainless steel to allow enclosure of up to 80 cm^2 leaf area, and sealed with a closed cell foam gasket of 1 cm width. A large, clear topped chamber was used for the short-term photosynthesis–respiration experiment, allowing enclosure of up to 130 cm^2 leaf area, and constructed as described by Sharkey, Berry & Raschke (1985,and see Field, Ball & Berry 1989). The large leaf area of both chambers maximized the difference in concentration and isotopic composition between incoming and outgoing chamber air, reducing errors in calculated $\delta^{13}C_{Rl}$ and photosynthetic discrimination.

For both long-term and short-term respiration experiments, a thermocouple was placed within the chambers to measure leaf temperature, and a 120 L buffer volume used to stabilize the CO_2 concentration of air entering the leaf chamber. The flow rate of air through the leaf chamber was 250 or 300 μmol s^{-1}. The relationship between leaf area and boundary layer conductance was measured for the chamber using wet filter paper and applied to determine boundary layer conductance for each leaf. Leaf area within the chamber was measured from digitized images of the leaf and imaging software (Scion Image; Scion Corp., Frederick, MD, USA), and gas exchange variables were recalculated with the corrected leaf area.

After measurement of R_c, a disc was cut from the portion of the leaf within the chamber for measurement of oxygen consumption rate (R_o) using an oxygen electrode (Hansatech, King's Lynn, UK). The precision of the oxygen electrode is $\pm$ 0.001 μmol mol^{-1} O_2. However, with an empty chamber, R_o values were quite variable for the first 5 min after the chamber was sealed (perhaps as the air within the chamber mixed). Thereafter, the net O_2 flux was 0.00 $\pm$ 0.01 μmol m^{-2} s^{-1}. Average R_o for each leaf was calculated between 5 and 6 min after the chamber was sealed. The temperature within the oxygen electrode leaf chamber was controlled using a water bath, and was altered during each measurement day to track ambient leaf temperature (as measured by the LI6400). Leaf temperature varied between 22 and 31 °C, and the electrode was re-zeroed after

each change in chamber temperature. The *RQ* was calculated from the ratio of CO_2 produced to O_2 consumed ($RQ = R_c/R_o$).

The LI6400 reference and sample infrared gas analyzers were matched with an empty leaf chamber prior to the start of measurements. Measurements for the short-term respiration experiment commenced when R_c stabilized (i.e. the coefficient of variation for the CO_2 partial pressure differential between the sample and the reference analysers was below 1%), and usually within 15 min of the plant being moved to the dark. R_c was recorded at 1 min intervals and tended to be very high initially, then reduced with time in the dark. R_c for some leaves, particularly those in the dark for more than 30 min before measurements commenced, tended to stabilize over the 20 min measurement period, and the average R_c over the last four measurements was used to calculate *RQ*. For most leaves, the initial rapid drop in R_c reduced to a linear decrease with time, so that R_c was estimated at the time of R_o measurement (for *RQ* calculation) from a linear regression of R_c against time over the linear part of the decline curve (the last four to six measurements). The linear model fit the data well, with between 72 and 99% of variation in R_c explained by time since the start of the dark period for an individual leaf. Using extrapolated R_c for *RQ* estimation, rather than average R_c over the last four measurements, results in values for *RQ* being 0.05 lower (on average). The values of *RQ* presented in the Results section refer to those estimated by the extrapolation technique.

R_c and $\delta^{13}C_{Rl}$ measurements were made as described earlier for four plants in the long-term respiration experiment between 45 min and 4.6 h after the start of the dark period. The plants were returned to the growth cabinet after measurements, and re-measured between 23 and 26 h and between 48 and 52 h after the start of the dark period. During these three time periods, two leaf discs were removed from each of the remaining four plants within the growth cabinet for R_o measurement. As the leaves were used repeatedly for R_c and $\delta^{13}C_{Rl}$ measurements, R_o measurements were necessarily made on different leaves. This meant that only an average *RQ* (i.e. the ratio of average R_c to average R_o, with eight individual R_o measurements used to calculate average R_o) could be calculated for each time period.

For the short-term photosynthesis–respiration experiment, compressed air from a cylinder was used, rather than buffered glasshouse air, to reduce temporal variability in $\delta^{13}C$ of air entering the leaf chamber. The clear topped chamber allowed measurements to be made at ambient light levels, which were very low (102–439 μmol m^{-2} s^{-1} PAR) during the experimental period. The CO_2 partial pressure of the cylinder air was 425 μmol mol^{-1}, and $\delta^{13}C$ was –8.3‰. The cylinder air had extremely low water vapor content, meaning that any water vapour in the leaf chamber was derived from leaf transpiration, and that air saturation deficits were generally low. The boundary layer conductance for the chamber was measured using wet filter paper, as described earlier. The flow rate into the leaf chamber was controlled by a mass flow controller at between 400 and 2070 mL min^{-1} (212–1104 μmol s^{-1}), depending on the net CO_2 flux, and gas exchange variables were recalculated from LI6400 measurements recorded every 20 s using corrected leaf areas, boundary layer conductance and flow rates. The time taken after the decrease in flow rate (coinciding with the switch from light to dark) for air within the large chamber to turnover and CO_2 fluxes to stabilize (i.e. the coefficient of variation for the CO_2 partial pressure differential between the sample and the reference analysers was below 1%) was between 9 and 22 min. Further details about the gas exchange measurement technique will be outlined in a subsequent paper.

Isotopic measurements

The ratio of ^{13}C to ^{12}C of CO_2 entering and leaving the leaf chamber was determined using *TDL* absorption spectroscopy (TGA100A; Campbell Scientific, Inc.). This instrument measures the mixing ratio of the individual isotopologues (Bowling *et al.* 2003), but we recalculated the values as isotope ratios in the familiar delta notation, that is, relative to the Vienna Pee Dee belemnite (VPDB) standard. The carbon isotope composition of CO_2 ($\delta^{13}C$) is then

$$\delta^{13}C = \frac{R_s}{R_{VPDB}} - 1, \quad (1)$$

where R_s and R_{VPDB} are the $^{13}C/^{12}C$ ratios of the sample and the VPDB standard. $\delta^{13}C$ is reported in parts per thousand (‰). Two primary calibration cylinders with total CO_2 mixing ratios of 352.02 and 566.65 μmol mol^{-1}, and $\delta^{13}C$ of –8.44 and –17.06‰, respectively, (measured by National Oceanic and Atmospheric Administration-Climate Monitoring and Diagnostic Laboratory) were used to calibrate two working standards with CO_2 concentrations of 336.65 and 553.74 μmol mol^{-1}, and $\delta^{13}C$ of –30.48 and –30.41‰, respectively. Mole mixing ratios of $^{12}CO_2$ and $^{13}CO_2$ for the high and low CO_2 working standards were calculated as 334.62, 3.63 and 550.39, 5.97 μmol mol^{-1}, respectively. Calibration of the *TDL* requires that the working standards span the range in expected mixing ratios of each isotopologue, not the isotope ratio per se (Bowling *et al.* 2003). Our calibration tanks span the expected range of isotope mixing ratios for the glasshouse air and leaf-respired CO_2.

Mixing ratios of total CO_2 ($[CO_2]_t$) were calculated from the mixing ratios of individual isotopologues by (Griffis *et al.* 2004)

$$[CO_2]_t = \frac{[^{12}CO_2] + [^{13}CO_2]}{1 - f_{other}}, \quad (2)$$

where f_{other} is the fraction of CO_2 containing all isotopologues other than $^{12}C^{16}O^{16}O$ and $^{13}C^{16}O^{16}O$, and assumed to be 0.00474 (Griffis *et al.* 2004).

The *TDL* measures at 10 Hz, with an SD of approximately 1.2‰. However, for the current application, a manifold was used to switch between each of the two working standards and the leaf chamber inlet (reference) and outlet

(sample) lines to allow a mean mixing ratio to be measured over 15 s at the end of a 30 s period (for each working standard) or a 1 min period (for each of the reference and sample air streams). This means that each measured value for air entering and leaving the leaf chamber is an average of 150 individual measurements. All air streams passed through a low-flow Nafion counterflow water trap (PD625 dual configurations, Campbell Scientific, Inc.), prior to entry into the instruments optical cell at a flow rate of approximately 200 mL min^{-1} (controlled by a critical flow orifice). The optical cell pressure was typically 22.10 ± 0.04 mbar during measurement of the standards, and 22.11 ± 0.04 during measurement of the sample and reference lines. Repeated measurements of each working standard produced mean SDs of 0.09 μmol mol^{-1} for $[CO_2]_t$ and 0.09‰ for $\delta^{13}C$. Further details about the coupled LI6400-*TDL* measurement technique, including lag times between the LI6400 and the *TDL* and error analysis will be provided in a subsequent paper.

Calculating $\delta^{13}C_{Rl}$, $\Delta^{13}C_A$ and $\delta^{13}C_A$

The stable carbon isotope ratio of CO_2 respired by the leaves ($\delta^{13}C_{Rl}$) was calculated by mass balance from the $\delta^{13}C$ and concentration of CO_2 entering ($\delta^{13}C_e$ and C_e, respectively) and leaving ($\delta^{13}C_o$ and C_o, respectively) the leaf chamber (Evans *et al.* 1986):

$$\delta^{13}C_{Rl} = \frac{\delta^{13}C_o - \delta^{13}C_e(1-p)}{p}, \quad (3)$$

where $p = (C_o - C_e)/C_o$. A single value of $\delta^{13}C_{Rl}$ was calculated from $\delta^{13}C_e$, $\delta^{13}C_o$, C_o and C_e measured in dry air by the *TDL*. Similarly, photosynthetic discrimination ($\Delta^{13}C_A$) and fractionation ($\delta^{13}C_A$) were calculated (Evans *et al.* 1986):

$$\Delta^{13}C_A = \frac{-\xi(\delta^{13}C_o - \delta^{13}C_e)}{1 + \delta^{13}C_o - \xi(\delta^{13}C_o - \delta^{13}C_e)} \quad (4)$$

and

$$\delta^{13}C_A = \frac{\delta^{13}C_o - \Delta^{13}C_A}{1 + \Delta^{13}C_A}, \quad (5)$$

where $\xi = C_e/(C_e - C_o)$.

Phloem sap sampling

Sucrose transported within the phloem in the stem in darkness (assumed to be isotopically the same as sucrose in the measured leaf; Badeck *et al.* 2005) was sampled for $\delta^{13}C$ analysis as described by Barbour *et al.* (2000) & Cernusak, Wong & Farquhar (2003). Samples were taken within 15 min after the completion of $\delta^{13}C_{Rl}$ measurements for all three experiments. Samples were dried in pre-weighed tin capsules at 70 °C for 48 h, then re-weighed prior to analysis on an isotope ratio mass spectrometer (Europa Scientific 20/20; Europa Scientific, Crewe, UK) interfaced to a Dumas elemental analyser (Europa Scientific ANCA-SL, Europa Scientific) at the Waikato Stable Isotope Unit, University of Waikato, New Zealand. The SD for the repeated analysis of an internal standard, commercial sugar, was ± 0.14‰. Calibration versus VPDB was achieved using a certified secondary standard from CSIRO, Canberra, ACT, Australia.

RESULTS

Precision of $\delta^{13}C_{Rl}$ measurement

The large leaf chambers allowed a large difference in concentration between CO_2 entering and leaving the leaf chamber. However, when buffered glasshouse air was used, high transpiration rates limited minimum flow rates that could be used without creating vapor pressure and condensation problems. This problem was overcome with the use of essentially dry cylinder air in the short-term photosynthesis–respiration experiment. A range in p (as defined in Eqn 3) between 0.034 and 0.129 was observed, with higher p values found at higher R_c. A Monte Carlo analysis (following Barbour, Andrews & Farquhar 2001), including 10 000 individual calculations using Eqn 3 and the SD of $[CO_2]_t$ and $\delta^{13}C$ (0.09 μmol mol^{-1} and 0.09‰, respectively) produced an SD of individual measurements of $\delta^{13}C_{Rl}$ between 0.53 and 1.99‰ (for $p = 0.129$ and $p = 0.034$, respectively).

Data from the first 5 h of the long-term respiration experiment were used to assess the degree of noise associated with the system, and between replicate plants. Figure 1 shows that the large buffer volume effectively smoothed temporal variability in $\delta^{13}C_e$, so that $\delta^{13}C_e$ ranged between –8.69 and –8.21‰ over 5 h. A Monte Carlo analysis (as previously described, but limited to 1000 individual calculations of $\delta^{13}C_{Rl}$) produced an SD of between 0.5 and 1.2‰, with an average of 0.9‰ over the 5 h of measurements. Over 9–15 min, the temporal variability in $\delta^{13}C_{Rl}$, expressed as an SD, was between 0.2 and 1.7‰, with an average of 0.8‰. Repeated measures of $\delta^{13}C_{Rl}$ on the same leaf over 5 h produced an SD of between 0.8 and 2.0‰, with an average SD of 1.7‰. Including variability between four replicate plants grown in the same environment, $\delta^{13}C_{Rl}$ was found to vary between –25.6 and –22.1, with an average and SD of –24.3 and 1.0‰, respectively. The advantage of the high temporal resolution of *TDL* measurements is revealed when the SE of the average $\delta^{13}C_{Rl}$ is calculated; $\delta^{13}C_{Rl}$ for four replicate plants over 5 h is –24.3 ± 0.1‰.

Temporal variability in $\delta^{13}C_e$ and C_e was reduced by using cylinder air in the short-term photosynthesis–respiration experiment. However, this did not result in an appreciable reduction in the Monte Carlo SD compared to the long-term respiration experiment. SDs (over 1000 individual calculations for each set of measured values in Eqn 3) varied from 0.5 to 1.3‰, with an overall average of 0.9‰.

Long-term respiratory response

After 24 h in the light, both R_c and R_o were high (> 1.5 and > 2 μmol m^{-2} s^{-1}, respectively) but declined with time when

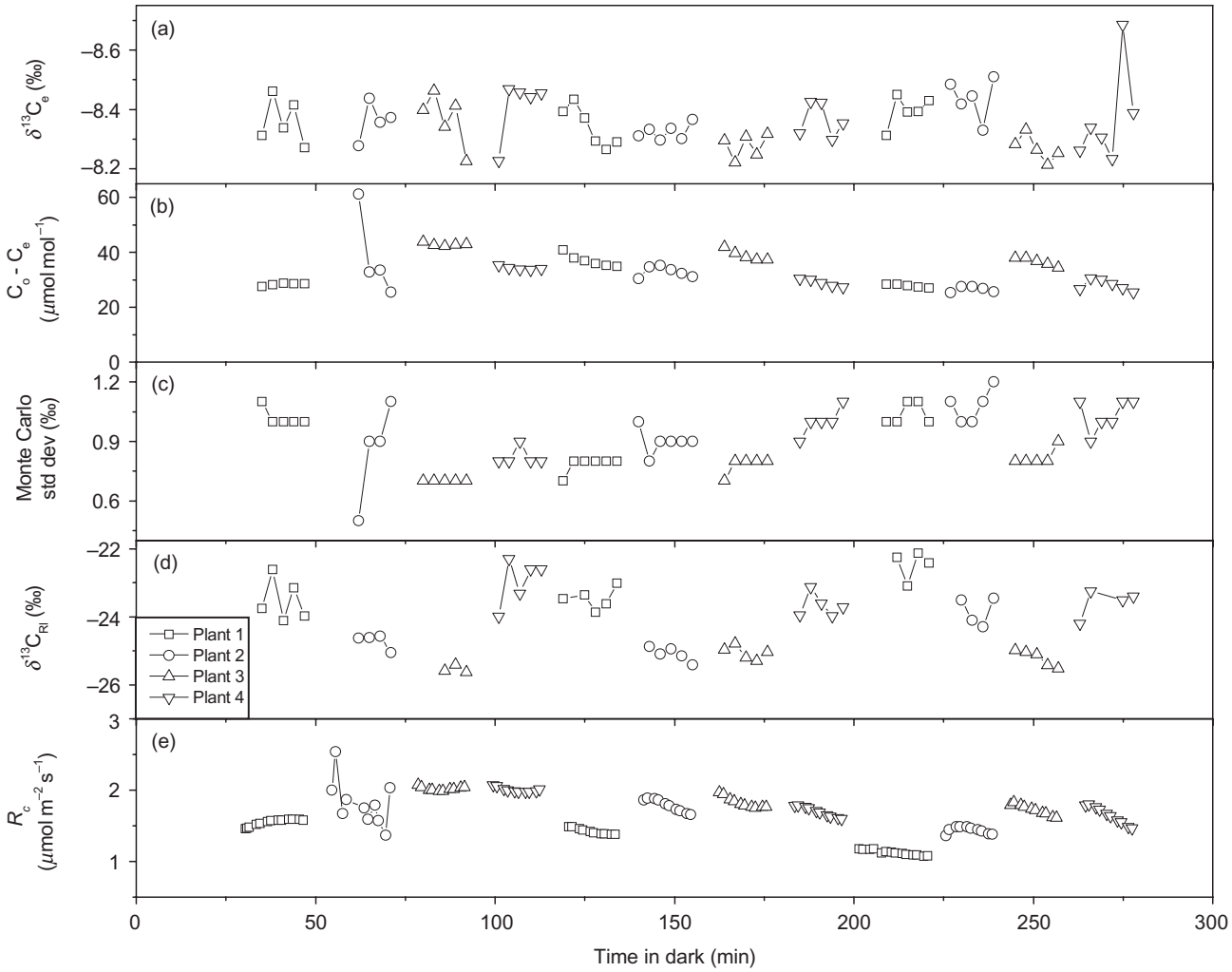

Figure 1. Isotope compositions and respiratory CO_2 fluxes for four replicate plants during the first 5 h of the long-term respiration experiment. Panel (a) shows the isotope composition of CO_2 in the inlet air stream ($\delta^{13}C_e$; buffered glasshouse air); panel (b) shows the difference in CO_2 mol fraction between the dry chamber inlet and outlet air streams ($C_o - C_e$, as measured by the *TDL*); panel (c) shows the Monte Carlo-derived SD of 1000 estimates of the carbon isotope composition of leaf-respired CO_2 [using Eqn 3 and the precision of $\delta^{13}C$ and $[CO_2]$ measurement]; panel (d) shows measured values of the carbon isotope composition of leaf-respired CO_2 ($\delta^{13}C_{Rl}$), and panel (e) shows the rate of respiratory CO_2 production (R_c, as measured by the LI6400).

the plants were moved into the dark (Table 1). R_c averaged 0.68 μmol m^{-2} s^{-1} after both 24 and 48 h in the dark, but R_o increased from 1.1 to 1.7 μmol m^{-2} s^{-1} from 24 to 48 h in the dark. *RQ* decreased from 0.99 after 3 h to 0.62 after 24 h to 0.52 after 48 h (Table 1). This suggests that carbohydrates were the main respiratory substrate for at least 5 h after plants had been placed in the dark, but then other, more reduced, substrates became important. $\delta^{13}C_{suc}$ increased from –30.5 to –27.6‰ over the first 5 h of the dark period (data not shown), but then remained constant at about –27.5‰ to 51 h in the dark (Table 1). $\delta^{13}C_{Rl}$ was fairly constant, or slightly increasing with time, over the first 5 h of the dark period (Fig. 1). After the plants had been in the dark for more than 20 h, $\delta^{13}C_{Rl}$ was more depleted and stable (–28.5 and –29.3‰ at 24 and 48 h, respectively, Table 1). The apparent fractionation between sucrose and respired CO_2 declined from 4.5‰ at 5 h to –1.1‰ at 26 h to –1.6‰ at 48 h (Table 1). $\delta^{13}C_{Rl}$ tended to increase with increasing *RQ* in the long-term (Fig. 2), confirming relationships presented by Tcherkez *et al.* (2004).

Short-term respiratory response

In the short-term respiration experiment, the rate of CO_2 production, R_c, tended to decline rapidly in the first 20 min after the leaves had been placed in the dark, then either continued to decline more slowly with time or stabilized (Fig. 3). Table 2 shows values of R_c used for calculation of *RQ*. As *RQ* was > 0.80 for all plants, carbohydrates were assumed to be the main respiratory substrate for all plants in the short-term respiration experiment. Stomatal conductance (g_s) was typically high for well-watered plants, and tended to decline with time in the dark. Table 2 presents average g_s during the last 6 min of R_c measurements for

Table 1. Gas exchange and stable carbon isotope ratios of leaf-respired CO_2 and phloem sap sucrose from castor bean plants grown under well-watered conditions for the long-term respiration experiment, after 2–50 h in the dark following 24 h of illumination

Variable	2–5 h	24 h	48 h
D (kPa)	0.9 ± 0.1	1.34 ± 0.26	1.09 ± 0.12
T_l (°C)	28.5 ± 0.5	27.6 ± 0.7	29.4 ± 0.1
g_s (mol m^{-2} s^{-1})	0.14 ± 0.04	0.07 ± 0.02	0.12 ± 0.02
R_c (μmol m^{-2} s^{-1})	2.01 ± 0.09	0.69 ± 0.05	0.68 ± 0.03
R_o (μmol m^{-2} s^{-1})	2.03 ± 0.25	1.09 ± 0.21	1.30 ± 0.22
RQ	0.99 ± 0.09	0.62 ± 0.03	0.52 ± 0.03
$\delta^{13}C_{Rl}$ (‰)	–24.2 ± 0.4	–28.5 ± 0.5	–29.3 ± 0.1
$\delta^{13}C_{suc}$	–28.7 ± 0.3	–27.4 ± 0.2	–27.7 ± 0.1
e_d	4.5 ± 0.7	–1.1 ± 0.6	–1.6 ± 0.6

Values are averages ± SE from measurements on four replicate plants. R_o was measured on four replicate plants under the same environmental conditions, to allow repeated gas exchange measurements on Plants 1–4.
D, leaf-to-air saturation deficit; T_l, leaf temperature; g_s, stomatal conductance; R_c, respiratory CO_2 production; R_o, respiratory O_2 consumption; RQ, respiratory quotient R_c/R_o; $\delta^{13}C_{Rl}$, $\delta^{13}C$ of leaf-respired CO_2; $\delta^{13}C_{suc}$, $\delta^{13}C$ of phloem sap; e_d, apparent fractionation between assumed respiratory substrates and leaf-respired CO_2.

each leaf, and was significantly lower for droughted compared with well-watered plants ($P < 0.05$). These values for g_s, although high for leaves in the dark, are consistent with g_s measured at low leaf-to-air saturation deficit for castor bean (Barbour & Buckley, in press).

The carbon isotope ratio of phloem sap sucrose ($\delta^{13}C_{suc}$) varied between –29.9 and –23.7‰ (Table 2). Two of the five droughted plants had quite enriched $\delta^{13}C_{suc}$ (–23.7 and –24.1‰). These plants also had very low g_s in the dark (Table 2). Half of the plants (three well-watered plants and two droughted plants) had rapidly declining values of $\delta^{13}C_{Rl}$ between 10 and 20 min after the start of the dark period (Fig. 3). This decline was as great as 8‰ in the case of Plant 4 of the well-watered treatment, but substantial variation was observed in the rate of decrease of $\delta^{13}C_{Rl}$ with time in the dark. Linear regressions of $\delta^{13}C_{Rl}$ on time revealed slopes between 0 and –0.62‰ min^{-1}, with a tendency towards steeper slopes with increasing cumulative irradiance (Table 2). The missing point for Plant 2 relates to a problem with the manifold switching between air streams, which was corrected at the end of the 3 min cycle. Subsequent measurements were not affected by this problem.

$\delta^{13}C_{Rl}$ measured 20–60 min after plants were placed in the dark, after values had stabilized, varied between –23.7 and –20.4‰. $\delta^{13}C_{Rl}$ increased with increasing $\delta^{13}C_{suc}$, with 79% of variation in $\delta^{13}C_{Rl}$ explained by variation in $\delta^{13}C_{suc}$ when values from both short-term experiments were included ($P < 0.001$, Fig. 4a). $\delta^{13}C_{Rl}$ was unrelated to RQ for either well-watered or droughted plants (Fig. 4b). The strong relationship between $\delta^{13}C_{suc}$ and $\delta^{13}C_{Rl}$ points to a rather constant apparent fractionation of $\delta^{13}C_{Rl}$ over $\delta^{13}C_{suc}$ across the treatments, of 5.1 ± 0.2‰.

Interestingly, apparent fractionation after 20–60 min in the dark decreased with increasing leaf temperature (Fig. 5). When the data presented by Tcherkez *et al.* (2003) from French bean were recalculated and plotted with current data from castor bean, it was apparent that e_d was higher for castor bean, although the slope of the relationship was similar.

Short-term photosynthetic-respiratory response

Watering regime had a clear effect on the gas exchange of plants in the short-term photosynthesis–respiration experiment. Droughted plants, with an average soil water content of 19%, were found to have g_s between 0.003 and 0.021 mol m^{-2} s^{-1} in the dark (about 11% of well-watered plants, Table 3). Values for g_s were lower than for the short-term respiration experiment because of generally higher air saturation deficits (Tables 2 & 3). Photosynthetic rates tended to be slightly higher for well-watered plants compared with droughted plants (Fig. 6). Higher stomatal conductances and (generally) higher photosynthetic rates resulted in higher $\Delta^{13}C_A$ and more negative $\delta^{13}C_{suc}$, on average, for well-watered plants (Table 3). $\delta^{13}C_{Rl}$ was more depleted for the well-watered plants compared with the droughted plants ($P = 0.02$). However, as in the previous experiment, $\delta^{13}C_{Rl}$ was positively related to $\delta^{13}C_{suc}$, and e_d was negatively related to T_l. Interestingly, $\delta^{13}C_{Rl}$ was not significantly related to photosynthetic fractionation ($\delta^{13}C_A$, expressed relative to the VPDB standard) immediately prior to the start of the dark period (Fig. 4a), probably reflecting the small amount of carbon fixed during this time because of low light and low g_s.

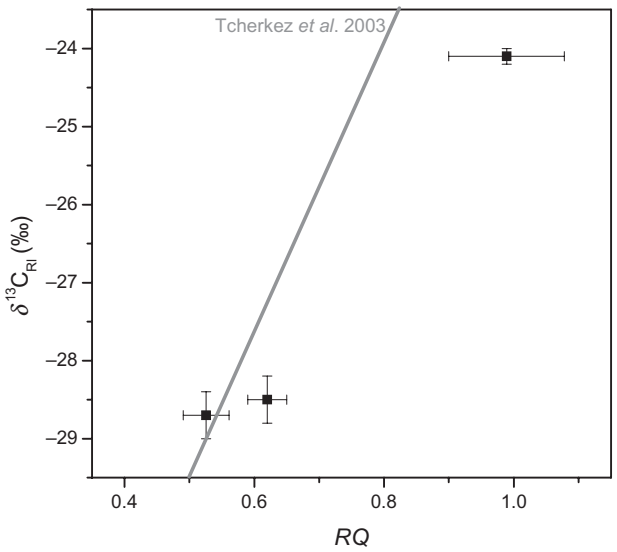

Figure 2. The relationship between the respiratory quotient (RQ) and the isotope composition of leaf-respired CO_2 ($\delta^{13}C_{Rl}$) for the long-term respiration experiment. Values are averages of four replicate plants. Also shown is the linear relationship between the two values fitted to data presented by Tcherkez *et al.* (2003) for comparison.

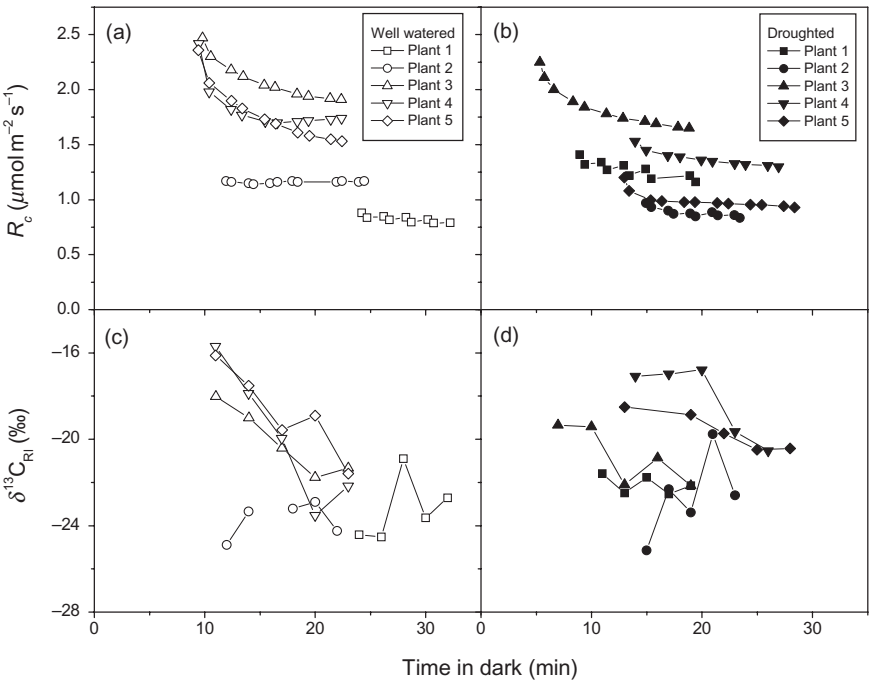

Figure 3. Rates of CO_2 efflux (a & b) and stable carbon isotope composition of respired CO_2 (c & d) from castor bean leaves for the short-term respiration experiment. Plants were grown under two water availabilities: well watered (a & c) and droughted (b & d). Conditions inside the leaf chamber during measurements are listed in Table 2. R_c, respiratory CO_2 production; $\delta^{13}C_{Rl}$, $\delta^{13}C$ of leaf-respired CO_2.

DISCUSSION

Precision of the combined LI6400-*TDL* system for $\Delta^{13}C_A$ and $\delta^{13}C_{Rl}$ measurement

The SD of $\delta^{13}C$ measurement with the *TDL* (0.09‰) is similar or lower than typically reported for traditional mass spectrometric analysis of CO_2 in air (e.g. 0.1‰, Cernusak *et al.* 2004; 0.2‰, Ghashghaie *et al.* 2001; 0 25‰, Ocheltree & Marshall 2004). Calculation of $\delta^{13}C_{Rl}$ using an open system necessarily includes the combined errors associated with measurement of both inlet and outlet $\delta^{13}C$. To compare the precision of the LI6400-*TDL* system with more traditional mass spectrometry systems, we ran a Monte Carlo analysis for three examples from recently published studies in which $\delta^{13}C_{Rl}$ or $\Delta^{13}C_A$ were measured, and compared these values to the SD estimated from the same calculation using the system described in the current paper.

Tcherkez & Farquhar (2003, 2005) measured $\delta^{13}C_{Rl}$ in a closed gas exchange system flushed with CO_2-free air prior to sealing. In this system, the CO_2 molar fraction was allowed to build up to 300 μL L^{-1}, with all CO_2 derived from leaf respiration (i.e. Eqn 3 does not apply). In this case, the precision of the system is simply the precision of mass spectrometer measurement, or 0.2‰.

Applying an off-line mass spectrometry system with a very large leaf chamber (400–800 cm^2), Cernusak *et al.* (2004) report ξ varying between 1.5 and 3.0, C_e between 533 and 967 μmol mol^{-1}, and $\delta^{13}C_e$ of –33.1‰. Using these values, the reported SD of $\delta^{13}C$ measurement (0.1‰), and estimating the SD of measured CO_2 partial pressures to be 0.1 μmol mol^{-1}, we made 10 000 Monte Carlo calculations of $\Delta^{13}C_A$ with Eqn 4. The SD of 10 000 Monte Carlo estimates of $\Delta^{13}C_A$ varied between 0.13 and 0.28‰.

Cousins, Badger & von Caemmerer (2006) applied an online mass spectrometry system to measure $\Delta^{13}C_A$. Using their reported values for ξ (between 4.9 and 29.9, depending on light level) and $\Delta^{13}C_A$ (between 3.6 and 11.0‰), and assuming $\delta^{13}C_e$ is –33.1‰, and the SDs of CO_2 partial pressures and $\delta^{13}C$ are the same as for the Cernusak *et al.* (2004) experiment, the SD of 10 000 Monte Carlo calculations using Eqn 4 varied between 0.44 and 2.80‰. Applying the same calculations to $\Delta^{13}C_A$ measurements made during the short-term photosynthesis–respiration experiment described here, we obtain SDs between 0.28 and 1.02‰.

Tcherkez *et al.* (2005) also employed an open gas exchange system for online measurement of $\Delta^{13}C_A$. Using a large leaf chamber (typical leaf surface area of 100 cm^2), values for ξ were typically 3.2, and with an SD of 0.05 μmol mol^{-1} and 0.2‰ for CO_2 partial pressure and isotope composition, respectively, 10 000 Monte Carlo calculations of Eqn 4 produce an SD for $\Delta^{13}C_A$ of 0.6‰.

The SD of individual $\delta^{13}C_{Rl}$ measurements calculated by Monte Carlo analysis using the coupled LI6400-*TDL* system described here varied between 0.5 and 2.0‰, depending on the difference in CO_2 mixing ratios between inlet and outlet air streams. Therefore, we conclude that the LI6400-*TDL* system has a precision between the off-line system of Cernusak *et al.* (2004), the closed-chamber online system of Tcherkez *et al.* (2003) and the online system of Cousins *et al.* (2006). However, it is worth noting that the higher precision of the Tcherkez *et al.* (2003) system comes from the closed nature of the system, which necessarily involves exposing the leaf to CO_2-free air, and that the higher precision of the Cernusak *et al.* (2004) system is due to the very large leaf area enclosed by the chamber. The disadvantage of using a very large leaf chamber is the slow

Table 2. Gas exchange and stable carbon isotope ratios of leaf-respired CO_2 ($\delta^{13}C_{RI}$) and phloem sap sucrose ($\delta^{13}C_{suc}$) from castor bean plants grown under well-watered and droughted conditions for the short-term respiration experiment, after 25–35 min in the dark

Growth env.	Plant no.	Cum. PAR (mol m^{-2})	D (Kpa)	T_l (°C)	g_s (mol m^{-2} s^{-1})	R_o (μmol m^{-2} s^{-1})	R_c (at R_o) (μmol m^{-2} s^{-1})	RQ	$\delta^{13}C_{RI}$ (‰)	$\Delta\delta^{13}C_{RI}:\Delta t$ (‰ min^{-1})	$\delta^{13}C_{suc}$ (‰)	e_d (‰)
Well watered	1	5.561	1.40	23.0	0.015	0.83	0.74	0.89	–23.2	NS	–29.9	6.7
Well watered	2	5.697	0.67	21.7	0.066	0.33	1.16	0.87	–23.7	NS	–30.0	6.3
Well watered	3	11.199	0.50	28.4	0.273	1.99	1.84	0.92	–21.5	–0.31**	–26.9	5.4
Well watered	4	13.501	1.76	30.3	0.049	1.48	1.73	1.17	–22.8	–0.62**	–26.7	3.9
Well watered	5	13.993	0.80	29.0	0.132	1.32	1.43	1.08	–21.6	–0.41**	–27.0	5.4
Droughted	1	5.616	1.00	2.5	0.033	1.36	1.09	0.80	–22.3	NS	–28.2	5.9
Droughted	2	5.795	1.39	21.1	0.009	0.75	0.78	1.04	–22.6	–0.23*	–28.5	5.9
Droughted	3	7.692	2.41	28.7	0.017	1.36	1.57	1.15	–21.7	NS	–26.2	4.5
Droughted	4	10.829	3.29	29.3	0.002	1.33	1.26	0.95	–20.5	–0.32**	–23.7	3.2
Droughted	5	13.008	3.42	31.2	0.003	0.99	0.90	0.91	–20.4	–0.15**	–24.1	3.7

Also shown are cumulative photosynthetically active radiation (PAR) prior to the being moved into the dark (Cum. PAR) and the slope of the relationship between $\delta^{13}C_{RI}$ and time ($\Delta\delta^{13}C_{RI}:\Delta t$), with asterisks indicating the significance of the relationship.

*$P < 0.10$; **$P < 0.05$.

NS, non-significant relationship. D, leaf-to-air saturation deficit; T_l, leaf temperature; g_s, stomatal conductance; R_c, respiratory CO_2 production; R_o, respiratory O_2 consumption; RQ, respiratory quotient R_c/R_o; $\delta^{13}C_{RI}$, $\delta^{13}C$ of leaf-respired CO_2; $\delta^{13}C_{suc}$, $\delta^{13}C$ of phloem sap; e_d, apparent fractionation between assumed respiratory substrates and leaf-respired CO_2.

turnover time for the chamber air, meaning that dynamic leaf responses at high temporal resolution cannot be measured.

We believe the combined LI6400-*TDL* system has a number of advantages over other systems including (1) high temporal resolution; (2) realistic O_2 and CO_2 concentrations within the leaf chamber [unlike the mass spectrometer system described by Cousins *et al.* (2006), the O_2 partial pressure is the same as ambient air, and unlike the closed system described by Tcherkez *et al.* (2003), ambient CO_2 was used]; (3) relative ease of control of environmental conditions inside the leaf chamber using the LI6400; (4) real-time visualization of isotopic fluxes that allows quality control methods such as assessment of isotopic stability and leak testing during the measurement period; and (5) no need to correct for potentially interfering masses such as N_2O.

Variation in $\delta^{13}C_{suc}$ and $\delta^{13}C_{RI}$ from the long-term dark experiment

$\delta^{13}C_{suc}$ increased from –30.5 to –27.6‰ over the first 5 h of the dark period. This change may reflect a change from export of recently fixed carbon, with depleted $\delta^{13}C$ resulting from high C_i/C_a in the high humidity and relatively low-light growth chamber, to export of stored carbohydrates, with a more enriched $\delta^{13}C$ from the lower C_i/C_a under lower humidity in the glasshouse. The C_i/C_a for castor bean photosynthesizing in glasshouse conditions was likely to be about 0.77 (giving $\delta^{13}C_{suc}$ of about –27‰), while C_i/C_a at 70% relative humidity and 400 μmol m^{-2} s^{-1} PAR would be closer to 0.90 (giving $\delta^{13}C_{suc}$ of about –30‰; M.M. Barbour, unpublished data from controlled environment experiments). In support of this suggestion, $\delta^{13}C_{suc}$ sampled after 5 h in the dark is the same as $\delta^{13}C_{suc}$ sampled from well-watered plants grown in the low-humidity glasshouse. An alternative explanation is that over time, starch is progressively re-mobilized for export from the leaves, and that starch is more enriched in ^{13}C than sucrose (Tcherkez *et al.* 2004).

The data from the long-term dark experiment confirm the decline in $\delta^{13}C_{RI}$ over a number of days in the dark, and the positive relationship between $\delta^{13}C_{RI}$ and RQ, observed for *P. vulgaris* plants (Tcherkez *et al.* 2003). This supports the suggestion (Ghashghaie *et al.* 2001; Tcherkez *et al.* 2003; Pataki 2005) that the enriched $\delta^{13}C_{RI}$ at quasi steady state and high RQ near the start of a dark period reflects release of enriched carbon from positions 3 and 4 of glucose via the action of PDH, while after 24 and 48 h in the dark, a switch to other respiratory substrates has occurred, because both RQ and $\delta^{13}C_{RI}$ are lower.

Variation in $\delta^{13}C_{RI}$ in the quasi steady state

In both the short-term respiration experiment and the short-term experiment during a switch from photosynthesis to respiration, CO_2 fluxes and $\delta^{13}C_{RI}$ values stabilized after

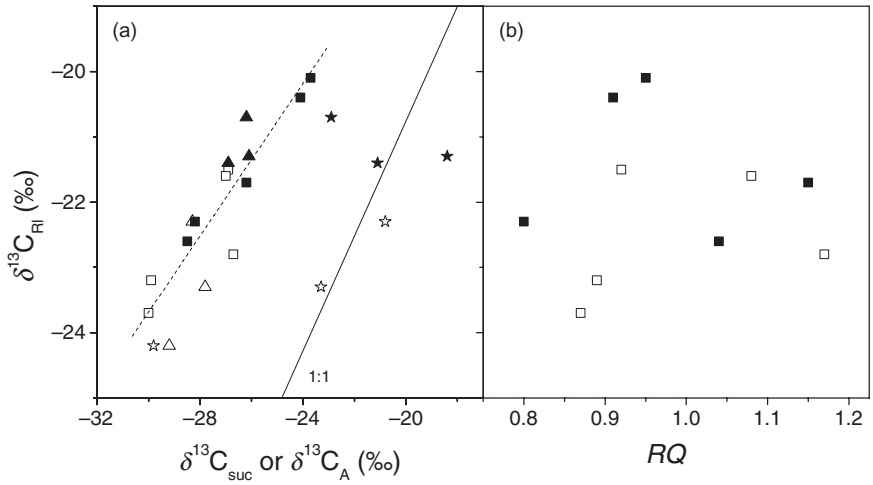

Figure 4. The relationships between $\delta^{13}C$ of leaf-respired CO_2 (at quasi steady state, between 20–60 min after the start of the dark period) and $\delta^{13}C$ of phloem sucrose ($\delta^{13}C_{suc}$) or photosynthetic ^{13}C fractionation immediately prior to the start of the dark period ($\delta^{13}C_A$) (a) and the respiratory quotient (*RQ*) (b). Data from both the short-term experiments are included, with open symbols representing values for well-watered plants and closed symbols values from droughted plants. In both panels, square symbols represent data from the short-term respiration experiment, and in (a), triangular symbols represent $\delta^{13}C_{suc}$, and stars represent $\delta^{13}C_A$ from the short-term photosynthesis–respiration experiment. The 1:1 line is shown in (a), as well as the fitted regression between $\delta^{13}C_{suc}$ and $\delta^{13}C_{Rl}$ across all plants; $\delta^{13}C_{Rl} = 0.58\ \delta^{13}C_{suc} - 6.18$, $r^2 = 0.79$.

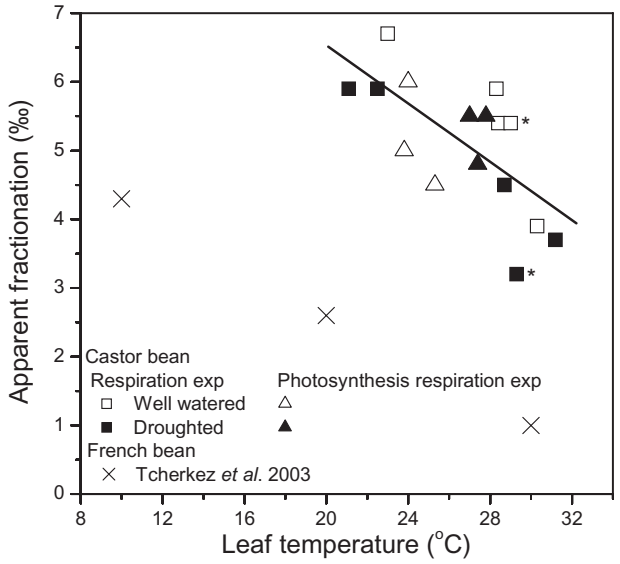

Figure 5. Change in apparent fractionation (i.e. difference between $\delta^{13}C$ of sucrose and leaf-respired CO_2) with changing leaf temperature. The French bean data are recalculated from fig. 1C of Tcherkez *et al.* (2003), over the first 2 h of a dark period following a light period of 8–10 h. The castor bean values are taken at quasi steady state (20–60 min after the start of a dark period). Data from both the short-term experiments are included, with open symbols representing values for well-watered plants and closed symbols values for droughted plants. Square symbols represent data from the short-term respiration experiment, and triangular symbols represent data from the short-term photosynthesis–respiration experiment. Two plants for which quasi steady state had not been reached ($\delta^{13}C_{Rl}$ not stable) are marked *. The fitted regression between $\delta^{13}C_{suc}$ and $\delta^{13}C_{Rl}$ across all plants is shown; $e_d = 10.77\ T_l - 0.21$, $r^2 = 0.45$, $p = 0.004$.

20–35 min in the dark. In quasi steady state, $\delta^{13}C_{Rl}$ was positively related to $\delta^{13}C$ of phloem sap sucrose ($r^2 = 0.79$), although $\delta^{13}C_{Rl}$ was 5.1‰ more enriched than $\delta^{13}C_{suc}$. This result compares well with previous observations of apparent fractionation: e_d determined between leaf carbohydrates and $\delta^{13}C_{Rl}$ was 6‰ in *P. vulgaris* (Duranceau *et al.* 1999), 4 and 3‰ for *N. sylvestris* and *H. annuus*, respectively (Ghashghaie *et al.* 2001), and 3.9‰ on average for four tropical rain forest species (Xu *et al.* 2004). The similarity in e_d between previous measurements and those in the current study suggest that published values of e_d are not an artifact

Table 3. Gas exchange and stable carbon isotope ratios of leaf-respired CO_2($\delta^{13}C_{Rl}$) and phloem sap sucrose ($\delta^{13}C_{suc}$) from castor bean plants grown under well-watered and droughted conditions for the short-term photosynthesis–respiration experiment, after 40–60 min in the dark

Variable	Well-watered	Droughted
SWC (%)	88 ± 5	19 ± 2
PAR (μmol m^{-2} s^{-1})	186 ± 48	299 ± 64
D (kPa)	0.40 ± 0.21	2.27 ± 0.68
T_l (°C)	24.4 ± 0.5	27.4 ± 0.2
g_s (mol m^{-2} s^{-1})	0.081 ± 0.022	0.009 ± 0.006
R_c (μmol m^{-2} s^{-1})	0.73 ± 0.03	0.80 ± 0.09
$\Delta^{13}C_A$ (‰)	20.7 ± 2.2	16.5 ± 2.1
$\delta^{13}C_{suc}$ (‰)	–28.4 ± 0.5	–26.4 ± 0.2
$\delta^{13}C_{Rl}$ (‰)	–23.3 ± 0.5	–21.1 ± 0.2
e_d (‰)	5.2 ± 0.4	5.3 ± 0.2

Values are averages ± SEs of measurements from three replicate plants. Also listed are values for soil water content (SWC), average photosynthetically active radiation (PAR) inside the leaf chamber (PAR) and photosynthetic discrimination against ^{13}C ($\Delta^{13}C_A$) prior to the start of the dark period.

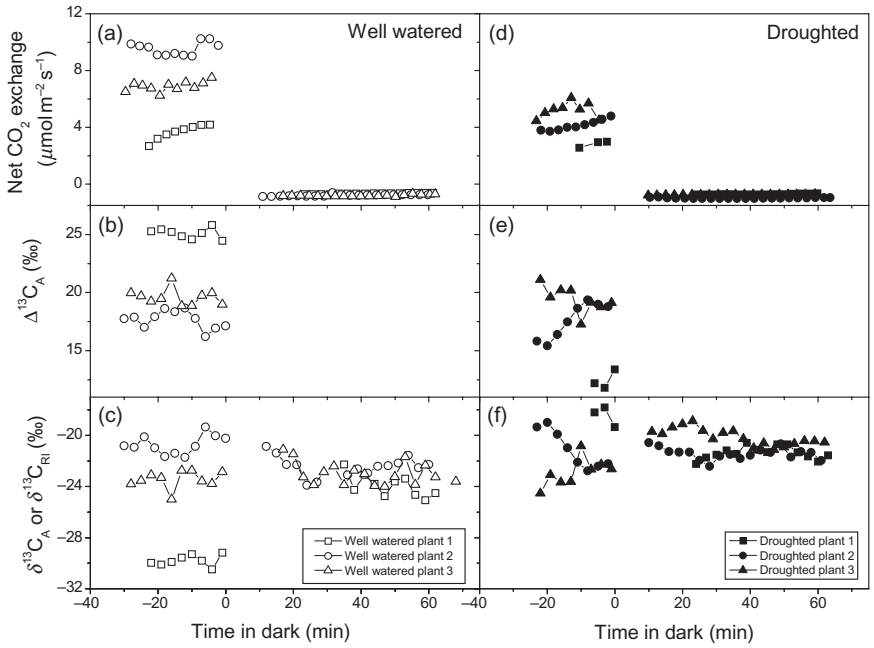

Figure 6. The rates of net CO_2 exchange (panels a & d), photosynthetic CO_2 discrimination ($\Delta^{13}C_A$, panels b & e) and photosynthetic ^{13}C fractionation or isotope composition of leaf-respired CO_2 ($\delta^{13}C_A$ and $\delta^{13}C_{Rl}$, respectively; panels c & f) for three plants grown under well-watered (panels a–c) or droughted (panels d–f) conditions during short-term photosynthesis–respiration experiment. Photosynthetic data are presented as both $\Delta^{13}C_A$ and $\delta^{13}C_A$ to allow the influence of $\delta^{13}C$ of recently fixed carbohydrates on $\delta^{13}C_{Rl}$ to be assessed. Conditions inside the leaf chamber during measurements are listed in Table 2.

of leaf respiration in chambers with unrealistically low or high CO_2 concentrations (Tu & Dawson 2005). $\delta^{13}C_{Rl}$ was not significantly related to $\delta^{13}C$ of carbon fixed immediately prior to the start of the dark period when plants were exposed to low light levels, but rather $\delta^{13}C_{Rl}$ was related to the more long-term, integrative signal of phloem sap sucrose.

An interesting observation was that e_d decreased as leaf temperature increased for both short-term experiments. This relationship was also observed for French bean by Tcherkez *et al.* (2003). Because *RQ* also decreased with increasing leaf temperature (from 10 to 32 °C) in the Tcherkez experiment, the relationship was interpreted as a switch from carbohydrates to other more reduced respiratory substrates. In the short-term respiration experiment, *RQ* was unrelated to leaf temperature (leaf temperature varied only 10 °C; between 21 and 31 °C), and *RQ* was relatively invariable, so that changes in respiratory substrates do not provide an explanation. However, R_c tended to be higher at higher leaf temperatures (as is typical), so that it seems possible that pools of intermediates were used up more quickly at high temperatures, leading to a more rapid decline in $\delta^{13}C_{Rl}$. This view is in accord with data interpretation presented by Ghashghaie *et al.* (2003). Further experiments with more careful control of leaf temperature and measurements of intermediate pool sizes and $\delta^{13}C$ are required to test this hypothesis.

Variation in $\delta^{13}C_{Rl}$ at the start of the dark period

CO_2 respired after high levels of illumination (within 20 min) was strongly ^{13}C enriched in the short-term respiration experiment, coinciding with higher respiration rates associated with LEDR (Atkin *et al.* 2000). LEDR and coincident enrichment in $\delta^{13}C_{Rl}$ was not observed in the short-term photosynthesis–respiration experiment, and $\delta^{13}C_{Rl}$ was not related to photosynthetic ^{13}C fractionation prior to the start of the dark period. These effects are probably due to the low light levels prior to the start of the dark period.

While it has been shown that the height of the LEDR peak is correlated to the cumulative photosynthesis rate during the previous light period (Hoefnagel, Atkin & Wiskich 1998), the metabolic origin of such an effect is not well known (Hill & Bryce 1992; Atkin, Evans & Siebke 1998). It has been suggested that this effect is related to the inhibition of respiration by light causing an accumulation of organic acids like malate (Cornic 1973). A plausible explanation for the strong ^{13}C enrichment in the first 20 min of the dark period is decarboxylation of the mitochondrial malate pool. Malate (and oxaloacetate) in the cytosol contains carbon from HCO_3^- initially fixed by phosphoenolpyruvate carboxylase (PEPC) during the light period (Fig. 7). Malate may be transported to the mitochondria and decarboxylated by the NAD^+-dependent malic enzyme in the dark, generating NADH. PEPC is known to discriminate against $H^{13}CO_3^-$ by about 2.2‰ (Brugnoli & Farquhar 2000), while the hydration equilibrium of CO_2 to HCO_3^- favours ^{13}C (fractionation of 9‰). As a result, light-produced cytosolic malate is enriched in ^{13}C compared to triose phosphates produced by the Calvin cycle. The contribution of malate decarboxylation to leaf respiratory CO_2 efflux would thus strongly enrich leaf-respired CO_2 and would be highest at the start of the dark period, then slowly decline as PEPC activity decreased and as mitochondrial respiration is up-regulated.

We nevertheless recognize that malic enzyme-catalysed decarboxylation is almost certainly associated with a kinetic isotope effect. Indeed, it has been shown that the chicken liver NADP-dependent malic enzyme fractionates by 34‰

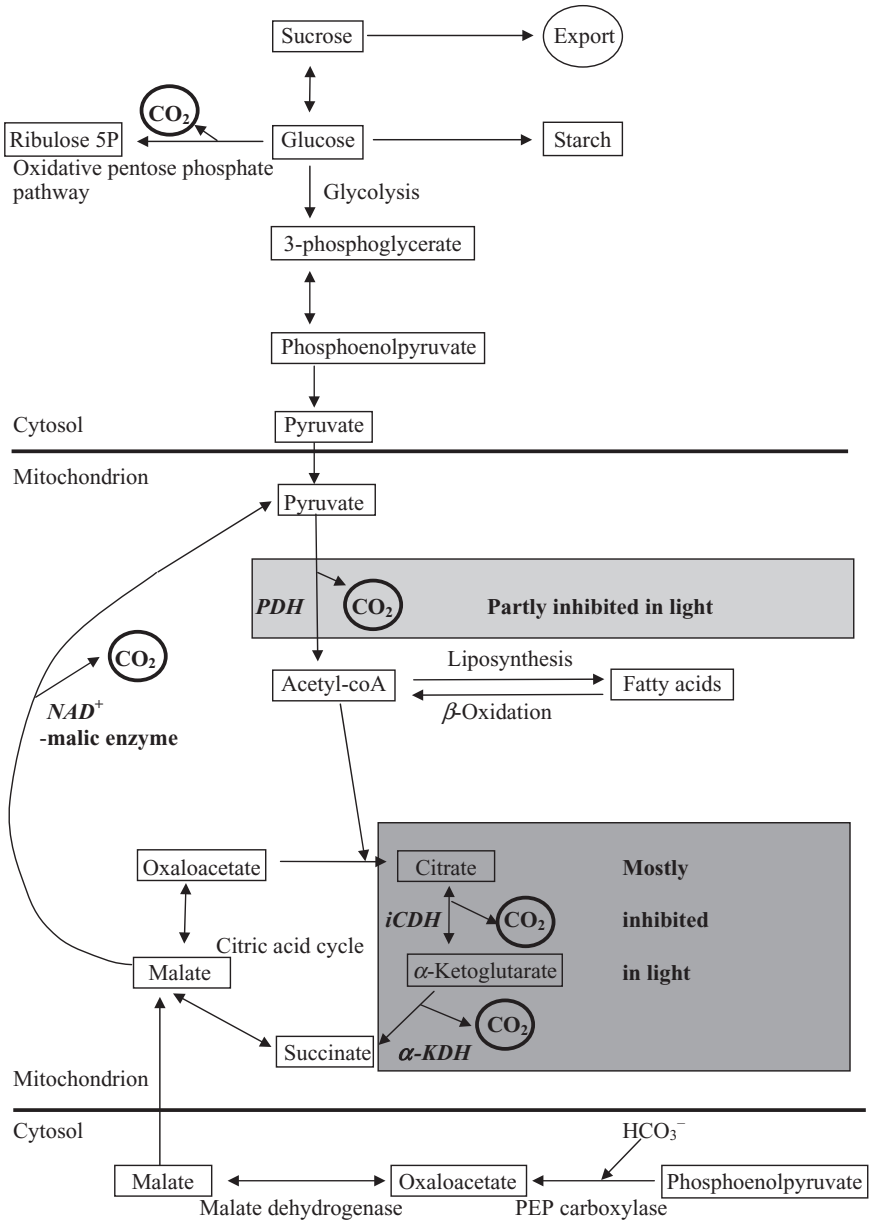

Figure 7. The biochemical pathways involved in leaf respiration in the dark. The pathways that are inhibited in the light and likely somewhat inhibited at the start of the dark period are indicated (Tcherkez *et al.* 2005).

(Edens, Urbauer & Cleland 1997) against ^{13}C. Unless all the malate molecules are consumed after illumination, this may eliminate the ^{13}C enrichment in C-4 of malate. Although very attractive, the malate decarboxylation hypothesis needs further evidence, namely, the rate of the metabolic fluxes associated with decarboxylation reactions just after illumination, and a compound-specific isotope analysis.

CONCLUSIONS

The *TDL* coupled to a gas exchange system provides a technique ideal for measurement of environmentally driven and temporally variable carbon isotope composition of leaf-respired CO_2. The present results confirm earlier observations that leaf-respired CO_2 is enriched in ^{13}C compared with likely respiratory substrates, but also provide the first evidence of variable and highly ^{13}C-enriched respired CO_2 over the first 10 min of a dark period for leaves previously in high light. This enrichment correlates with higher respiration rates, suggesting that LEDR is associated with the decarboxylation of heavier metabolites, among which malate is a plausible candidate. To test this hypothesis, measurements of the carbon isotope composition of purified metabolites are required, and this will be addressed in subsequent work.

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