RESEARCH PAPER

Malate as a key carbon source of leaf dark-respired CO₂ 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 CO₂. The carbon isotopic composition of leaf dark-respired CO₂ (i.e. δ¹³C_R) shows daily enrichments up to 14.8‰ under different environmental conditions. However, the reasons for this ¹³C enrichment in leaf dark-respired CO₂ are not fully understood, since daily changes in δ¹³C of putative leaf respiratory carbon sources (δ¹³C_RS) are not yet clear. Thus, we exposed potato plants (Solanum tuberosum) to different temperature and soil moisture treatments. We determined δ¹³C_R with an in-tube incubation technique and δ¹³C_RS with compound-specific isotope analysis during a daily cycle. The highest δ¹³C_RS values were found in the organic acid malate under different environmental conditions, showing less negative values compared to δ¹³C_R (up to 5.2‰) and compared to δ¹³C_RS of soluble carbohydrates, citrate and starch (up to 8.8‰). Moreover, linear relationships between δ¹³C_R and δ¹³C_RS among different putative carbon sources were strongest for malate during daytime (r²=0.69, P≤0.001) and nighttime (r²=0.36, P≤0.001) under all environmental conditions. A multiple linear regression analysis revealed δ¹³C_RS of malate as the most important carbon source influencing δ¹³C_R. Thus, our results strongly indicate malate as a key carbon source of ¹³C enriched dark-respired CO₂ 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 dissipate carbon sources for the production of intermediates and reducing equivalents in support of metabolic processes, thereby continuously releasing CO₂ via plant respiration (Hopkins, 2006). Leaf-respired CO₂ is mainly derived from oxidative decarboxylation reactions catalysed by enzymes from the Krebs cycle.
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 (Ci) in the substomatal cavity, which is regulated by other physiological parameters such as net assimilation rate (A), and stomatal conductance (g). 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. 13C/12C) 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; Wügener et al., 2010), which differs among functional groups (Priault et al., 2009; Werner et al., 2009).

13C/12C is thereby linked to the carbon isotopic composition of putative leaf respiratory carbon sources (i.e. 13C/12C) 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 13C/12C and 13C/12C. More negative 13C/12C 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 13C/12C and 13C/12C have not yet been investigated under controlled conditions. Other studies have demonstrated less negative 13C/12C and 13C/12C 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 13C/12C values have been found under dry conditions for Mediterranean trees and herbs such as Quercus ilex and Tuberaria 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 13C/12C and 13C/12C under controlled conditions have yet to be tested.

Moreover, 13C/12C 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 13C/12C 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 13C/12C 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 anapleurotic 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 13C/12C (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 13C/12C 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 13C/12C? How are 13C/12C and 13C/12C influenced by temperature and soil moisture conditions? Our main objectives were (i) to analyse the relationship between 13C/12C and 13C/12C influenced by temperature and soil moisture conditions? Our main objectives were (i) to analyse the relationship between 13C/12C and 13C/12C influenced by temperature
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 (Okohum, 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⁻²s⁻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 50–60% 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) with a 0.1 mm single sieving 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 ≤ 1.4‰, 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 (A_n), intercellular CO₂ concentration (C_i), and stomatal conductance (g_s). 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 \( R_{sample} \) is the \(^{13}C/^{12}C \) ratio of the sample material and \( R_{standard} \) is that of the international standard VPDB (Vienna Pee Dee Belemnite).

**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 (δ¹³CBulk) and starch, an elemental analyser (Flash EA 1112 Series) coupled to a DeltaplusXP-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 = 0.12‰.

**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 0.1-μm filter papers (Whatman, Maidstone, UK) and subsequently lyophilized. All samples designated for carbohydrate analyses were filtered with 0.1 μm filter papers (Whatman, Maidstone, UK) and subsequently lyophilized.

To determine δ¹²C 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 Krumen 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\(^{-1}\) (Boschker 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 \(\delta^{13}C_{RS}\) and concentration measurements were affected by partial co-elution of fructose with other compounds. To correct \(\delta^{13}C_{RS}\) 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 \(\delta^{13}C_{RS}\) 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 \(\delta^{13}C\) in organic acid standards was SD<0.4‰. Low citrate concentrations from \(T_{\text{low}}\) samples (<45 ng C μl\(^{-1}\)) impeded the analytical accuracy of the \(\delta^{13}C_{RS}\) 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 \(\delta^{13}C\) (by EA-IRMS) for all carbohydrates and organic acids measured in this study. Differences between \(\delta^{13}C\) 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, \(\delta^{13}C\) 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 (\(A_n\), \(C_i\), \(g_s\), 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 \(T_{\text{high}}\) and dry conditions, and highest values (5.4 μmol m\(^{-2}\) s\(^{-1}\)) under \(T_{\text{high}}\) 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 \(T_{\text{high}}\) than under \(T_{\text{low}}\) under both soil moisture conditions. The intercellular \(CO_2\) 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 \(T_{\text{low}}\) and dry conditions and highest \(C_i\) (332.8 μmol mol\(^{-1}\)) under \(T_{\text{high}}\) 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 \(T_{\text{high}}\) and wet conditions, whereas the temperature influence under wet conditions tended to cause higher \(g_s\) values under \(T_{\text{high}}\) than under \(T_{\text{low}}\). 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 \(T_{\text{low}}\) and wet conditions and lowest values under \(T_{\text{high}}\) and dry conditions (Tables 1, 2), indicating different stress levels created by the four treatments.

**Carbon isotopes in potato leaves**

**Daily cycles of \(\delta^{13}C_{R}\) and \(\delta^{13}C_{\text{leaf}}\)**

\(\delta^{13}C\) values of leaf dark-respired \(CO_2\) (\(\delta^{13}C_{R}\)) 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 \(\delta^{13}C_{R}\) values under \(T_{\text{high}}\) were up to 4.7‰ more negative compared to those under \(T_{\text{low}}\), independent of soil moisture conditions, whereas nighttime \(\delta^{13}C_{R}\) values of both temperature treatments were very similar, particularly in the second night. Dry soil moisture conditions caused less negative \(\delta^{13}C_{R}\) 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 \(\delta^{13}C_{R}\) values was highest under \(T_{\text{low}}\) and wet conditions, at 5.7‰, and lowest under \(T_{\text{high}}\) and dry conditions, at 2.5‰.
The bulk leaf material reflects all environmental conditions experienced during the whole growth period. $\delta^{13}C_{leaf}$ of all treatments showed no changes during the sampling period and no interactions between treatments and time (Fig. 2B; Table 3). Under $T_{\text{high}}$, $\delta^{13}C_{leaf}$ 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 $\delta^{13}C_{leaf}$ ($P=0.005$, Table 3), independent of temperature treatments, with values up to 1.1‰ less negative under dry than under wet conditions mainly during nighttime.

$\delta^{13}C_{RS}$ of soluble carbohydrates, organic acids, and starch 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. Grey areas indicate nighttime. Means ±SE are given ($n=3$).

**Table 1. Environmental influences on physiological parameters**

Results of linear mixed effects models testing the effects of temperature (low, high) and soil moisture (wet, dry) on physiological parameters ($A_n$, net assimilation rate; $C_i$, intercellular $\text{CO}_2$ concentration; $g_s$, stomatal conductance; SWC, volumetric soil water content), total plant biomass, tuber weight, and tuber count during the sampling period. $P$-values are given for treatments and their interaction. Significant differences are given in bold ($P<0.05$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$A_n$</th>
<th>$C_i$</th>
<th>$g_s$</th>
<th>SWC</th>
<th>Plant biomass</th>
<th>Tuber weight</th>
<th>Tuber count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0.070</td>
<td>0.012</td>
<td>0.127</td>
<td>0.863</td>
<td>0.978</td>
<td>0.359</td>
<td>0.400</td>
</tr>
<tr>
<td>Soil moisture</td>
<td>0.020</td>
<td>0.010</td>
<td>0.001</td>
<td>0.002</td>
<td>0.008</td>
<td>0.023</td>
<td>0.233</td>
</tr>
<tr>
<td>Temp.:moisture</td>
<td>0.522</td>
<td>0.110</td>
<td>0.174</td>
<td>0.845</td>
<td>0.565</td>
<td>0.892</td>
<td>0.486</td>
</tr>
</tbody>
</table>

**Fig. 1.** Physiological parameters under different environmental conditions during the treatment period: (A) net assimilation rate ($A_n$, µmol m$^{-2}$ s$^{-1}$), (B) intercellular $\text{CO}_2$ concentration ($C_i$, µmol mol$^{-1}$), (C) stomatal conductance ($g_s$, mol m$^{-2}$ s$^{-1}$), (D) volumetric soil water content (SWC, m$^3$ m$^{-3}$). 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. Boxed areas indicate the sampling period. Means ±SE are given ($n=3$).

**Fig. 2.** Daily cycles of the carbon isotopic composition of (A) leaf dark-respired $\text{CO}_2$ ($\delta^{13}C_{RS}$) and (B) bulk leaves ($\delta^{13}C_{leaf}$) under different environmental conditions during the sampling period. 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. Grey areas indicate nighttime. Means ±SE are given ($n=3$).
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 $T_{\text{high}}$ compared to those under $T_{\text{low}}$ 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 $\delta^{13}C_{RS}$ ($r^2=0.74$, $P\leq0.001$) and concentration values ($r^2=0.8$, $P\leq0.001$), 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 $T_{\text{high}}$ than under $T_{\text{low}}$ (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).

Concentrations of soluble carbohydrates, organic acids, and starch

Concentrations of glucose of all treatments (Fig. 4B), ranging from 27 to 95 μmol g DW$^{-1}$, showed no temporal variations ($P=0.927$, Table 3). In contrast, concentrations of sucrose

Table 2. Biomass and tuber analyses after sampling period

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$T_{\text{low}}$</th>
<th>$T_{\text{high}}$</th>
<th>$T_{\text{low}}$</th>
<th>$T_{\text{high}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total biomass (g)</td>
<td>10.6 ± 1.4</td>
<td>10.3 ± 1.0</td>
<td>8.1 ± 0.5</td>
<td>8.1 ± 0.5</td>
</tr>
<tr>
<td>Tuber weight (g)</td>
<td>513.9 ± 18.4</td>
<td>481.4 ± 15.9</td>
<td>430.3 ± 24.2</td>
<td>430.3 ± 24.2</td>
</tr>
<tr>
<td>Tuber count (no.)</td>
<td>21.3 ± 2.7</td>
<td>19.3 ± 1.2</td>
<td>19.3 ± 2.3</td>
<td>19.3 ± 2.3</td>
</tr>
</tbody>
</table>

Table 3. Environmental influences on leaf dark-respired CO$_{2}$ 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$_{2}$ ($\delta^{13}C_{\text{R}}$), 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 ($P\leq0.05$).

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

Concentration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Fructose</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Malate</th>
<th>Citrate</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>0.663</td>
<td>0.352</td>
<td>0.142</td>
<td>0.011</td>
<td>n.a.</td>
<td>0.002</td>
</tr>
<tr>
<td>Soil moisture</td>
<td>0.001</td>
<td>0.001</td>
<td>0.031</td>
<td>0.999</td>
<td>0.052</td>
<td>0.001</td>
</tr>
<tr>
<td>Time</td>
<td>0.016</td>
<td>0.927</td>
<td>0.001</td>
<td>0.035</td>
<td>0.110</td>
<td>0.001</td>
</tr>
<tr>
<td>Temp:moisture</td>
<td>0.475</td>
<td>0.705</td>
<td>0.462</td>
<td>0.796</td>
<td>n.a.</td>
<td>0.001</td>
</tr>
<tr>
<td>Temp:time</td>
<td>0.901</td>
<td>0.847</td>
<td>0.113</td>
<td>0.387</td>
<td>n.a.</td>
<td>0.324</td>
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<tr>
<td>Moisture:time</td>
<td>0.831</td>
<td>0.629</td>
<td>0.063</td>
<td>0.889</td>
<td>0.895</td>
<td>0.071</td>
</tr>
</tbody>
</table>

n.a., not available
(Fig. 4C) in the range of 23 to 159 μmol g DW⁻¹ showed clear daily variations (P≤0.001, Table 3), with highest concentrations for all treatments by the end of the day, except for Tₜₕ and dry conditions. Glucose concentrations were significantly higher under dry than under wet conditions (P≤0.001, Table 3), while converse results were observed for sucrose (P=0.031, 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, Table 3). In contrast to soluble carbohydrates, malate concentrations were significantly higher under Tₜₕ than under Tₜₙ (P=0.011, Table 3), but were not affected by soil moisture treatments (P=0.999, Table 3). Citrate concentrations under Tₜₕ of ~15 μmol g DW⁻¹ were the lowest of all measured putative carbon 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≤0.001, Table 3), independent of any treatment. The average starch concentration of 243 μmol g DW⁻¹ under Tₜₙ 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 Tₜₕ compared to those under Tₜₙ (P≤ 0.001, Table 3).

### Table 4. Relationships between δ¹³C of leaf dark-respired CO₂ and δ¹³C of respiratory carbon sources

Linear regression analyses relating δ¹³C of leaf dark-respired CO₂ to δ¹³C of putative respiratory carbon sources and to δ¹³C of bulk leaves (δ¹³Cᵢₑₜᵢₜ) across all environmental conditions for daytime (0h, 16h, 24h), for nighttime (2h, 4h, 8h, 26h, 28h, 32h), and for the total daily cycle (sampling period over 32h). Results for fructose are affected by co-elution with other compounds. Generic regression equation y=mx+b was used. r² values are given, stars indicate P-values. All correlation coefficients were positive.

<table>
<thead>
<tr>
<th>Putative carbon sources</th>
<th>Daytime</th>
<th>Nighttime</th>
<th>Daily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fructose</td>
<td>0.35***</td>
<td>0.34***</td>
<td>0.12***</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.54***</td>
<td>0.34***</td>
<td>0.13***</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.59***</td>
<td>0.20***</td>
<td>0.04*</td>
</tr>
<tr>
<td>Malate</td>
<td>0.69***</td>
<td>0.36***</td>
<td>0.26***</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.67***</td>
<td>0.28**</td>
<td>0.17**</td>
</tr>
<tr>
<td>Starch</td>
<td>0.48***</td>
<td>0.16**</td>
<td>0.06*</td>
</tr>
<tr>
<td>δ¹³Cᵢₑₜᵢₜ</td>
<td>0.63***</td>
<td>0.33***</td>
<td>0.20***</td>
</tr>
</tbody>
</table>

*, P≤0.05; **, P≤0.01; ***, P≤0.001

### Linear relationships between δ¹³Cᵣ and δ¹³Cᵣₛ

Linear regression analyses were performed to understand the biochemical link between δ¹³Cᵣ and δ¹³Cᵣₛ across all treatments (Table 4; Supplementary Fig. S1). δ¹³Cᵣₛ of malate explained most of the daily variation of δ¹³Cᵣ (r²=0.26, P≤0.001), while the explanatory power of fructose, glucose, and citrate was lower. The lowest linear relationships during the daily cycle were found between δ¹³Cᵣ and δ¹³Cᵣₛ of sucrose and starch. Due to the high daily variations in δ¹³Cᵣ, we carried out the same analysis separately for daytime and nighttime. Daytime linear relationships were generally stronger than during nighttime, with δ¹³Cᵣ strongly related to δ¹³Cᵣₛ of malate, citrate, and δ¹³Cᵢₑₜᵢₜ (r²>0.6, P≤0.001), but lower related to δ¹³Cᵣₛ of soluble carbohydrates and starch. During nighttime, δ¹³Cᵣₛ of malate explained 36% of the variation in δ¹³Cᵣ, but δ¹³Cᵣₛ of

![Fig. 3. Daily cycles of the carbon isotopic composition of different leaf respiratory carbon sources (δ¹³Cᵣₛ) 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 Tₜₙ (low temperature; closed symbols), Tₜₕ (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).](https://academic.oup.com/jxb/article-abstract/66/19/5769/695866)
of fructose and glucose, as well as δ13Cleaf showed similarly high explanatory power.

**Influence of environmental drivers and carbon sources on δ13C**

Furthermore, a stepwise (backward) multiple linear regression analysis was performed to identify environmental drivers and carbon sources influencing δ13C (Table 5). Daytime/nighttime showed the strongest positive effect on δ13C (β=0.73, P<0.001), while δ13CRS of malate was the carbon source that affected δ13C most (β=0.4, P<0.001). By comparison, the influence of δ13CRS of starch and soil moisture conditions on δ13C values was minor.

### Discussion

This study clearly demonstrates that different temperature and soil moisture conditions influence δ13C of leaf dark-respired CO₂ (δ13C), δ13C of different putative leaf respiratory carbon sources (δ13CRS), 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 δ13C 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 δ13Cleaf, with up to 2.2‰ more negative δ13Cleaf values under Thigh conditions compared to those under Tlow conditions (Fig. 2B). This is in agreement with a study showing more negative δ13C 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 δ13Cleaf value with increasing temperature (Fig. 2A), but due to our long-term treatment we found also more negative δ13CRS values (Fig. 3). On the other hand, dry conditions in both of the temperature treatments caused less negative δ13Cleaf, δ13C, and δ13CRS values compared to those under wet conditions, which is consistent with previous studies under controlled conditions (Duranceau et al., 1999; Ghashghaie et al., 2001).

The isotopic results under the different environmental conditions can be directly linked to the leaf gas exchange observed during the 32h sampling period (day 15 of the treatment period). Increasing temperature caused lower Aᵣ values under both soil moisture conditions (Fig. 1A; Table 1), indicating that plants under Thigh 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, Aᵣ 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 $T_{\text{high}}$ 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 $T_{\text{high}}$ conditions. However, this was only observed in plants under $T_{\text{high}}$ and well-watered conditions, when SWC was high. Subsequently, lower carbon fixation and higher CO$_2$ diffusion into the stomatal cavities under $T_{\text{high}}$, in comparison to $T_{\text{low}}$, caused an increase of $C_t$ (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 $C_t$ and less negative $\delta^{13}C$ values (Table 6).

Plants under $T_{\text{high}}$ and dry conditions showed the lowest 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 ($T_{\text{high}}$ and dry conditions) were rather similar to those under lowest stress level ($T_{\text{low}}$ 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 $T_{\text{high}}$ and dry conditions, resulting in a moderate reduction of $C_t$, while at the same time $g_s$ strongly reduces CO$_2$ diffusion into the stomatal cavities, causing an increase of $C_t$. Consequently, this led to intermediate $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values under $T_{\text{high}}$ 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 $T_{\text{low}}$ 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 $T_{\text{high}}$ or dry conditions were used for maintenance of biochemical processes rather than for carbon storage. Additionally, this indicates that plants under $T_{\text{high}}$ 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).

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**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 $T_{\text{low}}$ and wet conditions.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>$A_n$</th>
<th>$g_s$</th>
<th>$C_t$</th>
<th>$\delta^{13}C_R$</th>
<th>$\delta^{13}C_{RS}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{low}}$ dry</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\uparrow)</td>
<td>(\uparrow)</td>
</tr>
<tr>
<td>$T_{\text{high}}$ wet</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\uparrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
</tr>
<tr>
<td>$T_{\text{high}}$ dry</td>
<td>(\uparrow)</td>
<td>(\downarrow)</td>
<td>(\downarrow)</td>
<td>(\rightarrow)</td>
<td>(\rightarrow)</td>
</tr>
</tbody>
</table>
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 (\( r^2=0.26, P \leq 0.001 \)). The strength of this relationship increased for \( \delta^{13}C_R \) and \( \delta^{13}C_{RS} \) of malate when considering daytime (\( r^2=0.69, P \leq 0.001 \)) and nighttime (\( r^2=0.36, P \leq 0.001 \)) 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 (Urbanczyk-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 \( ^{13}C \)-enriched 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
Malate as a key carbon source of leaf dark-respired CO$_2$ in potato and soil moisture conditions on a daily basis in a C$_3$ plant. Overall, we found that $\delta^{13}C_R$ values generally reflect changes in $\delta^{13}C_{RS}$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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 CO$_2$ 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. Finally, 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 $\delta^{13}C$ of malate and $\delta^{13}C$ of leaf dark-respired CO$_2$.

Supplementary data

Supplementary data are available at JXB online.

**Supplementary Figure S1.** Linear regressions between $\delta^{13}C$ of leaf dark-respired CO$_2$ ($\delta^{13}C_R$) and $\delta^{13}C$ of different putative respiratory carbon sources ($\delta^{13}C_{RS}$) across all environmental conditions for daytime, for nighttime, and for the total daily cycle.

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Prosopis velutina. 

Recent carbon: from beech leaves to soil CO₂ efflux. Neuf Phytole 184, 950–961.


