RESEARCH PAPER

Changes and their possible causes in $\delta^{13}C$ of dark-respired CO$_2$ and its putative bulk and soluble sources during maize ontogeny

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Received 8 January 2016; Accepted 8 February 2016

Editor: Howard Griffiths, University of Cambridge

Abstract

The issues of whether, where, and to what extent carbon isotopic fractionations occur during respiration affect interpretations of plant functions that are important to many disciplines across the natural sciences. Studies of carbon isotopic fractionation during dark respiration in C$_3$ plants have repeatedly shown respired CO$_2$ to be $^{13}C$ enriched relative to its bulk leaf sources and $^{13}C$ depleted relative to its bulk root sources. Furthermore, two studies showed respired CO$_2$ to become progressively $^{13}C$ enriched during leaf ontogeny and $^{13}C$ depleted during root ontogeny in C$_3$ legumes. As such data on C$_4$ plants are scarce and contradictory, we investigated apparent respiratory fractionations of carbon and their possible causes in different organs of maize plants during early ontogeny. As in the C$_3$ plants, leaf-respired CO$_2$ was $^{13}C$ enriched whereas root-respired CO$_2$ was $^{13}C$ depleted relative to their putative sources. In contrast to the findings for C$_3$ plants, however, not only root- but also leaf-respired CO$_2$ became more $^{13}C$ depleted during ontogeny. Leaf-respired CO$_2$ was highly $^{13}C$ enriched just after light–dark transition but the enrichment rapidly decreased over time in darkness. We conclude that (i) although carbon isotopic fractionations in C$_4$ maize and leguminous C$_3$ crop roots are similar, increasing phosphoenolpyruvate-carboxylase activity during maize ontogeny could have produced the contrast between the progressive $^{13}C$ depletion of maize leaf-respired CO$_2$ and $^{13}C$ enrichment of C$_3$ leaf-respired CO$_2$ over time, and (ii) in both maize and C$_3$ leaves, highly $^{13}C$ enriched leaf-respired CO$_2$ at light-to-dark transition and its rapid decrease during darkness, together with the observed decrease in leaf malate content, may be the result of a transient effect of light-enhanced dark respiration.

Key words: Carbon isotope discrimination, leaves versus roots, light-enhanced dark respiration, maize, ontogeny, respired CO$_2$.

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Introduction

Carbon isotopic fractionations associated with plant respiration have received increasing attention because of their possible influences on efforts to elucidate factors associated with ecosystem carbon balances via carbon isotopic analyses. A significant example in which respiration may play a role is the 13C depletion of autotrophic relative to heterotrophic tissue in C3 plants (Badeck et al., 2009; Terwilliger and Huang, 1996; reviewed in Cernusak et al., 2009; Ghashghaie and Badeck, 2014; Werner and Gessler 2011). Differences in carbon isotopic composition (δ13C values) are found not only between organs such as autotrophic leaves and heterotrophic roots but also during the ontogeny of organs as leaves pass from heterotrophic to autotrophic stages. Understanding the reasons for these differences would improve the interpretation of factors affecting plant carbon use from δ13C values of organic matter.

Respiration has been implicated in the differences in δ13C values between mature leaves and roots of herbaceous C3 plants because leaf-respired CO2 was found to be 13C enriched whereas root-respired CO2 was 13C depleted relative to their respective organs and soluble substrates therein (Klumpp et al., 2005; reviewed in Cernusak et al., 2009; Ghashghaie and Badeck, 2014, but see references in the latter review for woody plants). Furthermore, δ13C values of both respired CO2 and its putative sources are related to variables such as species and environmental conditions, and assessing the contributions of respiration to the aforementioned apparent fractionations would clarify these relationships.

Respiration may also be implicated in differing trends in δ13C values between plant organs during their ontogeny. The only studies we know of that compare changes in δ13C values of respired CO2 between organs during early ontogeny are for the C3 legumes Phaseolus vulgaris L. (bean) (Bathellier et al., 2008) and Arachis hypogaea L. (peanut) (Ghashghaie et al., 2015). In both studies, the leaf-respired CO2 became progressively and greatly 13C enriched (approximately 7‰ increase in δ13C) for both species while respired CO2 from roots became 13C depleted (approximately 3‰o and 4‰ decrease in bean and peanut δ13C, respectively) with respect to their bulk tissues. Leaves and roots both became 13C depleted as their development progressed. These results are consistent with several mechanisms under the broad categories of relative fluxes through biochemical pathways and substrate use for respiration and growth (Ghashghaie et al., 2001, 2003; Werner et al., 2011) that could cause relationships of respiration to changes in δ13C values of different plant fractions during ontogeny.

Several biochemical activities have been shown to influence δ13C values of respired CO2 in C3 plants. First, the heterogeneous 13C distribution within hexose molecules (Gilbert et al., 2009; Hobbie and Werner, 2004; Rossmann et al., 1991), as well as the relative activities of metabolic pathways releasing 13C-enriched CO2 (pyruvate dehydrogenase reaction) or 13C-depleted CO2 (Krebs cycle and pentose phosphate pathway), has been shown to contribute to the δ13C values of respired CO2 in C3 leaves (Tcherkez et al., 2003) and roots (Bathellier et al., 2009). However, fragmentation fractionation based on the heterogeneous 13C distribution was not the sole cause of apparent respiratory fractionation. Second, the decarboxylation of malate driven by light-enhanced dark respiration (LEDR) at the light–dark transition results in highly 13C-enriched CO2 respired by C3 leaves in comparison with respiration after longer periods of being in darkness (Barbour et al., 2007; Gessler et al., 2009; Werner et al., 2009).

A third variable that could contribute to differences in δ13C values of respired CO2, its putative sources among organs, and during ontogeny is the proportion of CO2 that is fixed by phosphoenolpyruvate carboxylase (PEPc) relative to all CO2 fixed by a plant organ. PEPc discriminates slightly against 13C, leading to 13C enrichment of the carbon it fixes and 13C depletion of residual carbon that is a component of the total respired CO2. In contrast, during the light reactions of photosynthesis in chlorophyll-containing organs, Rubisco more readily fixes 13C than 12C. PEPc fixes CO2 regardless of whether or not a plant organ is capable of photosynthesizing. The carbon fixed can be a source of anaplerotic replenishment of metabolites.

Two lines of results support the hypothesis that the proportion of CO2 fixed by PEPc contributes to variations in the δ13C values of respired CO2 and associated organs. First, the δ13C values of C3 leaf tissue have been demonstrated to increase with increasing fixed CO2 in the dark (Nalborczyk, 1978) and increasing ratios of PEPc/total carboxylase activities (Le Roux-Swarthout et al., 2000). Second, mass balance simulations of anaplerotic CO2 fixation by PEPc resulted in decreasing δ13C values of root-respired CO2 with increasing ratios of PEPc activity/gross respiration rate (Badeck et al., 2005).

The potential impact of PEPc on the δ13C values of respired CO2 has not yet been investigated. The high PEPc activities and near elimination of fractionation by Rubisco during photosynthesis (Farquhar, 1983) make C4 plants offer potentially decisive opportunities for understanding carbon isotopic fractionations during respiration and their consequences for other carbon pools. Nonetheless, efforts to examine respiratory carbon isotope fractionation in C4 plants have been few and have yielded conflicting results (Schnyder and Lattanzi, 2005; Sun et al., 2012; Werth and Kuzyakov, 2005; Zhu and Cheng, 2011; reviewed in Ghashghaie and Badeck, 2014).

The main objectives of the present work were to examine (i) whether the differences in carbon isotope compositions between respired CO2 and tissue of leaves and roots, and the isotopic pattern during early ontogeny observed in two C3 species (bean and peanut), can also be observed in a C4 plant; (ii) possible influences of LEDR on this fractionation; and (iii) whether PEPc activity in leaves covaries with respiratory fractionation.

This is the first time that respiratory carbon isotope fractionation has been reported in different organs of a C4 plant during early ontogeny. We compare our results with those already reported for two C3 species, bean (Bathellier et al., 2008) and peanut (Ghashghaie et al., 2015).

Materials and methods

Plant materials, growth conditions, and experimental designs

In the present study, the term ‘non-germinated’ refers to caryopses (for maize) or seeds (for the C3 dicots) before the start of imbibition.

Maize (Zea mays L.) caryopses were sown in small pots filled with vermiculite as culture support to facilitate extraction of the
roots. Plants were grown in a greenhouse with a 16 h photoperiod maintained at a minimum photosynthetic photon flux density of 500 μmol m\(^{-2}\) s\(^{-1}\) by supplementary lighting from high-pressure sodium lamps. Temperature and leaf-to-air vapour pressure deficit were maintained at approximately 25.5/18.5 °C and 1.4/1.2 kPa day/night, respectively. The pots were watered twice a day with a commercial nutrient solution (Hydrokani C2, Hydro, France). The δ\(^{13}\)C value of CO\(_2\) in the greenhouse was \(-9.5 \pm 0.2\,\%\text{SE} (n=3)\).

Four experiments were conducted on maize plants grown under the above conditions. The first experiment examined carbon isotopic fractionations between respiring CO\(_2\) and putative bulk and labile sources in different organs (leaves, roots, stems including coleoptiles, and caryopses) from the beginning of germination until plants had at least four fully expanded leaves. The leaves were ranked by numbers reflecting their order of appearance. The BBCH scale (Meier, 2001) was used to number the developmental stages of plants (Table 1). Autotrophy acquisition was not measured, but the emergence of the first green leaf was used as an indirect criterion of the onset of autotrophy.

The second and third experiments were performed on mature plants during 2h periods of darkness. δ\(^{13}\)C values of leaf-respired CO\(_2\) were measured in the second experiment in order to evaluate possible effects of LEDR. For the third experiment, changes in leaf malate content during darkness were measured to determine whether they were associated with the δ\(^{13}\)C results of the second experiment. In the fourth experiment, PEPc activities of different leaf ranks at different developmental stages were measured in order to determine whether these activities corresponded to changes in δ\(^{13}\)C values in the first experiment.

δ\(^{13}\)C analyses of respired CO\(_2\)

In the first experiment, respired CO\(_2\) was obtained from organs sampled at each developmental stage and placed in flasks completely darkened with aluminium foil covers (25 or 50 ml depending on the sample size). The flasks were first flushed with N\(_2\) (150 ml min\(^{-1}\)) for long enough (approximately 5 min) to remove all the CO\(_2\), then closed with septum caps (GRACE, aluminium cap 20 mm with O-ring PTFE/Butyl Pharmalix, Epernon, France). The needle of a micro-gas chromatograph (Micro-GC, Agilent 490, Les Ulis, France) was automatically introduced into a flask through the septum to sample the air inside, then injected into the Micro-GC (injection volume 2-4 μl every 3 min) to measure the CO\(_2\) concentration in the air sample. After 40-80 min of darkness (depending on the sample size and respiration rate), when the CO\(_2\) concentration was high enough (minimum of 500 μl l\(^{-1}\)) in each flask, the air was sampled manually through the septum for carbon isotopic analysis using gas-tight syringes (SGE Analytical Science, Trajan brand, Australia) (0.5 to 1 ml depending on the CO\(_2\) concentration) and introduced into a gas chromatograph (GC; HP 5890, Les Ulis, France) coupled to a stable isotope ratio mass spectrometer (IRMS) (Optima Isochrom, Fisons Instruments, Manchester, UK). The syringes were flushed with helium five times and then with sample air inside a given flask three times before obtaining each sample to be injected into the GC. The air inside the same flask was sampled and measured 2-3 times to ensure the values were stable.

δ\(^{13}\)C values of respirated CO\(_2\) (δ\(^{13}\)C\(_R\)) were analysed from entire first small leaves (ranks 1 to 3, with the leaf cut at the ligule but only the middle part of the older, bigger leaves (ranks >3). The leaf sheath was sampled together with the stem and the coleoptile. In order to examine the possibility of fractionation due to LEDR, leaf-respired CO\(_2\) was analysed in the second experiment during a 2h period of darkness in different leaf ranks of plants at stages 13 and 14 (stages are described in Table 1). In this case, the flasks were flushed with N\(_2\) initially and after each measurement for about 5 min, that is, each measured value for a given sample at a given time corresponds to the δ\(^{13}\)C\(_R\) at that time. The empty flasks (without plant samples inside) were leak-checked after being filled with N\(_2\) for approximately 2 h and their CO\(_2\) concentrations remained zero.

Table 1. Phenological stages of maize plants according to the BBCH scale (Meier, 2001).

<table>
<thead>
<tr>
<th>BBCH stage</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Caryopsis before the start of imbibition</td>
</tr>
<tr>
<td>3</td>
<td>Caryopsis imbibition completed</td>
</tr>
<tr>
<td>5</td>
<td>Radicle emerged from caryopsis</td>
</tr>
<tr>
<td>6</td>
<td>Radicle elongated, root hairs and/or side roots visible</td>
</tr>
<tr>
<td>7</td>
<td>Coleoptile emerged from caryopsis</td>
</tr>
<tr>
<td>9</td>
<td>Emergence: coleoptile penetrates soil surface (cracking stage)</td>
</tr>
<tr>
<td>10</td>
<td>Leaf rank 1 emerged through coleoptile</td>
</tr>
<tr>
<td>11</td>
<td>Leaf rank 1 unfolded, and leaf rank 2 still growing</td>
</tr>
<tr>
<td>12</td>
<td>Leaf rank 2 unfolded, and leaf rank 3 still growing</td>
</tr>
<tr>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>n</td>
<td>Leaf rank n unfolded, and leaf rank n+1 still growing</td>
</tr>
</tbody>
</table>

δ\(^{13}\)C analyses of tissues and water-soluble fractions

Immediately after the δ\(^{13}\)C\(_R\) analyses of the first experiment, plant organs were stored at –80 °C. They were then lyophilized, weighed, and ground to fine powder with a ball mill (Retsch MM200, Batch-Block Scientific, Ilkirch, France). Aliquots of approximately 800 μg powder from each sample were transferred into tip cups (Courtage Analyse Service, Mont St Aignan, France) for carbon isotopic analyses of bulk organic matter (δ\(^{13}\)C\(_{OM}\)).

To measure the carbon isotopic composition of putative respiratory substrates, the water-soluble fraction of OM (WSOM) was extracted according to the method of Tcherkez et al. (2003). In brief, 50 mg of powder was suspended with 1 ml of cold distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany) and kept in an ice slurry for 60 min except when removed to agitate in a vortex mixer every 10 min. The mixtures were then centrifuged at 14000 g for 15 min at 5 °C; the supernatant containing WSOM was retrieved, heated at 100 °C for 5 min, and then kept on ice for 30 min to precipitate the heat-denatured proteins, which were removed after centrifugation at 14000 g for 15 min at 5 °C. Aliquots of 200 μl of protein-free WSOM were poured into tin cups and oven-dried at 50 °C for isotope analysis.

Carbon isotopic compositions of samples of plant organic matter (OM and WSOM) were analysed using an elemental analyser (Vario Pyrocube, Elementar, Villeurbanne, France) connected to the IRMS (Isoprime100, Elementar, Villeurbanne, France). Two laboratory standards (glutamic acid and acetanilide, both calibrated using glutamic acid from IAEA, USGS40) were also measured to check for any drift of the IRMS (six USGS40 and six acetanilide per day, and one internal glutamic acid every six samples). Carbon isotope composition was then calculated as the per mil (i.e. ‰) deviation of the carbon isotope ratio (\(^{13}\)C/\(^{12}\)C, called R) from the international standard (Vienna Pee Dee Belemnite): \( \Delta^{13}C = 10^{3}(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}} \).

Extraction and assay of malate

For the third experiment, the method described by Crecelius et al. (2003) was adapted to measure the malate content of the leaves. Leaf ranks 3 and 5 were sampled from each of three control plants that had been subjected to greenhouse light for 8 h and from each of the remaining plants, which had been in darkness for intervals ranging from 4 to 100 min. About 500 mg of fresh leaf material (middle section of each leaf after removal of the main ribs) of each rank were excised, immediately plunged into liquid nitrogen, and then stored at -80 °C. They were then ground into fine powder under liquid nitrogen, diluted with 1.2 ml of 10% perchloric acid and 60 mg Polyclar AT, agitated with a vortex mixer, shaken for 45 min on ice, and centrifuged for 6 min at 15 500 g at 5 °C. The supernatants were drawn up into Eppendorf tubes, and the remaining pellets were...
again diluted with 400 μl of 2% perchloric acid and centrifuged. The supernatants from both centrifugation steps were then mixed. The pH of the whole supernatant was adjusted to 7 by adding 5 M KOH and 1 M triethanolamine. Next, 100 mg of Polycar A tube added and, after homogenization and recording the volume for every tube, the mixtures were shaken for 45 min at room temperature. The mixtures were then centrifuged for 10 min at 20 800 g at 5 °C and their supernatants were collected for malate determination. To do this, 20 μl of each of these final extracts was added to an assay mixture containing 10 μl of distilled water, 850 μl of buffer pH 10 (0.1 M glycyglycine and 50 mM glutamate), 20 μl of 33 mM NAD, and 50 μl of glutamate-oxaloacetate transaminase (50 U). Malate content was determined by complete malate oxidation with malate dehydrogenase (MDH) and blocking the back reaction by transforming oxaloacetate to aspartate with glutamate-oxaloacetate transaminase. The formation of NADH terminated after 20 min incubation at room temperature. The difference between both absorption measurements corresponds to the malate content in the sample, which was calculated with the Lambert–Beer equation as described in Crecelius et al. (2003).

**Extraction and assay of PEPc specific activity**

For the fourth experiment, the method described by Crecelius et al. (2003) was adapted for extraction and assay of PEPc specific activity. Different leaf ranks of six plants per developmental stage were sampled separately after an 8 h photoperiod in the greenhouse. Up to 500 mg samples of leaf mass were obtained from two plants per sample for each leaf rank (n=3) at each developmental stage studied. Leaf samples (middle section of each leaf after removal of the main ribs) were plunged into liquid nitrogen and then stored at –12.16 °C. Frozen leaves were ground to a fine powder under liquid nitrogen and the powder was mixed with 6–80 °C. Frozen leaves were ground to a fine powder under liquid nitrogen and then stored at –12.16 ± 0.02‰, also became progressively more 13C depleted compared with the non-germinated caryopsis OM (–12.68 ± 0.17‰ versus –12.16 ± 0.02‰). CO2 resired by maize leaves was 13C enriched (mean δ13C value for leaf ranks 1 and 2 was –10.21 ± 0.31‰), and the non-germinated caryopsis OM (–12.68 ± 0.17‰ versus –12.16 ± 0.02‰), also became progressively more 13C depleted with plant development. Root δ13C decreased by 0.68‰ per unit stage between stage 10 and 16 (R2=0.8, p<0.001), reaching –13.3 ± 0.25‰ at stage 12 (i.e. when leaf 2 was mature and following), and –16.5‰ at stage 15, remaining low and stable thereafter (Fig. 1C).

Initial δ13COM values of maize leaves and roots were the same (–13.10 ± 0.31‰ and –13.05 ± 0.38‰, respectively, measured at stage 11 and 9) and were slightly more 13C depleted (by 0.94‰ and 0.88‰; t-test, p=0.040 and 0.014, respectively) than the non-germinated caryopse. Thereafter, leaf δ13COM values decreased in parallel with leaf δ13C rich to a final mean of –14.5‰ at stage 14 (Fig. 1A). In contrast to leaves, root δ13COM values increased at stage 12, when two leaves had unfolded, and then subsequently decreased (model from stage 12 on: root δ13COM=0.19 stage–12.6; R2=0.34, p=0.012) (Fig. 1C). Stem/cotyledon δ13C rich was –12.47 ± 0.32‰ at coleoptile emergence (stage 7), then progressively increased until stage 12 (R2=0.50, p<0.001), reaching a peak of –10.45 ± 0.15‰ at stage 12, and decreased thereafter (R2=0.84, p<0.001), like leaf δ13C rich, reaching very low values of –15.5‰ at stage 18 (Fig. 1B). By contrast, stem/cotyledon δ13COM progressively decreased during ontogeny (from –11.9‰ at stage 8 to –14.6‰ at stage 18). With the exception of stage 18, the CO2 resired by stem/cotyledon was 13C enriched compared with respective OM (1.2 ± 0.15‰).

CO2 resired by maize caryopses was 2.36 ± 0.12‰ and 2.73 ± 0.13‰ 13C depleted compared with their OM and WSOM at all stages of ontogeny except imbibition, when all fractions had the same δ13C values (stage 3) (Fig. 1D). As for roots, caryopses δ13C rich values decreased after stage 12. Unlike roots, however, caryopses δ13C rich values increased again after stage 13. The δ13COM values of caryopses were stable throughout ontogeny, remaining the same as the δ13COM value of the non-germinated caryopses (equal means, p>0.65, t-test), except for a 13C depletion in OM at the very early stages (before root emergence), and at stage 13 when the caryopsis was almost empty.

δ13CWSOM values were, on average, 0.38‰, 0.73‰, 0.97‰, and 1.05‰ higher than δ13COM values of caryopses, roots, leaves, and stems/cotyledones, respectively (Fig. 1A–D). Consequently, the trends with ontogeny of δ13COM and δ13CWSOM were similar for a given organ. δ13COM and δ13CWSOM did not differ significantly from one another in

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**Results**

As expected for a C4 plant, the non-germinated caryopses of maize that were analysed in this study were 13C-enriched (δ13COM=–12.16 ± 0.02‰, n=12) compared with the non-germinated seeds of C3 species (–26.83 ± 0.22‰ and –29.05 ± 0.24‰, n=10, for bean and peanut, respectively) determined with previous studies (Bathellier et al., 2008; Ghashghaie et al., 2015).
roots from stage 13 on (Fig. 1C) and in caryopses from stage 7 on (Fig. 1D).

Changes in $\Delta_R$ of organs during ontogeny

Apparent respiratory carbon isotope fractionation ($\Delta_R$) for each organ was approximated as the difference between $\delta^{13}C$ of either WSOM or bulk OM and $\delta^{13}C$ of respired CO$_2$ (i.e. $\Delta_R$(WSOM)=[$\delta^{13}C$-WSOM]−$\delta^{13}C$$_R$ or $\Delta_R$(OM)=[$\delta^{13}C$-OM]−$\delta^{13}C$$_R$). Negative $\Delta_R$ (for $\Delta_R$(WSOM) or $\Delta_R$(OM)) values indicate $^{13}C$ enrichment and positive $\Delta_R$ values indicate $^{13}C$ depletion in respired CO$_2$ compared with its putative organic source of carbon. In the following section, $\Delta_R$ represents both $\Delta_R$(WSOM) and $\Delta_R$(OM).

There was a negative or negligible $\Delta_R$(OM) of leaves at all stages of ontogeny of maize (Fig. 2A). Previous studies have similarly shown $\Delta_R$ to be negative or negligible in leaves of C$_3$ legumes (Batheilier et al., 2008; Ghashghaie et al., 2015) (Fig. 2B). However, two fundamental differences were apparent between $\Delta_R$ trends in leaves of C$_4$ maize and C$_3$ legumes after onset of autotrophy (maize leaves were too small to be analysed at heterotrophic stages). First, at the earliest comparable autotrophic stage of growth (stage 11), leaves of maize exhibited their largest negative departures from 0 $\Delta_R$(OM) (−3.08±0.40‰) and leaves of legumes their smallest (from 0 at heterotrophic stages to approximately −2‰ at stage 11) (Fig. 2A, B). Second, maize leaf $\Delta_R$ increased to zero (i.e. decreased in absolute magnitude) with advanced ontogeny (Fig. 2A), whereas leaf $\Delta_R$ decreased progressively in C$_3$ legumes to values between approximately −8‰ (bean) and −11‰ (peanut) (Fig. 2B), that is, opposite trends in leaf $\Delta_R$ were observed over the course of ontogeny of C$_4$ maize and C$_3$ legumes.

The $\Delta_R$(OM) value of maize roots was zero at their emergence and then progressively increased (Fig. 2A), as was also the case in C$_3$ roots (Fig. 2B). At later stages, the $\Delta_R$(OM) values thus became positive (i.e. indicating progressive $^{13}C$ depletion in root-respired CO$_2$ compared with root OM) and slightly higher in maize roots compared with bean ($\Delta_R$(OM) was approximately +3‰ in maize roots and +2‰ in bean roots at stage 15; Fig. 2A, B, respectively). The $\Delta_R$(OM) values oscillated between 0 and −1‰ in peanut roots, never becoming positive (Fig. 2B).

Because our putative substrate for respiration (WSOM) was never $^{13}C$ depleted compared with the bulk OM of the respective organs, $\Delta_R$(WSOM) values were slightly lower in leaves and higher in roots of maize than were their respective $\Delta_R$(OM) values. Furthermore, $\Delta_R$(WSOM) values were positive in roots and negative or zero in leaves of C$_4$ maize (Fig. 2C), as well as in C$_3$ plants at comparable stages of growth (Fig. 2D). As for leaf $\Delta_R$(OM) (Fig. 2A), leaf $\Delta_R$(WSOM) values became less negative during ontogeny of maize plants until $\Delta_R$ was zero (Fig. 2C). In contrast to C$_3$ plants, for which opposite $\Delta_R$ trends were reported in leaves versus roots (Fig. 2B, D), $\Delta_R$ values in maize plants showed parallel changes for these organs (Fig. 2A, C).

$\Delta_R$(OM) in stems/coleoptiles was zero at coleoptile emergence, then decreased, reaching leaf $\Delta_R$(OM) values at stage 12; the $\Delta_R$(OM) values for both organs increased (i.e. absolute fractionation decreased) similarly thereafter (Fig. 2A). The stem/coleoptile $\Delta_R$(OM) values also changed in parallel with those of roots after stage 12 (Fig. 2A, grey squares). $\Delta_R$(WSOM) values were slightly higher than $\Delta_R$(OM) in stems/coleoptiles, changing similarly during ontogeny (Fig. 2C).

$\Delta_R$(OM) and $\Delta_R$(WSOM) of maize caryopses (Fig. 3) showed positive values (near zero at imbibition stage, and oscillating around +3‰ thereafter, i.e. indicating $^{13}C$-depleted respired CO$_2$); these values were almost superimposed on those of bean cotyledons. In contrast, peanut cotyledons had markedly negative $\Delta_R$(OM) values (i.e. reflecting $^{13}C$-enriched
respired CO$_2$, ranging from approximately $-2\%$ at imbibition to $-5\%$ until stage 12, and then becoming less negative to approach zero at later stages (Fig. 3A). $\Delta_{\text{WSOM}}$ values were slightly higher than $\Delta_{\text{OM}}$ in caryopses and changed similarly during ontogeny (Fig. 3B).

**LEDR effect on leaf $\delta^{13}C_R$**

LEDR was not directly measured (leaf respiration rate was not monitored during the period of darkness) but our results suggest it had an effect on leaf $\delta^{13}C_R$ values. Leaf $\delta^{13}C_R$ values were markedly high (ranging from approximately $-4$ to $-8\%$) throughout the first 20 min of darkness, decreasing exponentially towards a minimum of approximately $-14\%$ (Fig. 4). A model assuming a negative exponential change in the fraction of CO$_2$ released from two differently depleted sources ($r^2=0.91$) indicated a highly significant ($p<0.001$) time constant for the change between the sources, corresponding to a half-life of 22 min. $\delta^{13}C_R$ values varied between leaf ranks. CO$_2$ respired by leaf rank 2 was approximately $1.4\%$ more $^{13}C$ enriched on average than CO$_2$ respired by leaf rank 1 over time ($p<0.001$ for final values). One measurement on leaf rank 4 (the youngest fully developed leaf at the top of the plant at stage 14) was even more $^{13}C$ enriched ($-3.8\%$) at 25 min into the period of darkness (Fig. 4, grey square). Another measurement on samples taken from all leaf ranks of a plant measured together also had a high $\delta^{13}C_R$ value ($-6\%$) within the first 15 min of the period of darkness, but was in the range of the $\delta^{13}C_R$ values of leaf rank 1 by 50 min (Fig. 4, grey circles).

Changes in leaf malate content during darkness

The malate contents of leaves of maize plants that had been exposed to an 8 h light period significantly decreased when placed in the dark (when regression was done on all of the data together, $R^2=0.41$, $p<0.001$; Fig. 5). Although the malate contents of younger (rank 5) and older, more shaded leaves (rank 3) decreased at the same rate, malate contents were significantly higher in the younger than the older leaves at any given duration of darkness (ANCOVA, parallel line test, $p<0.05$).

Changes in PEPc activity in different leaf ranks during ontogeny

PEPc specific activity of the leaves, measured after an 8 h light period and calculated on the basis of leaf protein content, significantly increased during plant ontogeny regardless of leaf rank ($R^2=0.29$, $p=0.012$; Fig. 6A). At stage 15, when the largest range of leaf ranks was present, there was no correlation between PEPc specific activity and leaf rank ($R^2=0.13$, $p=0.16$).
Discussion

As expected from the well-studied differences in carbon isotopic fractionations attributable to photosynthetic pathways, δ^{13}C values of bulk organs were at least 15‰ less negative in C_4 maize than in C_3 bean or peanut (Bender, 1968). In addition, several trends in apparent fractionations associated with respiration were the same for C_4 maize as for C_3 legumes. When compared with its putative soluble substrata (WSOM) after the onset of leaf autotrophy, CO_2 was ^{13}C enriched when respired from leaves and ^{13}C depleted when respired from roots (i.e. leaf Δ_R was negative and root Δ_R positive) in both C_4 maize and the C_3 legumes (Fig. 2C, D). In addition, root Δ_R(WSOM) was initially very low (0≤root Δ_R(WSOM)<1‰) but increased (became more positive) during the ontogeny of all three species.
Nevertheless, some differences in trends in $\Delta_R$ and its $\delta^{13}C$ components occurred between species and during ontogeny, and these may be relevant to the elucidation of carbon balances from carbon isotope analyses of plants. During ontogeny, $\delta^{13}C$ values of CO$_2$ progressively decreased when respired from C$_4$ leaves (Fig. 1A) but increased when respired from C$_3$ leaves (Bathellier et al., 2008; Ghashghaie et al., 2015). Differences in carbon isotopic trends were sometimes greater between the two C$_3$ legume species than between photosynthetic pathway types, however. Root $\Delta_R$ values were always $\leq$ 0 in C$_3$ peanut but was always $\geq$ 0 in C$_4$ maize and C$_3$ bean. (Fig. 2A, B). Surprisingly, the CO$_2$ respired by maize caryopses and bean cotyledons was $^{13}$C depleted compared with its respective OM and WSCOM carbon sources (i.e. positive $\Delta_R$) but the respired CO$_2$ of peanut cotyledons was $^{13}$C enriched relative to its carbon sources (i.e. negative $\Delta_R$) (Fig. 3A, B). $\Delta_R(OM)$ and $\Delta_R(WSCOM)$ values of maize caryopses were similar to those of bean cotyledons and $\geq 0$ throughout ontogeny, whereas peanut cotyledons had lower $\Delta_R$ values, which were in all but one case $\leq 0$. We now evaluate possible explanations for the observed differences.

**Metabolic origin of $^{13}$C-enriched leaf-respired CO$_2$ and $^{13}$C-depleted root-respired CO$_2$**

The findings reported in this study add to the emerging consistent patterns of $^{13}$C-enriched leaf- and $^{13}$C-depleted root-respired CO$_2$ in C$_3$ and C$_4$ herbaceous plants that most probably should be linked to common metabolic causes. Unravelling the causes of this pattern and its variability will help to understand differences in $\delta^{13}C$ of CO$_2$ respired by different organs during periods of darkness (e.g. at night) and the contribution of fractionation during dark respiration to overall $\delta^{13}C$ of OM, and will pave the way for further progress in developing a non-invasive diagnostic system for monitoring metabolic activities.

As has been found in C$_3$ plants, the present results demonstrate that the respired CO$_2$ from leaves of C$_4$ maize are enriched in $^{13}$C relative to the OM and WSCOM of the leaves from which it evolved. One possible source of the $^{13}$C enrichment of leaf-respired CO$_2$ relative to OM in C$_3$ plants that may also operate in C$_4$ plants is the $^{13}$C-rich carbon atoms coming from positions C-3 and C-4 of glucose (Rossmann et al., 1997) during its decarboxylation by pyruvate dehydrogenase (Tcherkez et al., 2003). Heterogeneous $^{13}$C distributions with C-3 and C-4 positions richer in $^{13}$C have also been found in carbohydrates of C$_4$ plants, although the contrast between positions C-3 and C-4 versus C-1, C-2, C-5, and C-6 has been smaller than in C$_3$ plants (Gilbert et al., 2009; Hobbie and Werner, 2004; Rossmann et al., 1991). Nevertheless, it is unlikely that the particularly marked $^{13}$C enrichment in leaf-respired CO$_2$ in both plant types at the light–dark transition period can be explained by decarboxylation of pyruvate dehydrogenase alone. Studies of C$_4$ leaves have clearly linked this enrichment to a residual effect of LEDR, and our results suggest a similar role of LEDR in C$_4$ plants (discussed below) (Barbour et al., 2007; Gessler et al., 2009; Werner et al., 2009).

As has been documented for C$_3$ plants, we found CO$_2$ respired from roots to be $^{13}$C depleted relative to its respective bulk OM and WSCOM. A metabolic origin of this fractionation has been suggested for C$_3$ bean and C$_4$ maize roots based on studies using positionally labelled glucose and pyruvate (Batlle-Auba et al., 2009) found $^{13}$C-depleted CO$_2$ to be released from roots as a result of high activity of the pentose phosphate pathway (PPP). Dieuaide-Noubhani et al. (1995, 1997) found the PPP activity of maize roots to be virtually the same as that for C$_3$ bean roots reported by Bathellier et al. (2009). These results suggest a similar root respiratory metabolism in C$_3$ and C$_4$ plants, with high PPP activity releasing $^{13}$C-depleted CO$_2$ via decarboxylation of the C-1 position of glucose. Indeed, the C-1 position of glucose is $^{13}$C depleted relative to the whole glucose molecule (Rossmann et al., 1991). In addition, 6-phosphogluconate dehydrogenase fractionates against $^{13}$C by approximately 9.6% during decarboxylation of C-1 of glucose in PPP (Rendina et al., 1984).
contributing to the observed $^{13}$C depletion in root-respired CO$_2$ in both plant types. The anaplerotic pathway was also reported to be high in maize root tips (Alonso et al., 2007; Dieuaida-Noubhani et al., 1995; Edwards et al., 1998), but its potential effect on root $^{13}$C$_R$ has not yet been measured. Root respiration (including the anaplerotic pathway) should be investigated in more C$_4$ and C$_3$ species under different conditions, together with labelling experiments, for better understanding of its metabolic origin.

In conclusion, our results combined with those of earlier studies suggest that any fractionation processes related to the heterogeneous distribution of carbon isotopes in photosynthates, discrimination by decarboxylation enzymes, PPP activity, and occurrence of decarboxylation of $^{13}$C-rich carbon during LEDR are the same in C$_4$ maize as in C$_3$ plants.

**Opposite trends in leaf $^{13}$C$_R$ between C$_4$ maize and C$_3$ legumes during ontogeny**

The opposite trends in leaf $^{13}$C$_R$ in C$_4$ maize and in C$_3$ legumes (i.e. progressive $^{13}$C enrichment in C$_4$ versus progressive $^{13}$C depletion in maize leaf-respired CO$_2$; see Bathellier et al., 2008 and Fig. 1A, respectively) during ontogeny could be associated with the acquisition of autotrophy in both plant types (which is considered to start when the first leaf becomes green). Improving the understanding of this phenomenon will contribute to characterization of the effects of time of carboxylation in terms of plant development on differences in $^{13}$C between organs.

In the case of maize leaves, changes in C$_4$ photosynthetic and/or respiratory metabolism (i.e. changes in enzyme activities and substrate availabilities) during leaf unfolding could affect leaf $^{13}$C$_R$. For instance, PEPc activity, along with an increase in the C$_4$ carbon concentration mechanism, could increase during maize leaf development, leading to the observed $^{13}$C-depletion pattern in leaf $^{13}$C$_R$. Our results show an increase in PEPc specific activity in maize leaves during ontogeny (Fig. 6A) and are in agreement with those reported by Deléens and Brulfert (1983), who also observed an increase in PEPc activity in different leaf ranks of maize seedlings during autotrophy acquisition. Perchorowicz and Gbbs (1980) showed that PEPc activity was lower in the basal section (non-mature tissues) than in the centre and top sections (mature parts) of maize leaves. More recently, Pick et al. (2011) also observed a progressive increase in C$_4$ enzyme activities from the base to the top section of maize leaves. The increase in PEPc activity in maize leaves with plant development could also increase the fixation of $^{13}$C-rich HCO$_3^-$ in darkness, leaving behind $^{13}$C-depleted CO$_2$, which is then released through the respiratory flux (as in heterotrophic tissues).

In contrast, in C$_3$ plants, CO$_2$ fixation is exclusively by Rubisco activity in heterotrophic leaves but decreases relative to CO$_2$ fixation as a consequence of Rubisco activity with leaf autotrophy acquisition (i.e. the ratio PEPc/total carboxylase activity decreases) (Le Roux-Swarthout et al., 2000).

Therefore, the opposite changes in PEPc and Rubisco activities during leaf development could be a potential cause of the observed opposite changes in $^{13}$C$_R$ of C$_4$ versus C$_3$ leaves. However, to test this hypothesis, the levels of PEPc activity, the nature of CO$_2$ fixed by PEPc (respiratory or ambient), and the fate of the fixed carbon need to be studied. Labelling experiments should be conducted to unravel the impact of PEPc activity on leaf $^{13}$C$_R$ and its changes when leaves switch from heterotrophic to autotrophic stages.

In the case of roots, similar decreasing trends in root $^{13}$C$_R$ of C$_4$ maize (Fig. 1C) and C$_3$ legumes (Bathellier et al., 2008; Ghashghaie et al., 2015) during ontogeny could be related to similar increasing activities of the PPP and probably the anaplerotic pathway in heterotrophic root tissues during plant development, both pathways being involved in $^{13}$C depletion of root-respired CO$_2$. The activity of these pathways should be determined during plant ontogeny in both plant types to confirm this hypothesis.

In summary, we hypothesize that the opposite trends in the isotopic signature of leaf-respired CO$_2$ between maize and C$_3$ legume species during early ontogeny are related to opposite trends in PEPc content and activity, while root-respired CO$_2$ follows a similar trajectory.

**Decreasing $^{13}$C of leaf and root tissues during ontogeny**

The decreasing trend in leaf and root $^{13}$C$_{OM}$ following the onset of autotrophy observed in maize in this study has already been reported for many C$_4$ species (for leaves: Kennedy et al., 2004; Lamade et al., 2009; Terwilliger 1997, 2001a, b; for leaves and roots: Bathellier et al., 2008; Ghashghaie et al., 2015). The present results in maize suggest that it may be a general pattern regardless of photosynthetic pathway type. Changes in substrata for growth as a consequence of the onset of autotrophy could have caused the decline in $^{13}$C$_{OM}$. The source of carbon for the initial emergence of both organs would have been the caryopsis reserves and might explain why $^{13}$C$_{WSOM}$ values were virtually identical to those of non-germinated caryopses. The use of $^{13}$C-depleted photoassimilates once leaves became autotrophic could have progressively changed the $^{13}$C values of leaf WSOM and then of leaf OM. Photoassimilates transported from leaves to roots could then have changed root $^{13}$C (both OM and WSOM) but with some delay compared with leaves, as reported by Deléens et al. (1984) in maize seedlings.

This explanation is consistent with findings by Deléens-Provent and Schwebel-Dugué (1987), who followed $^{13}$C$_{OM}$ in maize leaves during seedling development in a culture room exposed to air containing $^{13}$C-depleted CO$_2$ of approximately –24‰. Their results suggested that the first three leaves of maize plants originated from caryopsis reserves and that they had a more C$_4$-type photosynthetic activity than subsequent leaves, which clearly had C$_3$ metabolism. The sugars from the caryopsis reserves were indeed richer in $^{13}$C than those arising from photosynthesis that thus labelled the autotrophic leaves. $^{13}$C-enriched sugars may also have led to $^{13}$C$_{WSOM}$ in emerging coleoptiles and roots that was higher than $^{13}$C$_{OM}$ of non-germinated caryopses. The later values remained high and stable until later stages (Fig. 1B, C), while leaf $^{13}$C$_{WSOM}$...
was high at leaf appearance but decreased rapidly afterwards because of a higher contribution of photoassimilates (Fig. 1A).

The observed parallel decreases in leaf $\delta^{13}C_R$, $\delta^{13}C_{WSOM}$, and $\delta^{13}C_{OM}$ (Fig. 1A) also suggested a change in predominant carbon sources from caryopsis reserves to photoassimilates for respiration. After having labelled the recent photoassimilates of maize seedlings, Deléens and Brullért (1983) demonstrated that the recent autotrophically fixed carbon was quickly used for respiration and to a lesser extent for the building up of leaf matter, while the heterotrophic carbon primarily located in the caryopsis was no longer involved in the respiratory pathway after establishment of leaf autotrophy. The time interval for this change could vary with growth conditions, caryopsis reserve pool sizes, growth rate, and maintenance needs.

In conclusion, C$_4$ maize exhibited similar trends in $\delta^{13}C_{OM}$ during early ontogeny to C$_3$ plants, which supports explanations related to the use of seed reserves first and new photo-synthates subsequently for growth and respiration.

Different $\Delta_R$ in leaves versus roots but parallel changes during ontogeny

Although $\Delta_R$ in leaves and roots had opposite signs, they changed in parallel during ontogeny (Fig. 3). This is in contrast to the results reported in C$_3$ plants, for which the leaf and root $\Delta_R$ values changed in opposite directions, with leaf $\Delta_R$ becoming more negative (the opposite of the changes seen in maize leaves) but root $\Delta_R$ more positive (similar to maize roots) during ontogeny (Batheiller et al., 2008; Ghashghaie et al., 2015). Differences in respiratory metabolism between heterotrophic and autotrophic tissues on the one hand, and between C$_3$ and C$_4$ leaves on the other, could underlie the observed differences in $\Delta_R$ between organs and between the two plant types.

As the decarboxylation of glycine during photorespiration in C$_3$ leaves fractionates against $^{13}C$ (released CO$_2$ is $^{13}C$ depleted compared with C-1 of glycine; Lanigan et al., 2008; Tcherkez 2006), it contributes to a trend for $^{13}C$ enrichment of the overall net assimilated carbon. This effect will be absent in C$_4$ plants. Yet per se it is not relevant for fractionation during dark respiration.

However, an effect of photorespiration on $^{13}C$-enrichment in the C-2 position of glucose can also be expected, according to the model of Tcherkez et al. (2004). This effect should increase with an increasing rate of photorespiration, a trend confirmed by Gilbert et al. (2011), who showed that the difference between $\delta^{13}C$ of the C-1 and C-6 positions and the C-2 and C-5 positions of glucose increased with increasing $\delta^{13}C$ of samples (under drought conditions, thus with high photorespiration). However, this effect is expected to be small according to the model and probably cannot explain all of the observed difference between C-2/C-5 and C-1/C-6 and the inverse pattern found in sugars of C$_4$ plants. Thus, Gilbert et al. (2011) proposed that additional fractionation steps may contribute, which need to be unravelled.

The isotopic signature in the C-2/C-5 positions was 2‰ less negative and 1‰ more negative than that in the C-1/C-6 positions in C$_3$ and C$_4$ plants, respectively (Gilbert et al., 2011). This can lead to opposite effects in apparent respiratory fractionation if substrates are diverted from the Krebs cycle after the first decarboxylation step. If Krebs cycle activity would favour the preferential release of C-2/C-5, fragmentation fractionation would contribute a trend for enriched respiratory CO$_2$ in C$_3$ plants and for its depletion in C$_4$ plants. If the carbon originating from C-2/C-5 was decarboxylated to a higher degree than carbon originating from C-1/C-6, this would lead to a positive shift in apparent respiratory fractionation in C$_3$ plants and a negative shift in C$_4$ plants.

It should be kept in mind that these potential effects of fragmentation fractionation will appear in concert with further fractionation steps within respiratory metabolism that need to be studied with labelling and fluxomics approaches (Tcherkez et al., 2012).

$\Delta_R$ in maize caryopses and bean cotyledons were similar to each other but not to values in peanut cotyledons

The similarity in $\Delta_R$ of maize caryopses and bean cotyledons and their dissimilarity to $\Delta_R$ of peanut cotyledons points to metabolic differences in reserve mobilization independent of the photosynthetic pathways.

Although the maize caryopsis $\delta^{13}C_R$ was high (approximately $-12.5\%$) when the imbibition stage was completed (stage 3), and close to the non-germinated caryopsis $\delta^{13}C_{OM}$ (stage 4), it rapidly decreased and remained low after stage 4 (oscillating between $-14$ and $-16\%$) (Fig. 1D). This pattern was the opposite of that reported by Deléens (1990), who observed remarkably negative caryopsis $\delta^{13}C_R$ values ($-15.8\%$) in different maize genotypes during the first hours of imbibition, followed by an increase ($^{13}C$ enrichment), reaching higher values (approximately $-12\%$) one day after imbibition and remaining high and stable thereafter. Deléens (1990) suggested that the maize caryopsis lipids were used as the respiratory substrate at the beginning of imbibition, releasing $^{13}C$-depleted CO$_2$ ($^{13}C$ of maize caryopsis lipids was approximately $-16.9\%$), but then the caryopsis starch reserve was rapidly remobilized and used as the respiratory substrate, thus evolving $^{13}C$-enriched respired CO$_2$ (very close to $\delta^{13}C_{OM}$ of non-germinated caryopses). In the present study, the caryopsis $\delta^{13}C_R$ was not measured during the first hours of imbibition but after a few days (at stage 3), when it is probable that the carbohydrate reserves were being used as a respiratory substrate (caryopsis $\delta^{13}C_R$ was thus $^{13}C$ enriched, similar to the observation of Deléens, 1990). However, the subsequent $^{13}C$ depletion (after stage 4) observed in the present study could indicate $^{13}C$-depleted substrate use or isotope fractionation against $^{13}C$ during caryopsis respiratory metabolism at later stages. Smith (1971) observed only a slight increase in $\delta^{13}C_R$ in 13-day-old maize seedlings compared with soaked caryopses ($-13.5\%$ and $-12.9\%$, respectively).

$\Delta_R$ was similar in maize caryopses and bean cotyledons (with almost superimposed $\Delta_R$ values during ontogeny, despite the
differences in $\delta^{13}\text{C}_{\text{OM}}$ and $\delta^{13}\text{C}_R$ in C$_4$ versus C$_3$ plants; Fig. 3). $\Delta_R$ values were thus positive (i.e. $^{13}\text{C}$-depleted CO$_2$ respired by maize caryopses and by bean cotyledons compared with their respective OM), as in heterotrophic roots. However, this was not the case for peanut cotyledons, in which $\Delta_R$ was negative (i.e. $^{13}\text{C}$-enriched CO$_2$ respired by peanut cotyledons compared with the OM), similar to the $\Delta_R$ of autotrophic tissues (leaves). Therefore, the difference in respiratory metabolism between autotrophic and heterotrophic tissues cannot solely be the origin of the observed isotopic differences. Instead, similar biochemical composition (maize caryopses contain $>$70% starch and bean cotyledons contain approximately 60% carbohydrates) and similar respiratory metabolism (i.e. similar use of caryopsis or cotyledon reserves for respiration, export, and growth) could have led to the similar $\Delta_R$ values in maize caryopsis and bean cotyledons.

$^{13}\text{C}$-enriched sugars (arising from starch degradation) were probably exported for shoot and root development, while $^{12}\text{C}$-depleted proteins were probably used for caryopsis/cotyledon respiration in both maize (Deléens, 1990) and bean (Bathellier et al., 2008). Contrasting biochemical composition of peanut non-germinated seeds (containing approximately 48% lipids and only around 17% carbohydrates) and different use of the reserves could have affected the $\delta^{13}\text{C}_R$, probably by influencing the relative metabolic activities (Ghashghaie et al., 2015). The relative activities of the anaplerotic pathway, the PPP, and gluconeogenesis could change the $\delta^{13}\text{C}_R$ of heterotrophic tissues. Degradation of lipids (gluconeogenesis) with potential fragmentation fractionation in fatty acids (with carboxyl positions $^{13}\text{C}$ enriched relative to methyl positions) and the potential isotope fractionation in the glyoxylate cycle could be the origin of the $^{13}\text{C}$-enriched respired CO$_2$ in peanut cotyledons (Ghashghaie et al., 2015). By contrast, the anaplerotic CO$_2$ fixation in heterotrophic tissues could lead to $^{13}$C depletion in net respired CO$_2$ (because PEPC discriminates against $^{12}$C). Both the PPP and the anaplerotic pathway, which have been shown to be high in bean and maize roots (see above), could also be high in bean cotyledons and maize caryopses, leading to the observed $^{13}$C-depleted respired CO$_2$ in these organs. In order to better understand the factors that determine root and caryopsis $\delta^{13}\text{C}_R$, PEPC and PPP activities and $\delta^{13}\text{C}_R$ should be conjointly analysed.

Changes in respiration rate, biomass, and the biochemical composition of each organ should also be followed, to determine the related energy demand for respiration and growth during ontogeny of plants with different non-germinated reserve types.

In summary, $\Delta_R$ of seeds/caryopses during germination appears to be related to the mix of reserve metabolites and the biochemical pathways used for their mobilization across C$_3$ and C$_4$ plants. The hypothesized effects of differences in relative fluxes through the respective pathways, as well as their potential changes during germination, need to be investigated further.

LEDR effect on $\delta^{13}\text{C}_R$ also occurs in C$_4$ maize leaves

Initially, we did not test for an LEDR effect in maize leaves. Only after the highly $^{13}$C-enriched leaf-respired CO$_2$ was observed in some cases did we follow its changes during a period of darkness. Although the respiration rate was not determined, the strong $^{13}$C enrichment in leaf-respired CO$_2$ (up to approximately –4‰) with remarkable $^{13}$C depletion during the period of darkness (i.e. more than 10‰ $^{13}$C depletion in less than 2 h of darkness; Fig. 4) suggested an LEDR effect similar to that reported for C$_3$ leaves (Barbour et al., 2007; Gessler et al., 2009; Werner et al., 2009). Characterizing the effect of LEDR on leaf $\delta^{13}\text{C}_R$ is a prerequisite to interpreting short-term changes in respiration after the onset of darkness.

The observed shift in $\delta^{13}\text{C}_R$ of maize leaves during the period of darkness was as large as in C$_3$ leaves. However, the $^{13}$C depletion in C$_3$ leaf $\delta^{13}\text{C}_R$ was more rapid (a sharp decrease during the first 20 min of darkness). This could be related to higher PEPC activity and malate levels in C$_4$ leaves. In addition, the decrease in leaf malate content during the period of darkness was in agreement with an LEDR effect in maize leaves too. The LEDR effect was significantly higher in younger leaves (rank 5, compared with older leaves at rank 3), probably because the younger leaves (on the upper part of the plant) were exposed to higher levels of light than the older leaves (at the bottom of the plant), which were shaded by the upper leaves. Respiration rate and isotopic measurements should be investigated in more leaf ranks under variable light levels to better analyse the impact of light on LEDR and its effect on leaf $\delta^{13}\text{C}_R$.

In summary, maize leaves exhibit short-term changes in $\delta^{13}\text{C}_R$ that are consistent with decarboxylation of $^{13}$C-rich carbon during LEDR.

**Conclusion**

This is the first time that the $\delta^{13}\text{C}_R$ of different plant organs during early ontogeny has been investigated in a C$_4$ plant species. The results showed that, as in C$_3$ plants, $\Delta_R$ values in leaves had opposite signs to $\Delta_R$ of roots in C$_4$ maize seedlings. However, in contrast to C$_3$ plants, where leaf $\Delta_R$ decreased after the onset of autotrophy while root $\Delta_R$ changed little, leaf and root $\Delta_R$ changed in parallel after C$_4$ maize leaves became autotrophic. These results suggest that C$_3$ and C$_4$ roots had similar respiratory metabolism but that this was not the case for leaves. Indeed, $\Delta_R$ was almost zero in C$_3$ leaves at their emergence and became more negative (i.e. $^{13}$C enrichment in respired CO$_2$ relative to source carbon) during ontogeny. In contrast, $\Delta_R$ was negative in maize leaves at their emergence and changed during ontogeny in the opposite direction to that seen in C$_3$ leaves, approaching zero at later stages. This opposite trend in $\Delta_R$ during leaf ageing in C$_4$ versus C$_3$ plants could be due to a progressive change from C$_3$ to C$_4$ pathway functioning in maize leaves (demonstrated by the increase in PEPC specific activity during ontogeny) as opposed to increasing C$_3$ photosynthetic activity in C$_3$ leaves during leaf development.

This is also the first time that an LEDR effect has been shown in C$_4$ leaves, with changes in leaf $\delta^{13}\text{C}_R$ of at least 10‰ over time in darkness together with a decrease in leaf malate content.
Such studies analysing the variation in $\delta^{13}C_R$ of different plant types and different organs at different developmental stages could be relevant for the discrete identification of different carbon sources and sinks through studies of ecosystem $\delta^{13}C_R$ (Brüggemann et al., 2011). The often-applied assumption of identical $\delta^{13}C_{OM}$ and $\delta^{13}C_R$ needs to be scrutinized before using it in the examination of multi-source systems.

**Acknowledgements**

The authors are grateful the Laboratoire d’Ecologie, Systématique et Evolution (ESE, CNRS-UMR 8079) at the University of Paris-Sud (Orsay) for financial support for isotope analyses, the platform ‘Metabolisme-Métabolome’ at IPS2 (Orsay) for technical assistance for carbon isotope analyses on plant material, and the Laboratoire de Biogéochimie et Ecologie des Milieux Continentaux (BIOEMCO, INRA, Thiverval-Grignon) for providing plant culture facilities as well as for technical assistance for isotope analyses on respired CO$_2$. We also acknowledge the two anonymous referees as well as the editor for their valuable comments and suggestions.

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