Malate as a key carbon source of leaf dark-respired CO$_2$ across different environmental conditions in potato plants

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Abstract

Dissimilation of carbon sources during plant respiration in support of metabolic processes results in the continuous release of CO$_2$. The carbon isotopic composition of leaf dark-respired CO$_2$ (i.e. $\delta^{13}C_{R}$) shows daily enrichments up to 14.8‰ under different environmental conditions. However, the reasons for this $^{13}C$ enrichment in leaf dark-respired CO$_2$ are not fully understood, since daily changes in $\delta^{13}C$ of putative leaf respiratory carbon sources ($\delta^{13}C_{RS}$) are not yet clear. Thus, we exposed potato plants (Solanum tuberosum) to different temperature and soil moisture treatments. We determined $\delta^{13}C_{R}$ with an in-tube incubation technique and $\delta^{13}C_{RS}$ with compound-specific isotope analysis during a daily cycle. The highest $\delta^{13}C_{RS}$ values were found in the organic acid malate under different environmental conditions, showing less negative values compared to $\delta^{13}C_{R}$ (up to 5.2‰) and compared to $\delta^{13}C_{RS}$ of soluble carbohydrates, citrate and starch (up to 8.8‰). Moreover, linear relationships between $\delta^{13}C_{R}$ and $\delta^{13}C_{RS}$ among different putative carbon sources were strongest for malate during daytime ($r^2=0.69$, $P\leq0.001$) and nighttime ($r^2=0.36$, $P\leq0.001$) under all environmental conditions. A multiple linear regression analysis revealed $\delta^{13}C_{RS}$ of malate as the most important carbon source influencing $\delta^{13}C_{R}$. Thus, our results strongly indicate malate as a key carbon source of $^{13}C$ enriched dark-respired CO$_2$ in potato plants, probably driven by an anapleurotic flux replenishing intermediates of the Krebs cycle.

Key words: Compound-specific isotope analysis (CSIA), drought, organic acids, plant respiration, stable carbon isotopes, sugars, temperature, tricarboxylic acid (TCA) cycle.

Introduction

The investigation of plant respiration as a major process in plant biochemistry has expanded our understanding of carbon cycling in autotrophic organisms. Plants dissimilate carbon sources for the production of intermediates and reducing equivalents in support of metabolic processes, thereby continuously releasing CO$_2$ via plant respiration (Hopkins, 2006). Leaf-respired CO$_2$ is mainly derived from oxidative decarboxylation reactions catalysed by enzymes from the Krebs cycle.
(KC) and from interacting anabolic and catabolic reactions (Voet and Voet, 2011).

Using stable isotopes, the pathway of carbon can be traced from photosynthetic carbon fixation to respiratory carbon loss. On the one hand, C₃ plants discriminate heavily against ¹³C due to photosynthetic isotope fractionation, leading to general ¹³C depletion in plant biomass of about 20% in comparison to atmospheric CO₂ (Farquhar et al., 1989). The exact magnitude of photosynthetic carbon isotope discrimination depends on the intercellular CO₂ concentration (C) in the substomatal cavity, which is regulated by other physiological parameters such as net assimilation rate (Aₙ) and stomatal conductance (gₙ). Environmental conditions such as light, temperature, soil moisture, and air humidity will influence these parameters and with them the photosynthetic carbon isotope discrimination. On the other hand, the carbon isotopic composition of leaf dark-respired CO₂ (i.e. δ¹³C_R) has clearly been shown to be less negative than leaf metabolites in several plant species (Ghashghaie et al., 2003; Bowling et al., 2008; Werner and Gessler, 2011; Ghashghaie and Badeck, 2014). In a daily cycle, leaf dark-respired CO₂ follows a progressive ¹³C enrichment during the day and a gradual ¹³C depletion during the course of the night (Hymus et al., 2005; Prater et al., 2006), resulting in a strong temporal variability of up to 14.8% (Barbour et al., 2007; Werner et al., 2009; Wegener et al., 2010), which differs among functional groups (Priault et al., 2009; Werner et al., 2009).

δ¹³C_R is thereby linked to the carbon isotopic composition of putative leaf respiratory carbon sources (i.e. δ¹³C_RS) such as carbohydrates (soluble mono- and di-saccharides, and starch) and organic acids. Previous studies showed that environmental drivers such as temperature and soil moisture influence δ¹³C_R and δ¹³C_RS. More negative δ¹³C_R values with increasing temperature have been observed with short-term changes in leaf temperature during darkness in Phaseolus vulgaris (Tcherkez et al., 2003), while long-term effects of higher temperatures on δ¹³C_R and δ¹³C_RS have not yet been investigated under controlled conditions. Other studies have demonstrated less negative δ¹³C_RS and δ¹³C_RS values under dry conditions compared to those under wet conditions (Duranceau et al., 1999; Ghashghaie et al., 2001). Similar observations were made in field experiments (Sun et al., 2009; Dubbert et al., 2012). Conversely, more negative δ¹³C_RS values have been found under dry conditions for Mediterranean trees and herbs such as Quercus ilex and Tuberaaria guttata compared to those under wet conditions (Unger et al., 2010), which have been explained with accompanied increases in temperatures and vapour pressure deficit. Nevertheless, the combined effects of temperature and soil moisture on δ¹³C_R and δ¹³C_RS under controlled conditions have yet to be tested.

Moreover, δ¹³C_R is determined by various post-photosynthetic carbon isotope fractionation processes at pivotal branching points in respiratory pathways, carbon isotope effects on enzymatic reactions, and changes in respiratory substrates (for a detailed review see Werner and Gessler, 2011). The ¹³C enrichment in leaf dark-respired CO₂ itself is thought to be a result of fragmentation fractionation processes based on heterogeneous intramolecular carbon isotope distribution in respiratory carbon sources (Tcherkez et al., 2004). For instance, C-3 and C-4 positions of glucose are known to be enriched in ¹³C compared to the other molecule positions due to an isotope effect of the aldolase reaction (Rossmann et al., 1991; Gleixner and Schmidt, 1997). Breakdown of glucose during glycolysis produces pyruvate with a ¹³C enriched C-1 position (former C-3 and C-4 positions of glucose). Thereafter, the pyruvate dehydrogenase reaction (PDH) releases the C-1 position as ¹³C enriched CO₂, whereas the more ¹³C depleted acetyl-CoA residue is used in the KC (Priault et al., 2009; Werner and Gessler, 2011). Thus, a PDH dominated respiratory pathway may lead to ¹³C enrichment in leaf dark-respired CO₂.

However, the knowledge about δ¹³C_R is often based on light-acclimated leaves, which have been transferred into darkness to allow respiratory measurements. This approach holds an unpreventable bias known as ‘light-enhanced dark respiration’ (LEDR), which needs to be taken into account when interpreting daytime δ¹³C_R values. LEDR is a short-term light-dark transition period, describing an increase in the amount of leaf dark-respired CO₂ shortly upon darkening for about 20 min, which depends on light intensity (Atkin et al., 1998). On the one hand, LEDR may be influenced by reassembly of the KC, which is thought to be only partially active under light conditions (Tcherkez et al., 2005; Sweetlove et al., 2010; Werner and Gessler, 2011; Werner et al., 2011). On the other hand, LEDR may be driven by a breakdown of a light-accumulated malate pool, causing ¹³C-enriched leaf dark-respired CO₂ (Barbour et al., 2007; Gessler et al., 2009; Werner et al., 2009; Barbour et al., 2011; Werner and Gessler, 2011). Malate itself is also known to be ¹³C enriched compared to other carbon sources (Gleixner et al., 1998; Ghashghaie et al., 2001). The ¹³C enrichment in malate was attributed to an anaplerotic flux via the phosphoenolpyruvate carboxylase reaction (PEPC), which fixes ¹³C-enriched hydrogen carbonate and replenishes KC intermediates (Melzer and O’Leary, 1987; Savidge and Blair, 2004). Thus, a possible breakdown of malate by the mitochondrial malic enzyme reaction, or within the KC, may influence δ¹³C_R (Barbour et al., 2007; Werner et al., 2011). In addition, plants may also use to a certain extent more complex carbon sources such as lipids and proteins under severe environmental conditions or under prolonged darkness (Tcherkez et al., 2003; Usadel et al., 2008). However, the driving processes, the respiratory carbon sources, and the mechanisms causing changes in δ¹³C_R during day and night are not fully resolved thus far.

Hence, with this study we intend to assess two major research questions. What causes the high daily variations in δ¹³C_R? How are δ¹³C_R and δ¹³C_RS influenced by temperature and soil moisture conditions? Our main objectives were (i) to analyse the relationship between δ¹³C_R and δ¹³C_RS influenced by temperature and soil moisture conditions? Our main objectives were (i) to analyse the relationship between δ¹³C_R and δ¹³C_RS values and (ii) to determine changes in δ¹³C_R and δ¹³C_RS values, as well as in concentrations of the putative carbon sources under different environmental conditions. Therefore, we exposed potato plants to different controlled temperature and soil moisture conditions and measured δ¹³C_R with an in-tube incubation technique, as well as δ¹³C_RS and concentrations of soluble...
carbohydrates, organic acids and starch from leaves with compound specific isotope analysis (CSIA) on a daily basis.

Materials and methods

Plant material

Potato plants (Solanum tuberosum L. cv. Annabell) were grown from tubers of the same size in 5 l pots filled with bark humus soil (Ökohum, Herrenhof, Switzerland) in a greenhouse, with average temperatures of 20/16°C and vapour pressure deficits (VPD) of about 0.9/0.4 kPa (day/night). The plants were exposed to a 16 h daylight period supplemented by 400 W sodium-lamps (Powertone Son-T Plus, Philips, Amsterdam, Netherlands). Forty days after planting, plants were transferred into walk-in climate chambers for acclimatization for 2 weeks. The 16 h daylight in the climate chambers had an averaged photosynthetic photon flux density of 400 μmol m⁻² s⁻¹ at leaf level, thus plants were not fully light-saturated. Before the treatment period, soil water status was optimal for at least 3 d after watering, while an individual plant consumed about 300 ml water per day. 50 ml of a 0.4% fertilizer solution (v/v, Gesal, Zürich, Switzerland) was applied twice to all plants during the whole experiment of 70 d.

Treatments were applied during the last 15 d of the experiment. Plants were exposed to high temperature (T_high) of 28/23°C (day/night) and low temperature conditions (T_low) of 22/17°C, at a VPD of about 0.9/0.35 kPa for both temperature treatments. Three climate chambers were used for replication of each temperature treatment. Within each climate chamber there were two soil-moisture treatments with nine plants each. Dry soil moisture conditions were kept constantly at 30–60% of the daily water consumption of each individual plant, determined by weighing the entire pots. Plants under wet conditions were kept at 100%.

The final sampling period lasted 32 h during the last 2 d of the experiment, when dry soil conditions were established for both temperature treatments. Sampling was done on a daily basis every 2 h (nighttime) or 4 h (daytime). During sampling, individual plants had 3–6 ranks, with about four fully developed leaves per rank. Always the third-last fully developed leaf per rank was sampled at all points in time, but within 24 h only one sample was taken from each individual plant to avoid any stress response induced by sampling. Sampled leaf material was immediately frozen in liquid nitrogen and stored at −80°C. Subsequently, the leaf material was freeze-dried and milled to powder by a steel ball mill (MM200, Retsch, Haan, Germany) for all further isotopic and biochemical analyses. In addition to leaf sampling, air CO₂ samples from all six climate chambers were collected at the same points in time during the sampling period, showing a mean δ¹³C value of −12.2‰ and typical daily variations of SD ≤0.5‰; no differences between temperature treatments (P>0.05) and points in time (P>0.05; linear mixed effects model) were observed during the daily cycle.

Physiological measurements and biomass determination

Several leaf physiological parameters were determined with an infrared gas analyser (LI-6400, LI-COR, Lincoln, Nebraska, USA), including net assimilation rate (Aᵣ), intercellular CO₂ concentration (Cᵢ), and stomatal conductance (gₛ). All measurements were taken in the last 4 h of the daylight phase. To monitor volumetric soil water content (SWC), up to three soil moisture sensors (EC-5 and logger Em5b, Decagon Devices, Pullman, USA) were installed for each treatment. Shortly after the sampling period, total plant biomass was harvested, oven-dried (at 60°C), and weighed. The fresh tuber weight and tuber count (number of potatoes) were determined.

Carbon isotope and concentration analyses

δ¹³C values are expressed as described by Craig (1957) and modified by Coplen (2011):

\[ \delta^{13}C = \frac{R_{sample}}{R_{standard}} - 1 \]

where the sample ratio (R) for ¹³C/¹²C ratio of the sample material and R_standard is that of the international standard VPDB (Vienna Pee Dee Belemnite).

Determination of δ¹³C_RI

The in-tube incubation technique was used for the collection of leaf dark-respired CO₂ during daytime and nighttime (Werner et al., 2007). A leaf was placed in a 12 ml gas-tight exetainer (Labco, Lampeter, UK), which was immediately darkened with a lightproof casing to trigger leaf dark respiration. The tube was then flushed for 1 min with synthetic air until a CO₂-free atmosphere was established, which was monitored with an infrared gas analyser (LI-6262, LI-COR, Lincoln, Nebraska, USA). After an incubation time of 3 min in darkness, an aliquot of dark-respired CO₂ was transferred with a gas-tight syringe into a new exetainer filled with dry N₂. δ¹³CRI values were determined with an IRMS, using a modified Gasbench II (Thermo Fisher, Bremen, Germany) connected to a Delta+XP IRMS, similar to Zeeman et al. (2008). The transfer of the CO₂ sample into a new exetainer, as well as the IRMS measuring procedure, were both tested with air of known δ¹³C to ensure no isotope fractionation had occurred. Measurement precision of a quality control standard (three standards per 24 samples) was SD≤0.1‰.

Determination of δ¹³C in bulk leaves and leaf starch

Extraction of leaf starch was performed as described in previous studies (Wanek et al., 2001; Goettlicher et al., 2006; Richter et al., 2009). Leaf starch was isolated from 50 mg leaf material with methanol/chloroform/water (MCW, 12:5:3, v/v/v) at 70°C for 30 min. Samples were centrifuged (10 000 × g, 2 min) and supernatants removed, while the leaf-starch-containing pellets were washed with MCW and deionized water and dried at room temperature (RT). Pellets were then re-suspended in water and boiled at 99°C for 15 min to facilitate starch gelatinization. Subsequently, leaf starch was enzymatically digested with α-amylase (EC 3.2.1.1, Sigma-Aldrich, Buchs, Switzerland) at 85°C for 2 h, and cleaned with centrifugation filters to remove enzymes (Vivaspin, Sartorius, Göttingen, Germany). To determine δ¹³C of bulk leaves (δ¹³C_starch) and starch, an elemental analyser (Flash EA 1112 Series) coupled to a Delta+XP-IRMS was used (both Thermo Fisher, Bremen, Germany; Werner et al., 1999). Measurements of samples, blanks, and reference material followed the identical treatment principle described by Werner and Brand (2001). The long-term precision of a quality control standard for all sequences was SD≤0.1‰.

Isotopic and concentration analysis of soluble carbohydrates and organic acids

Water-soluble compounds were extracted from 100 mg leaf material with water at 85°C for 30 min, similar to Streit et al. (2013). Subsequently, soluble carbohydrates and organic acids were separated by ion-exchange chromatography (Wanek et al., 2001; Goettlicher et al., 2006; Richter et al., 2009), using Dowex 50WX8 in H⁺-form and Dowex 1X8 in NaCOO⁻-form (both 100–200 mesh, Sigma-Aldrich, Buchs, Switzerland). To avoid clogging of the HPLC column by polyphenols, all samples designated for carbohydrate analyses were filtered with 100 µm PTFE syringe filter (Infochroma AG, Zug, Switzerland) prior to HPLC measurements.

To determine δ¹³C_RS values and the concentrations of soluble carbohydrates and organic acids, a HPLC-IRMS system consisting of a high performance liquid chromatograph coupled to a Delta V Advantage IRMS by a LC IsoLink (all Thermo Fisher, Bremen, Germany).
was used according to Krümmen et al. (2004). Carbohydrates were separated on a 3 × 150 mm anion-exchange column CarboPac PA20 (Dionex, Olten, Switzerland) using 2 mM NaOH as the mobile phase and a flow speed of 250 μl min⁻¹ (Boscher et al., 2008; Rinne et al., 2012). Low column temperature of 20°C was used to prevent isomerization of hexoses (Rinne et al., 2012). This enabled chromatographic separation for sucrose and glucose, but fructose δ¹³C_RS and concentration measurements were affected by partial co-elution of fructose with other compounds. To correct δ¹³C_RS values and to calculate concentrations from the peak areas, interspersed standard solutions in a concentration range of 20–180 ng C μl⁻¹ were measured within each sequence. The measurement precision of δ¹³C_RS values in all carbohydrate standards was SD≤0.5‰. Below a concentration of 60 ng C μl⁻¹, the precision of fructose standards was lower for certain batches, and therefore these results were excluded.

Organic acids were separated on a 4.6 × 300 mm Allure Organic Acids column (Restek, Bellefonte, USA) at 5–10°C. The mobile phase was a 100 mM monopotassium phosphate buffer (pH 3) with a flow speed of 500 μl min⁻¹ (Hettmann et al., 2005). The measurement precision of δ¹³C in organic acid standards was SD≤0.4‰. Low citrate concentrations from T low samples (<45 ng C μl⁻¹) impeded the analytical accuracy of the δ¹³C RS values, therefore these samples were not taken into account.

All purification steps were verified for each batch of 24 samples using 2.5 mg standard solutions of known δ¹³C (by EA-IRMS) for all carbohydrates and organic acids measured in this study. Differences between δ¹³C values before and after purification were generally ≤0.2‰, indicating no significant isotope fractionation for any standard. Mean recovery was 101 ± 6% for fructose, 96 ± 6% for glucose, 89 ± 3% for sucrose, 91 ± 3% for malate, and 86 ± 3% for citrate.

**Determination of starch concentration**

For the extraction of leaf starch for concentration analyses we used a modified method of Critchley et al. (2001). Leaf starch was isolated with 1.12 M perchloric acid from 50 mg leaf material at RT for 15 min and centrifuged (10 min, 3000 × g, 4°C). The supernatant was removed and the leaf-starch-containing pellet was washed free from pigments with deionized water and ethanol. Pellets were then dried at RT, resuspended in water, and gelatinized. Subsequently, starch samples were enzymatically hydrolysed to glucose for 2 h at 37°C with a solution mix of α-amylase (EC 3.2.1.1, Sigma-Aldrich, Buchs, Switzerland) and α-amylglucosidase (EC 3.2.1.3, Roche, Rotkreuz, Switzerland) in 220 mM sodium acetate buffer (pH 4.8). The glucose concentration was determined at 340 nm with a 96-well microplate reader (EL×800, BioTek, Luzern, Switzerland) using a coupled enzymatic reaction (Hoch et al., 2002). Potato starch was used as a standard. Glucose concentrations are expressed in molarity of starch monomers.

**Data analysis**

R version 3.0.2 (R Core Team, 2013) was used for (multiple) linear regression analyses and linear mixed effects models (R package nlme). Models included fixed effects (temperature, soil moisture, sampling time) and random effects (climate chambers, individual plants). If applicable, δ¹³C values and concentrations were logarithmically transformed to ensure normal distribution. For the best-fit combination of the multiple linear regression analysis, variables were excluded if P≥0.05.

**Results**

**Physiological parameters and biomass**

Physiological parameters (A_n, C_n, g_s, and SWC) of potato plants exposed to four different treatments were monitored during the treatment period of 15 d (Fig. 1). The net assimilation rate declined during the treatment period under all four treatments (Fig. 1A). During the sampling period (Fig. 1A, day 15), A_n was significantly influenced by soil moisture (P=0.02, Table 1), with lowest values (1.9 μmol m⁻² s⁻¹) under T high and dry conditions, and highest values (5.4 μmol m⁻² s⁻¹) under T low and wet conditions, whereas the temperature influence on A_n was not significant (P=0.07, Table 1) but tended to cause lower A_n values under T high than under T low under both soil moisture conditions. The intercellular CO₂ concentration increased during the treatment period for all four treatments (Fig. 1B). During the sampling period (Fig. 1B, day 15), C_i was independently influenced by temperature (P=0.012, Table 1) and soil moisture (P=0.01, Table 1), with lowest C_i (247.5 μmol mol⁻¹) under T low and dry conditions and highest C_i (332.8 μmol mol⁻¹) under T high and wet conditions. Stomatal conductance during the treatment period was lower under dry treatments compared to those under wet treatments (Fig. 1C). During the sampling period (Fig. 1C, day 15), g_s was significantly influenced by soil moisture (P=0.001, Table 1), with lowest g_s (about 0.06 mol m⁻² s⁻¹) in plants of both dry treatments and highest g_s (0.22 mol m⁻² s⁻¹) in plants under T high and wet conditions, whereas the temperature influence under wet conditions tended to cause higher g_s values under T high than under T low. The volumetric soil water content was lower under dry conditions (~7–14%) compared to wet conditions (23–27.5%) for the last 9 d of the treatment period (Fig. 1D), including the sampling period (Fig. 1D, day 15), where SWC was significantly affected only by soil moisture treatments (P=0.002, Table 1). Generally, no significant interactions between temperature and soil moisture were observed for any parameter (Table 1). In addition, only soil moisture treatments affected plant biomass (P=0.008, Table 1) and tuber weight (P=0.023, Table 1) taken shortly after the sampling period, independent of temperature treatments. Highest values tended to be under T low and wet conditions and lowest values under T high and dry conditions (Tables 1, 2), indicating different stress levels created by the four treatments.

**Carbon isotopes in potato leaves**

**Daily cycles of δ¹³C_R and δ¹³C_usef**

δ¹³C values of leaf dark-respired CO₂ (δ¹³C_R) varied significantly over time (P≤0.001, Table 3) with values in the range of −21.9‰ and −32‰, declining strongly during nighttime and increasing again during the daytime for all four treatments (Fig. 2A). An interaction between temperature and time showed that the influence of temperature differed with time (P=0.014, Table 3). Daytime δ¹³C_R values under T high were up to 4.7‰ more negative compared to those under T low, independent of soil moisture conditions, whereas nighttime δ¹³C_R values of both temperature treatments were very similar, particularly in the second night. Dry soil moisture conditions caused less negative δ¹³C_R values compared to those under wet conditions during the daily cycle (P=0.013, Table 3), with a maximum difference of 2.7‰, independent of temperature treatments. On average, the difference between daytime and nighttime δ¹³C_R values was highest under T low and wet conditions, at 5.7‰, and lowest under T high and dry conditions, at 2.5‰.
The bulk leaf material reflects all environmental conditions experienced during the whole growth period. $\delta^{13}C_{\text{leaf}}$ of all treatments showed no changes during the sampling period and no interactions between treatments and time (Fig. 2B; Table 3). Under $T_{\text{high}}$, $\delta^{13}C_{\text{leaf}}$ values were up to 2.2‰ more negative compared to those under $T_{\text{low}}$, resulting in a significant temperature effect independent of soil moisture conditions ($P=0.022$, Table 3). Similarly, soil moisture showed a significant effect on $\delta^{13}C_{\text{leaf}}$ ($P=0.005$, Table 3), independent of temperature treatments, with values up to 1.1‰ less negative under dry than under wet conditions mainly during nighttime.

$\delta^{13}C_{\text{RS}}$ of soluble carbohydrates, organic acids, and starch Highest $\delta^{13}C$ values in putative leaf respiratory carbon sources ($\delta^{13}C_{\text{RS}}$) were found in the organic acid malate, while
soluble carbohydrates (fructose, glucose and sucrose) exhibited generally lowest $\delta^{13}C_{RS}$ values (Fig. 3). $\delta^{13}C_{RS}$ of soluble carbohydrates of all treatments were in the range of $-27.2\%$ and $-36.6\%$. More negative $\delta^{13}C_{RS}$ values of glucose and sucrose under T\textsubscript{high} compared to those under T\textsubscript{low} were found, independent of soil moisture conditions, while less negative $\delta^{13}C_{RS}$ values under dry conditions compared to those under wet conditions were observed, independent of temperature treatments (Fig. 3B, C; Table 3). Significant interactions between temperature and time for $\delta^{13}C_{RS}$ of glucose ($P=0.008$, Table 3) and sucrose ($P=0.003$, Table 3) showed that daily cycles differed between temperatures. Additionally, soil moisture conditions caused significant temporal variations during the daily cycle in $\delta^{13}C_{RS}$ of sucrose ($P=0.002$, Table 3).

We observed significant linear relationships between fructose and glucose for $\delta^{13}C_{RS}$ ($r^2=0.74$, $P=0.001$) and concentration values ($r^2=0.8$, $P=0.001$), while relationships between the other $\delta^{13}C_{RS}$ values and concentrations of different carbon sources were weaker (data not shown). However, the deviant results for $\delta^{13}C_{RS}$ of fructose in comparison to the other sugars are assumed to reflect peak overlap issues of this sugar (Tables 3, 4). This is clearly reflected also in the concentration results (Fig. 4A). Consequently, the fructose results will not be discussed further in detail.

$\delta^{13}C_{RS}$ of malate (Fig. 3D) in the range of $-24\%$ and $-29.3\%$ and $\delta^{13}C_{RS}$ of citrate (Fig. 3E) in the range of $-29.6\%$ and $-32.1\%$ showed no temporal variations ($P=0.198$ and $P=0.052$ for malate and citrate, respectively, Table 3). Significant interactions between temperature and soil moisture treatments were observed for $\delta^{13}C_{RS}$ of malate ($P=0.017$, Table 3), resulting in larger differences between $\delta^{13}C_{RS}$ values of soil moisture conditions under T\textsubscript{high} than under T\textsubscript{low} (Fig. 3D). Citrate showed less negative $\delta^{13}C_{RS}$ values under dry conditions than under wet conditions ($P=0.009$, Table 3).

$\delta^{13}C_{RS}$ of starch of all treatments (Fig. 3F), ranging from $-25.2\%$ and $-32.1\%$, was influenced by soil moisture conditions ($P=0.046$, Table 3), independent of temperature treatments, while temperature showed no significant effect ($P=0.107$, Table 3). In addition, soil moisture conditions caused significant temporal variations during the daily cycle in $\delta^{13}C_{RS}$ of starch ($P=0.032$, Table 3).

**Table 2. Biomass and tuber analyses after sampling period**

Total plant biomass (dry weight), tuber weight (fresh weight), and tuber count (number of potatoes) after the sampling period. Potato plants were treated with a combination of T\textsubscript{low} (low temperature), T\textsubscript{high} (high temperature), and wet or dry conditions. Means ±SE are given ($n=3$). Refer to Table 1 for statistical analysis.

| Treatments | T\textsubscript{low} wet | T\textsubscript{low} dry | T\textsubscript{high} wet | T\textsubscript{high} dry |
|------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Total biomass (g) | 10.6 ± 1.4 | 7.8 ± 1.2 | 10.3 ± 1 | 8.1 ± 0.5 |
| Tuber weight (g) | 513.9 ± 18.4 | 458.3 ± 15 | 481.4 ± 15.9 | 430 ± 24.2 |
| Tuber count (no.) | 21.3 ± 2.7 | 20.2 ± 1.7 | 19.3 ± 1.2 | 19 ± 2.3 |

**Table 3. Environmental influences on leaf dark-respired CO\textsubscript{2} and respiratory carbon sources**

Results of linear mixed effects models testing the effects of temperature (low, high) and soil moisture (wet, dry) on $\delta^{13}C$ values in different putative leaf respiratory carbon sources, bulk leaves ($\delta^{13}C_{\text{bulk}}$), and in leaf dark-respired CO\textsubscript{2} ($\delta^{13}C_{\text{CO}_2}$), as well as on concentrations of different carbon sources during the sampling period. Results for fructose are affected by co-elution with other compounds. $P$-values are given for treatments, time, and their interactions. Significant differences are given in bold ($P<0.05$).

### $\delta^{13}C$

| Parameter | Fructose | Glucose | Sucrose | Malate | Citrate | Starch | $\delta^{13}C_{\text{Leaf}}$ | $\delta^{13}C_{\text{RS}}$ |
|-----------|----------|---------|---------|--------|---------|-------|----------------|------------------|
| Temperature | 0.019 | 0.004 | 0.028 | 0.015 | n.a. | 0.107 | 0.022 | 0.044 |
| Soil moisture | 0.001 | 0.001 | 0.001 | 0.049 | 0.009 | 0.046 | 0.005 | 0.013 |
| Time | 0.001 | 0.195 | 0.081 | 0.198 | 0.052 | 0.001 | 0.066 | 0.001 |
| Temp.:moisture | 0.035 | 0.063 | 0.543 | 0.017 | n.a. | 0.270 | 0.165 | 0.875 |
| Temp.:time | 0.256 | 0.008 | 0.003 | 0.807 | n.a. | 0.113 | 0.812 | 0.014 |
| Moisture:time | 0.061 | 0.291 | 0.002 | 0.060 | 0.411 | 0.032 | 0.596 | 0.883 |

### Concentration

| Parameter | Fructose | Glucose | Sucrose | Malate | Citrate | Starch |
|-----------|----------|---------|---------|--------|---------|-------|
| Temperature | 0.663 | 0.352 | 0.142 | 0.011 | n.a. | 0.002 |
| Soil moisture | 0.001 | 0.001 | 0.031 | 0.999 | 0.052 | 0.001 |
| Time | 0.016 | 0.927 | 0.001 | 0.035 | 0.110 | 0.001 |
| Temp.:moisture | 0.475 | 0.705 | 0.462 | 0.796 | n.a. | 0.001 |
| Temp.:time | 0.901 | 0.847 | 0.113 | 0.387 | n.a. | 0.324 |
| Moisture:time | 0.831 | 0.629 | 0.063 | 0.899 | 0.895 | 0.071 |

n.a., not available
(Fig. 4C) in the range of 23 to 159 µmol g DW\(^{-1}\) showed clear daily variations \((P \leq 0.001, \text{ Table 3})\), with highest concentrations for all treatments by the end of the day, except for \(T_{\text{high}}\), and dry conditions. Glucose concentrations were significantly higher under dry than under wet conditions \((P \leq 0.001, \text{ Table 3})\), while converse results were observed for sucrose \((P = 0.031, \text{ Table 3})\). Generally, no effect of temperature on the concentration of any soluble carbohydrate was observed.

Malate concentrations of all treatments (Fig. 4D), ranging from 23 to 163 µmol g DW\(^{-1}\), showed a daily pattern with declining concentrations in the beginning of the night and an increase after 2–4 h in the dark \((P = 0.035, \text{ Table 3})\). In contrast to soluble carbohydrates, malate concentrations were significantly higher under \(T_{\text{high}}\) than under \(T_{\text{low}}\) \((P = 0.011, \text{ Table 3})\), but were not affected by soil moisture treatments \((P = 0.999, \text{ Table 3})\). Citrate concentrations under \(T_{\text{high}}\) of ~15 µmol g DW\(^{-1}\) were the lowest of all measured putative carbohydrate sources available for leaf dark respiration and showed no changes due to soil moisture treatments and time (Fig. 4E; Table 3).

Starch concentrations (Fig. 4F), ranging from 67 to 282 µmol g DW\(^{-1}\), showed significant temporal variations \((P \leq 0.001, \text{ Table 3})\), independent of any treatment. The average starch concentration of 243 µmol g DW\(^{-1}\) under \(T_{\text{low}}\) and wet conditions was clearly higher (~2.5 times) compared to those under other treatments. In addition, interactions between temperature and soil moisture treatments led to smaller differences between the values of wet and dry conditions under \(T_{\text{high}}\) compared to those under \(T_{\text{low}}\) \((P \leq 0.001, \text{ Table 3})\).

### Table 4. Relationships between δ\(^{13}\)C of leaf dark-respired CO\(_2\) and δ\(^{13}\)C of respiratory carbon sources

| Putative carbon sources | Daytime | Nighttime | Daily |
|-------------------------|---------|-----------|-------|
| Fructose                | 0.35*** | 0.34***   | 0.12*** |
| Glucose                 | 0.54*** | 0.34***   | 0.13*** |
| Sucrose                 | 0.59*** | 0.20***   | 0.04*  |
| Malate                  | 0.69*** | 0.36***   | 0.26*** |
| Citrate                 | 0.67*** | 0.28**    | 0.17** |
| Starch                  | 0.48*** | 0.16**    | 0.06*  |
| δ\(^{13}\)C\(_R\)leaf | 0.63*** | 0.33***   | 0.20*** |

*, \(P \leq 0.05\); **, \(P \leq 0.01\); ***, \(P \leq 0.001\)

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**Fig. 3.** Daily cycles of the carbon isotopic composition of different leaf respiratory carbon sources (δ\(^{13}\)C\(_R\)) under different environmental conditions during the sampling period: (A) fructose, (B) glucose, (C) sucrose, (D) malate, (E) citrate, and (F) starch. Potato plants were treated with a combination of \(T_{\text{low}}\) (low temperature; closed symbols), \(T_{\text{high}}\) (high temperature; open symbols), and wet (circles) or dry (triangles) conditions. Results for fructose are affected by co-elution with other compounds. Grey areas indicate nighttime. Means ± SE are given \((n = 2–3)\).
Table 5. Environmental drivers and carbon sources influencing δ\(^{13}\)C of leaf dark-respired CO\(_2\)

Result of stepwise (backward) multiple linear regression analysis showing the best-fit combination of independent environmental drivers (temperature, soil moisture, daytime/nighttime), time, and δ\(^{13}\)C of glucose, sucrose, malate, and starch as variables influencing δ\(^{13}\)C of leaf dark-respired CO\(_2\) (δ\(^{13}\)C\(_{leaf}\)) during the sampling period in potato leaves. Standardized β-coefficients and P-values are given.

| Drivers and carbon sources influencing δ\(^{13}\)C\(_R\) | Standardized β-coefficient | P-value |
|-------------------------------------------------|---------------------------|---------|
| Daytime/nighttime                               | 0.73                      | <0.001  |
| Malate                                          | 0.40                      | <0.001  |
| Starch                                          | 0.11                      | 0.019   |
| Soil moisture                                   | 0.14                      | 0.013   |

of fructose and glucose, as well as δ\(^{13}\)C\(_{leaf}\) showed similarly high explanatory power.

Influence of environmental drivers and carbon sources on δ\(^{13}\)C\(_R\)

Furthermore, a stepwise (backward) multiple linear regression analysis was performed to identify environmental drivers and carbon sources influencing δ\(^{13}\)C\(_R\) (Table 5). Daytime/nighttime showed the strongest positive effect on δ\(^{13}\)C\(_R\) (β=0.73, P≤0.001), while δ\(^{13}\)C\(_{RS}\) of malate was the carbon source that affected δ\(^{13}\)C\(_R\) most (β=0.4, P≤0.001). By comparison, the influence of δ\(^{13}\)C\(_{RS}\) of starch and soil moisture conditions on δ\(^{13}\)C\(_R\) values was minor.

Discussion

This study clearly demonstrates that different temperature and soil moisture conditions influence δ\(^{13}\)C of leaf dark-respired CO\(_2\) (δ\(^{13}\)C\(_R\)), δ\(^{13}\)C of different putative leaf respiratory carbon sources (δ\(^{13}\)C\(_{RS}\)), and concentrations of carbon sources during a daily cycle in potato leaves. Furthermore, our findings strongly indicate malate as a key carbon source of daytime and nighttime δ\(^{13}\)C\(_R\) across different environmental conditions.

Influence of temperature and soil moisture on isotopic compositions

After 2 weeks of treatment, we already found a clear temperature effect on δ\(^{13}\)C\(_{leaf}\), with up to 2.2‰ more negative δ\(^{13}\)C\(_{leaf}\) values under T\(_{high}\) conditions compared to those under T\(_{low}\) conditions (Fig. 2B). This is in agreement with a study showing more negative δ\(^{13}\)C values with increasing temperature for bulk leaves of Xanthium species (Smith et al., 1976). Similar to Tcherkez et al. (2003) under short-term temperature treatments, we observed more negative δ\(^{13}\)C\(_R\) value with increasing temperature (Fig. 2A), but due to our long-term treatment we found also more negative δ\(^{13}\)C\(_{RS}\) values (Fig. 3). On the other hand, dry conditions in both of the temperature treatments caused less negative δ\(^{13}\)C\(_{leaf}\), δ\(^{13}\)C\(_R\), and δ\(^{13}\)C\(_{RS}\) values compared to those under wet conditions, which is consistent with previous studies under controlled conditions (Duranceau et al., 1999; Ghasshgeia et al., 2001).

The isotopic results under the different environmental conditions can be directly linked to the leaf gas exchange observed during the 32 h sampling period (day 15 of the treatment period). Increasing temperature caused lower A\(_n\) values under both soil moisture conditions (Fig. 1A; Table 1), indicating that plants under T\(_{high}\) were beyond the photosynthetic optimum. This result is in agreement with earlier studies, showing that cold-adapted potato plants have reduced rates of photosynthesis with temperatures above 20°C (Levy and Veilleux, 2007). Additionally, A\(_n\) might be also influenced by leaf ageing, since
$A_n$ decreased under all treatments during the treatment period. On the other hand, $g_s$ tended to higher values with increasing temperature, but only under wet conditions (Fig. 1C; Table 1). An increase of $g_s$ under $T_{high}$ might be triggered by increasing transpiration rates, which could be a physiological response to compensate reduced rates of $A_n$ by cooling the leaf temperature under $T_{high}$ conditions. However, this was only observed in plants under $T_{high}$ and well-watered conditions, when $SWC$ was high. Subsequently, lower carbon fixation and higher CO$_2$ diffusion into the stomatal cavities under $T_{high}$, in comparison to $T_{low}$, caused an increase of $C_i$ (Fig. 1B) and more negative $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values (Table 6). Furthermore, dry soil moisture conditions caused reduced rates of $A_n$ and $g_s$ compared to those under wet conditions (Fig. 1A, C; Table 1), independent of temperature treatments. This can be explained with the severe drought stress, reflecting low $SWC$ values (Fig. 1D). Consequently, plants under dry conditions experienced reduced CO$_2$ diffusion into the stomatal cavities, leading to lower $C_i$ and less negative $\delta^{13}C$ values (Table 6).

Plants under $T_{high}$ and dry conditions showed the least performance during the sampling period compared to plants under other treatments, which is reflected in low $A_n$ values (Fig. 1A), plant biomass, tuber weight and tuber count (Table 2). $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ in these plants were expected to be the most positive compared to other treatments due to a severe drought caused by the double effect of high temperature and dry soil moisture. Instead, $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of the plants under the highest stress level ($T_{high}$ and dry conditions) were rather similar to those under lowest stress level ($T_{low}$ and wet conditions). This was particularly observed for $\delta^{13}C_{RS}$ of soluble carbohydrates and starch (Fig. 3). Again, this is an indicator of low $A_n$ under $T_{high}$ and dry conditions, resulting in a moderate reduction of $C_i$, while at the same time $g_s$ strongly reduces CO$_2$ diffusion into the stomatal cavities, causing an increase of $C_i$. Consequently, this led to intermediate $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values under $T_{high}$ and dry conditions (Table 6). In summary, our findings indicate that combined effects of temperature and soil moisture conditions on $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ could cancel out the individual effect of each driver.

Environmental influences on concentrations of putative carbon sources

Soil moisture and temperature affected concentrations of putative leaf respiratory carbon sources differently. Sucrose concentration decreased under dry conditions (Fig. 4C; Table 3), which is in contrast to the recent study by Lemoine et al. (2013). This may be explained by reduced rates of sucrose synthesis due to lowering of the sucrose phosphate synthase reaction (SPS) (Vu et al., 1998). The decrease in the enzyme activity is probably triggered by limited rates of phloem sugar transport observed under drought (Ruehr et al., 2009). This in turn could be an explanation for lower plant biomass and tuber weight/count in response to higher temperatures and dry conditions (Tables 1, 2). Subsequently, the increase of fructose and glucose concentrations under drought may also be a consequence of lower SPS activity (Fig. 4A, B; Table 3), since the demand for both hexoses for sucrose synthesis was reduced. Additionally, increasing fructose and glucose concentrations under drought might have osmotic functionality, maintaining metabolic activity (Lemoine et al., 2013).

On the other hand, malate concentrations increased with temperature (Fig. 4D; Table 3), which is most likely a consequence of higher PEPC activity (Chinthapalli et al., 2003). Higher malate concentrations may also support respiratory processes in the KC or regulation of stomatal opening (Finkemeier and Sweetlove, 2009). Moreover, decreased starch concentrations in leaves under treatments with higher environmental stress than $T_{low}$ and wet conditions (Fig. 4F) were similar to previous findings (Lemoine et al., 2013). The result also supports the assumption that reduced amounts of assimilated carbon due to lower $A_n$ under $T_{high}$ or dry conditions were used for maintenance of biochemical processes rather than for carbon storage. Additionally, this indicates that plants under $T_{high}$ or dry conditions were under severe environmental stress.

Malate as a key respiratory carbon source of daytime and nighttime $\delta^{13}C_R$

The daily cycle of $\delta^{13}C_R$ was highly variable, showing less negative daytime and more negative nighttime values, while $\delta^{13}C_{RS}$ values generally showed lower changes during the same period (Figs 2A, 3; Table 3). $\delta^{13}C_{RS}$ values of all treatments compared to $\delta^{13}C_R$ values were more negative for soluble carbohydrates (up to 9.3‰) and citrate (up to 4.1‰), but also less negative for starch (up to 4‰) and malate (up to 5.2‰) during the daily cycle (Figs 2A, 3). In particular, malate was strongly enriched in $^{13}C$, by up to 8.8‰, compared to all other putative carbon sources (Fig. 3). This was similar to a previous study investigating metabolites in potato leaves (Gleixner et al., 1998) and indicates a possible biochemical link between $^{13}C$ enriched leaf dark-respired CO$_2$ and $^{13}C$ enriched malate.

For a better understanding of the overall biochemical connections between $\delta^{13}C_R$ and different putative carbon sources, we carried out linear regression analyses, independent of environmental conditions (Table 4; Supplementary Fig. S1).

### Table 6. Coherence between leaf physiological parameters and $\delta^{13}C$ values. Leaf physiological parameters and $\delta^{13}C$ values during the sampling period in potato plants under different treatments compared to those in potato plants growing under $T_{low}$ and wet conditions.

The following variables were considered: $A_n$, net assimilation rate; $C_i$, intercellular CO$_2$ concentration; $g_s$, stomatal conductance; $\delta^{13}C_R$, $\delta^{13}C$ of leaf dark-respired CO$_2$; $\delta^{13}C_{RS}$, $\delta^{13}C$ of different putative respiratory carbon sources (fructose, glucose, sucrose, starch, and malate). Arrows indicate strong (↑, ↓), intermediate (→, ←), or no changes (–) due to the influence of treatment combinations ($T_{low}$, low temperature; $T_{high}$, high temperature; and wet or dry conditions).

| Treatments | $A_n$ | $g_s$ | $C_i$ | $\delta^{13}C_R$ | $\delta^{13}C_{RS}$ |
|------------|-------|-------|-------|----------------|------------------|
| $T_{low}$ dry | ↑     | ↑     | ↑     | ↑              | ↑                |
| $T_{high}$ wet | ↓     | ↓     | ↑     | ↑              | ↓                |
| $T_{high}$ dry | ↓     | ↓     | ↑     | →              | →                |
The daily linear relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate was stronger compared to all other putative carbon sources ($r^2=0.26$, $P<0.001$). The strength of this relationship increased for $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate when considering daytime ($r^2=0.69$, $P<0.001$) and nighttime ($r^2=0.36$, $P<0.001$) separately. Moreover, relationships of $\delta^{13}C_R$ with $\delta^{13}C_{RS}$ of malate were stronger than those of $\delta^{13}C_R$ with $\delta^{13}C$ of bulk leaves (reflects the average $\delta^{13}C$ value of all respiratory substrates), which was, however, not the case for most relationships of $\delta^{13}C_R$ with other carbon sources.

Please note that comparisons between daytime and nighttime relationships must be done carefully (Table 4) due to the bias caused by LEDR in daytime $\delta^{13}C_R$, which depends on the amount of assimilated carbon (Priault et al., 2009) and probably also on environmental conditions. LEDR is considered to be fuelled by malate (Atkin et al., 1998; Barbour et al., 2007; Gessler et al., 2009; Werner and Gessler, 2011). Consequently, the strong daytime relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate might be explained by a higher respiratory consumption of malate during the LEDR period, provoking less negative daytime $\delta^{13}C_R$ values (Fig. 2A). Furthermore, transferring light-acclimated leaves into darkness is suggested to lead to reassembly of the KC by activation of light-inhibited enzymatic reactions of the cycle (Tcherkez et al., 2005; Sweetlove et al., 2010; Werner and Gessler, 2011). During LEDR the KC might not be fully active, leading to changes in metabolic fluxes and isotope fractionations, which may not occur during nighttime when KC is fully reassembled (Werner et al., 2011). This could be an important factor, explaining light-dark differences in the relationships between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of different carbon sources in this study (Table 4).

In contrast to malate, $\delta^{13}C_{RS}$ of carbon storage compounds, such as starch and sucrose, were less related to $\delta^{13}C_R$ during daytime and nighttime (Table 4). This can particularly be explained for starch due to the fact that its isotopic composition is always a mix of fresh and old assimilates, constraining good relationships with the isotopic composition of recently respired $CO_2$. Moreover, the high daytime relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of citrate might be explained by the close biochemical relationship of citrate with malate via the mitochondrial malate dehydrogenase and citrate synthase (Voet and Voet, 2011). However, citrate was $^{13}C$ depleted and showed very low concentrations compared to other carbon sources (Figs 3E, 4E), contradicting the role of citrate as an important carbon source of $\delta^{13}C_R$.

We also observed regular decreases in malate concentrations in the beginning of the night across all environmental conditions (Fig. 4D), as observed in previous studies (Urbansczyk-Wochniak et al., 2005; Gessler et al., 2009), which may reflect the use of malate for respiratory processes shortly upon darkening, e.g. LEDR. It has also been suggested that malate accumulates during daytime (Barbour et al., 2007; Gessler et al., 2009; Werner and Gessler, 2011). However, low temporal variations in malate concentrations during daytime do not support this hypothesis.

Furthermore, the hypothesis that $\delta^{13}C_R$ is influenced by the putative carbon source malate across all treatments was also indicated by a stepwise multiple linear regression analysis (Table 5, $P$-values). The findings are in line with our other observations showing that (i) daytime and nighttime periods have a clear influence on $\delta^{13}C_R$ (Fig. 2A); (ii) $\delta^{13}C_{RS}$ of malate has the strongest influence on $\delta^{13}C_R$ compared to all other putative carbon sources; and (iii) influences of other environmental drivers and carbon sources are weaker and less significant compared to daytime/nighttime and malate. Overall, the findings strongly indicate $\delta^{13}C_{RS}$ of malate as a key carbon source of $\delta^{13}C_R$ during the daily cycle across all environmental conditions within this study.

A mechanistic explanation for the respiratory use of malate can be found within the amphibole functionality of the KC and associated reactions (malic enzyme, PDH; Fig. 5). Generally, the breakdown of glucose during glycolysis produces pyruvate. Leaf feeding experiments using position-specific $^{13}C$ labelled pyruvate have shown in different species that respiration of the C-1 position of pyruvate is higher compared to respiration of the C-2 and C-3 position of pyruvate during daytime (Priault et al., 2009; Wegener et al., 2010), as well as during nighttime (Werner et al., 2009). This clearly indicates that acetyl-CoA (C-2 and C-3 position of pyruvate) from the PDH reaction, which enters the KC, is used for biosynthesis of diverse metabolic compounds (e.g. amino acids or lipids), rather than for respiration (Fig. 5). If this is true, withdrawn KC intermediates must be refilled due to stoichiometric reasons to maintain the functionality of the KC. This could be achieved by an anapleurotic flux via PEPC, which has often been described as replenishing KC intermediates (Melzer and O’Leary, 1987; Savidge and Blair, 2004). The PEPC reaction produces $^{13}C$-enriched oxaloacetate, of which the greatest proportion is directly converted into malate via the malate dehydrogenase reaction. A breakdown of this malate pool within the KC or associated reactions (malic enzyme, PDH) would then produce $^{13}C$-enriched leaf dark-respired $CO_2$ (Fig. 5), explaining the close relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate found in this study. Moreover, malate is supposed to be a $^{13}C$ enriched leaf at the C-4 position via PEPC, while other positions of the molecule are $^{13}C$ depleted via glycolysis (Melzer and O’Leary, 1987; Savidge and Blair, 2004), causing dampening of the $^{13}C$ enrichment at the C-4 position when measuring $\delta^{13}C$ of the whole malate molecule (Fig. 3D). Therefore, slight changes in $\delta^{13}C$ of malate may indicate higher changes at the C-4 position, which can be decarboxylated by the malic enzyme reaction or within the KC and thus be highly relevant for variations in $\delta^{13}C_R$. In brief, our findings strongly suggest that $\delta^{13}C_{RS}$ of malate has a strong influence on $\delta^{13}C_R$ during daytime, as well as nighttime, across different environmental conditions in this study and that their biochemical link is driven by an anapleurotic flux via PEPC, replenishing KC intermediates.

Conclusions

Here we showed for the first time results of $\delta^{13}C$ of leaf dark-respired $CO_2$ and $\delta^{13}C$ of putative respiratory carbon sources under the combined influence of controlled temperature
and soil moisture conditions on a daily basis in a C3 plant. Overall, we found that δ13C_R values generally reflect changes in δ13C_RS values in putative respiratory carbon sources due to the influence of different temperature and soil moisture treatments on leaf physiological parameters. It is worth noting that the temperature in this study exceeded the photosynthetic optimum of the potato plants under T_high, unexpectedly leading to more negative δ13C values under T_high and dry conditions than those observed under T_low and dry conditions. This demonstrates that conclusions about the individual influence of an environmental driver on δ13C values should be drawn carefully and that verification of the isotopic results by gas exchange measurements is mandatory. Moreover, our findings indicate malate as a key respiratory carbon source of leaf dark-respired CO2 in potato plants. This could also be the case in plant species comparable with potato, but should not be generalized and transferred to respiratory processes in species of different functional groups such as trees or shrubs without verification. Please note that for exact quantification of the respiratory contribution of malate in comparison to other metabolites more knowledge about metabolic fluxes and turnover rates is necessary. For subsequent studies on this topic we recommend the inclusion of isotopic measurements of malate or of the organic acid pool, given the strong indications observed herein for a biochemical link between δ13C of malate and δ13C of leaf dark-respired CO2.

Supplementary data

Supplementary data are available at JXB online.
chromatography/isotope ratio mass spectrometry. Rapid Communications in Mass Spectrometry 22, 3902–3908.

Bowling DR, Pataki DE, Randerson JT. 2008. Carbon isotopes in terrestrial ecosystem pools and CO₂ fluxes. New Phytologist 178, 24–40.

Chinthapalli B, Murmu J, Raghavendra AS. 2003. Dramatic difference in the responses of phosphoenolpyruvate carboxylase to temperature in leaves of C₄ and C₃ plants. Journal of Experimental Botany 54, 707–714.

Coplen TB. 2011. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. Rapid Communications in Mass Spectrometry 25, 2538–2560.

Craig H. 1957. Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. Geochimica et Cosmochimica Acta 12, 133–149.

Critchley JH, Zeeman SC, Takaha T, Smith AM, Smith SM. 2001. A critical role for diapronotating enzyme in starch breakdown is revealed by a knock-out mutation in Arabidopsis. The Plant Journal 26, 89–100.

Dubbert M, Rascher KG, Werner C. 2012. Species-specific differences in temporal and spatial variation in δ¹³C of plant carbon pools and dark-respired CO₂ under changing environmental conditions. Photosynthesis Research 113, 297–309.

Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. 1999. δ¹³C of CO₂ respired in the dark in relation to δ¹³C of leaf carbohydrates in Phaseolus vulgaris L. under progressive drought. Plant, Cell & Environment 22, 515–523.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. Annual Review of Plant Physiology and Plant Molecular Biology 40, 503–537.

Finkemeier I, Sweetlove LJ. 2009. The role of malate in plant homeostasis. F1000 Biology Reports doi: 10.3410/B1-47.

Gessler A, Tcherkez G, Karyanto O, Keitel C, Ferro JP, Ghashghaie J, Kreuzwieser J, Farquhar GD. 2009. On the metabolic origin of the carbon isotope composition of CO₂ evolved from darkened light-acclimated leaves in Ricinus communis. New Phytologist 181, 374–386.

Ghashghaie J, Badeck FW. 2014. Opposite carbon isotope discrimination during dark respiration in leaves versus roots—a review. New Phytologist 201, 751–769.

Ghashghaie J, Badeck FW, Lanigan G, Nogues S, Tcherkez G, Deleens E, Cornic G, Griffiths H. 2003. Carbon isotope fractionation during dark respiration and photosorption in C₄ plants. Phytochemistry Reviews 2, 145–161.

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E. 2001. δ¹³C of CO₂ respired in the dark in relation to δ¹³C of leaf metabolites: comparison between Nicotiana sylvestris and Helianthus annuus under drought. Plant, Cell & Environment 24, 505–515.

Gleixner G, Schmidt H-L. 1997. Carbon isotope effects on the fructose-1,6-bisphosphate aldolase reaction, origin for non-statistical δ¹³C distributions in carbohydrates. Journal of Biological Chemistry 272, 5382–5387.

Gleixner G, Scrimgeour C, Schmidt H-L. 1997. Stable isotope distribution in the major metabolites of source and sink organs of Solanum tuberosum L.: a powerful tool in the study of metabolic partitioning in intact plants. Planta 207, 241–245.

Goettlicher S, Knohl A, Wanek W, Buemann N, Richter A. 2006. Short-term changes in carbon isotope composition of soluble carbohydrates and starch: from canopy leaves to the root system. Rapid Communications in Mass Spectrometry 20, 653–660.

Hettmann E, Gleixner G, Juchelka D. 2005. IRM-LC/MS: δ¹³C analysis of organic acids in plants. Application Note 30075, Thermo Fisher Scientific.

Hoch G, Popp M, Koerner C. 2002. Altitudinal increase of mobile carbon pools in Pinus cembra suggests sink limitation of growth at the Swiss treeline. Oikos 98, 361–374.

Hopkins WG. 2006. Photosynthesis and Respiration. New York: Chelsea House, 88–109.

Hymus GJ, Maseyk K, Valentini R, Yakir D. 2005. Large daily variation in δ¹³C-enrichment of leaf-respired CO₂ in two Quercus forest canopies. New Phytologist 167, 377–384.

Krummen M, Hilkert AW, Juchelka D, Duhr A, Schluter HJ, Pesch R. 2004. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. Rapid Communications in Mass Spectrometry 18, 2260–2266.

Lemoine R, La Camera S, Atanassova R, et al. 2013. Source-to-sink transport of sugar and regulation by environmental factors. Frontiers in Plant Science 4 doi: 10.3389/fpls.2013.00272.

Levy D, Veilleux RE. 2007. Adaptation of potato to high temperatures and salinity—a review. American Journal of Potato Research 84, 487–506.

Melzer E, O’Leary MH. 1987. Anapleurotic CO₂ fixation by phosphoenolpyruvate carboxylase in C₄ plants. Plant Physiology 84, 58–60.

Prater JL, Mortazavi B, Chanton JP. 2006. Diurnal variation of the δ¹³C of pine needle respired CO₂ evolved in darkness. Plant, Cell & Environment 29, 202–211.

Praulp T, Wegener F, Werner C. 2009. Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO₂ among functional groups. New Phytologist 181, 400–412.

R Core Team. 2013. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Richter A, Wanek W, Werner RA, et al. 2009. Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a comparison of methods. Rapid Communications in Mass Spectrometry 23, 2476–2488.

Rinne KT, Saurer M, Streit K, Siegwolf RTW. 2012. Evaluation of a liquid chromatography method for compound-specific δ¹³C analysis of plant carbohydrates in alkaline media. Rapid Communications in Mass Spectrometry 26, 2173–2185.

Rossmann A, Butzenlechner M, Schmidt H-L. 1991. Evidence for a nonstatistical carbon isotope distribution in natural glucose. Plant Physiology 96, 609–614.

Ruehr NK, Offermann CA, Gessler A, Winkler JB, Ferrio JP, Buchmann N, Barnard RL. 2009. Drought effects on allocation of recent carbon: from beech leaves to soil CO₂ efflux. New Phytologist 184, 950–961.

Savidge WB, Blair NE. 2004. Patterns of intramolecular carbon isotope heterogeneity within amino acids of autotrophs and heterotrophs. Oecologia 139, 178–189.

Smith BN, Oliver J, Millian CM. 1976. Influence of carbon source, oxygen concentration, light intensity, and temperature on δ¹³C/δ¹²C ratios in plant tissues. Botanical Gazette 137, 99–104.

Streit K, Rinne KT, Hagedorn F, Dawes MA, Saurer M, Hoch G, Werner RA, Buchmann N, Siegwolf RTW. 2013. Racing fresh assimilates through Larix decidua exposed to elevated CO₂ and soil warming at the alpine treeline using compound-specific stable isotope analysis. New Phytologist 197, 838–849.

Sun W, Resco V, Williams DG. 2009. Diurnal and seasonal variation in the carbon isotope composition of leaf dark–respired CO₂ in velvet mesquite (Prosopis velutina). Plant, Cell & Environment 32, 1390–1401.

Sweetlove LJ, Beard KFM, Nunes-Nesi A, Fernie AR, Ratcliffe RG. 2010. Not just a circle: flux modes in the plant TCA cycle. Trends in Plant Science 15, 462–470.

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 C₄ plants. Functional Plant Biology 31, 857–877.

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.

Unger S, Maguas C, Pereira JS, Aires LM, David TS, Werner C. 2010. Dissentangling drought-induced variation in ecosystem and soil respiration using stable carbon isotopes. Oecologia 163, 1043–1057.

Urbanczyk-Wochniak E, Baxter C, Kolbe A, Kopka J, Sweetlove LJ, Fernie AR. 2005. Profiling of diurnal patterns of metabolite and transcript abundance in potato (Solanum tuberosum) leaves. Planta 221, 931–939.

Usadel B, Blasio OE, Gibon Y, Retzlaff K, Hoehne M, Gunther M, Stitt M. 2008. Global transcript levels respond to small changes of
the carbon status during progressive exhaustion of carbohydrates in Arabidopsis rosettes. *Plant Physiology* **146**, 1834–1861.

**Voet D, Voet JG.** 2011. *Biochemistry*, 4th edition. New York: Wiley, 789–822.

**Vu JCV, Baker JT, Pennanan AH, Allen LH, Bowes G, Boote KJ.** 1998. Elevated CO₂ and water deficit effects on photosynthesis, ribulose bisphosphate carboxylase-oxygenase, and carbohydrate metabolism in rice. *Physiologia Plantarum* **103**, 327–339.

**Wanek W, Heintel S, Richter A.** 2001. Preparation of starch and other carbon fractions from higher plant leaves for stable carbon isotope analysis. *Rapid Communications in Mass Spectrometry* **15**, 1136–1140.

**Wegener F, Beyschlag W, Werner C.** 2010. The magnitude of diurnal variation in carbon isotopic composition of leaf dark respired CO₂ correlates with the difference between δ¹³C of leaf and root material. *Functional Plant Biology* **37**, 849–858.

**Werner C, Gessler A.** 2011. Diel variations in the carbon isotope composition of respired CO₂ and associated carbon sources: a review of dynamics and mechanisms. *Biogeosciences* **8**, 2437–2459.

**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.

**Werner C, Wegener F, Unger S, Nogues S, Priault P.** 2009. Short-term dynamics of isotopic composition of leaf-respired CO₂ upon darkening: measurements and implications. *Rapid Communications in Mass Spectrometry* **23**, 2426–2438.

**Werner RA, Brand WA.** 2001. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Communications in Mass Spectrometry* **15**, 501–519.

**Werner RA, Bruch BA, Brand WA.** 1999. ConFlo III—an interface for high precision δ¹³C and δ¹⁵N analysis with an extended dynamic range. *Rapid Communications in Mass Spectrometry* **13**, 1237–1241.

**Werner RA, Buchmann N, Siegwolf RTW, Kornexl BE, Gessler A.** 2011. Metabolic fluxes, carbon isotope fractionation and respiration—lessons to be learned from plant biochemistry. *New Phytologist* **191**, 10–15.

**Zeeman MJ, Werner RA, Eugster W, Siegwolf RTW, Wehrle G, Mohn J, Buchmann N.** 2008. Optimization of automated gas sample collection and isotope ratio mass spectrometric analysis of δ¹³C of CO₂ in air. *Rapid Communications in Mass Spectrometry* **22**, 3883–3892.