# Metabolic origin of the $\delta^{13}C$ of respired $CO_2$ in roots of *Phaseolus vulgaris*

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## Summary

- Root respiration is a major contributor to soil $CO_2$ efflux, and thus an important component of ecosystem respiration. But its metabolic origin, in relation to the carbon isotope composition ($\delta^{13}C$), remains poorly understood.
- Here, $^{13}C$ analysis was conducted on $CO_2$ and metabolites under typical conditions or under continuous darkness in French bean (*Phaseolus vulgaris*) roots. $^{13}C$ contents were measured either under natural abundance or following pulse-chase labeling with $^{13}C$-enriched glucose or pyruvate, using isotope ratio mass spectrometer (IRMS) and nuclear magnetic resonance (NMR) techniques.
- In contrast to leaves, no relationship was found between the respiratory quotient and the $\delta^{13}C$ of respired $CO_2$, which stayed constant at a low value (*c*. –27.5‰) under continuous darkness. With labeling experiments, it is shown that such a pattern is explained by the $^{13}C$-depleting effect of the pentose phosphate pathway; and the involvement of the Krebs cycle fueled by either the glycolytic input or the lipid/protein recycling. The anaplerotic phospho*eno*lpyruvate carboxylase (PEPc) activity sustained glutamic acid (Glu) synthesis, with no net effect on respired $CO_2$.
- These results indicate that the root $\delta^{13}C$ signal does not depend on the availability of root respiratory substrates and it is thus plausible that, unless the $^{13}C$ photosynthetic fractionation varies at the leaf level, the root $\delta^{13}C$ signal hardly changes under a range of natural environmental conditions.

## Introduction

Photosynthesis in $C_3$ plants discriminates against the heavy carbon isotope ($^{13}C$) so that plant organic matter is, on average, $^{13}C$-depleted by *c*. 20‰ compared with atmospheric $CO_2$. A carbon isotope discrimination occurs during both $CO_2$ diffusion (through the stomata and the mesophyll), and $CO_2$ carboxylation (by Rubisco) in the chloroplasts (Brugnoli & Farquhar, 2000). However, there are now several lines of evidence that isotopic fractionations during post-photosynthetic processes (such as respiration, root exudation, etc.) occur. Such fractionations may substantially modify the isotope composition of the carbon that was initially fixed by photosynthesis (for a review, see Badeck *et al.*, 2005).

In this respect, root respiration, which is responsible for a substantial loss for the carbon budget of plants (Amthor, 2000), is an important component of the plant isotopic mass balance (Jackson *et al.*, 1996; Bathellier *et al.*, 2008). At the ecosystem scale, isotopic approaches that aim at deconvoluting the different respiring compartments of ecosystems need more accurate understanding of respiratory processes, among which roots are key actors. In fact, soil respiration contributes up to 50–70% to ecosystem respiration (Lavigne *et al.*, 1997) and root respiration is a major component of such a $CO_2$

efflux (Kuzyakov & Larionova, 2006). Therefore, a better knowledge of the isotope composition (denoted as $\delta^{13}C$) of root-respired $CO_2$ may be helpful to disentangle variations of ecosystem respired $\delta^{13}C$.

In leaves, $CO_2$ respired in darkness is substantially $^{13}C$-enriched (up to 6‰) as compared with sucrose (Duranceau *et al.*, 1999, 2001; Ghashghaie *et al.*, 2001, 2003; Tcherkez *et al.*, 2003) or total soluble sugars (Ocheltree & Marshall, 2004; Xu *et al.*, 2004; Hymus *et al.*, 2005; Klumpp *et al.*, 2005; Barbour *et al.*, 2007). By contrast, root-respired $CO_2$ is $^{13}C$-depleted as compared with total soluble sugars or sucrose by *c.* 2‰ (Badeck *et al.*, 2005; Klumpp *et al.*, 2005; Bathellier *et al.*, 2008). Although the $\delta^{13}C$ value of respired $CO_2$ varies by up to 4–5‰ (independently on respiratory substrates such as sucrose), the $^{13}C$ depletion has an important recognized influence on the $^{13}C$ enrichment of root organic matter (Terwilliger & Huang, 1996; Badeck *et al.*, 2005; Bathellier *et al.*, 2008). On the other hand, much uncertainty remains about the causes of such a $^{13}C$ depletion and the source of its variations. In leaves, the $^{13}C$ enrichment of evolved $CO_2$ stems from both the nonstatistical distribution of carbon isotopes in glucose and the nature of the respiratory substrate (Tcherkez *et al.*, 2003). There is currently no such metabolic framework for roots. Data on the primary metabolism and metabolic fluxes associated with decarboxylations in root cells are also poorly known. Steady-state labeling experiments with $^{13}C$- and $^{14}C$-labeled glucose coupled to nuclear magnetic resonance (NMR) analysis on detached maize root tips indicated that the pentose phosphate pathway (PPP) accounted for 24% of the evolved $CO_2$ in these cells, while both the Krebs cycle and pyruvate dehydrogenase reaction (PDH) together contributed to 53%, and glucuronic acid decarboxylation to 17% (Dieuaide-Noubhani *et al.*, 1995). A substantial anaplerotic flux via phospho*enol*pyruvate carboxylase (PEPc) was also inferred by these authors from the labeling distribution in malate and glutamate. This observation was confirmed by Edwards *et al.* (1998) on the same material and more recently on *Catharanthus roseus* hairy roots (Sriram *et al.*, 2007). Interestingly, similar patterns were reported for PPP flux in heterotrophic cell cultures of tomato (Rontein *et al.*, 2002) and *Arabidopsis* (Kruger *et al.*, 2007), using the same method.

In heterotrophic plant cells, the PPP is thought to be an important source of reducing power (as it produces NADPH) for various biosynthetic processes, such as nitrate assimilation (for a review see Neuhaus & Emes, 2000), sulphur reduction (Droux, 2004) or lipid synthesis. Thus presumably, the isotopic signature of respired $CO_2$ in roots is related to the specific metabolic requirements, such as nitrate reduction and assimilation, which in turn depend on environmental conditions. However, the metabolic fluxes associated with these decarboxylating pathways (PPP, Krebs cycle, and PDH) under typical environmental conditions are currently not established and the relationship between the $^{12}C/^{13}C$ isotope effects of such decarboxylations and the isotope composition of evolved $CO_2$ is also unclear.

As an aid to clarifying the metabolic origin of the $\delta^{13}C$ value of root-respired $CO_2$, we investigated the respiratory metabolism of intact washed root systems (still attached to shoots) in French bean (*Phaseolus vulgaris*). $^{13}C$ measurements in $CO_2$ and metabolites under natural abundance (with isotope ratio mass spectrometry, IRMS) or following [$^{13}C$]Glc and [$^{13}C$]Pyr pulse-chase labeling (with IRMS and NMR) were carried out after a 10 h photoperiod (typical conditions) or under continuous darkness.

## Materials and Methods

### Plant material and growth conditions

Experiments were conducted on French bean (*Phaseolus vulgaris* L. cv. contender, Vilmorin, la Verpillière, France). Seeds were sown directly in vermiculite, in individual 1 l pots. Plants were grown in a glasshouse with a 16 h photoperiod and a minimum photosynthetic photon flux density maintained at approx. 500 μmol $m^{-2}$ $s^{-1}$ by supplementary lighting from high-pressure sodium light. Temperature and leaf-to-air vapor pressure deficit were maintained at approx. 25.5 : 18.5°C and 1.4 : 1.2 kPa day : night, respectively. Watering was achieved with a commercial nutrient solution (Hydrokani C2, Yara, Nanterre, France) twice during the photoperiod. The carbon isotope composition of $CO_2$ in the glasshouse was $-9.85 \pm 0.3$‰ ($n = 3$). Experiments were carried out on plants having a mature first trifoliate leaf, which correspond, in our growing conditions, to 3- to 4-wk-old plants. In such plants, root biomass was typically *c.* 7.5 g FW per plant.

### $^{13}C$-enriched molecules

The positional $^{13}C$-labeled molecules (99% $^{13}C$ in the considered position) were purchased from Eurisotop (Saint Aubin, France). Pyruvate was dissolved in distilled water and pH was corrected to 6.8 with NaOH. To obtain nonfully labeled solutions for the gas exchange experiments, the labeled compounds were mixed with industrial glucose ($\delta^{13}C = -9$‰) or pyruvate ($\delta^{13}C = -21$‰) from Sigma. The resulting overall composition of the glucose and pyruvate solutions was checked to be 200 and 600‰, respectively. The final concentration was 0.01 mol $l^{-1}$ in all cases.

### Carbon isotope analysis and gas exchange measurements

Dark-respired $CO_2$ was analyzed online with a closed system coupled to an elemental analyzer NA-1500 (Carlo-Erba, Milan, Italy) through a 15 ml loop (Tcherkez *et al.*, 2003). The closed system included a 400 ml respiration chamber, a magnesium perchlorate column (Fluka, USA), a membrane pump (KNF, type NMP015B, Germany), an infrared gas

analyzer (BINOS Leybold-Heraeus, Germany), the 15 ml sample loop and two soda lime columns. Initially, air flowed through the soda lime columns to remove $CO_2$ until its concentration reached equilibrium (15–20 min). The soda lime trap was then shunted, and $CO_2$ was accumulated in the system up to *c.* 400 µl $l^{-1}$. The air inside the loop was connected to a helium flux and flushed toward the elemental analyzer for gas chromatography (GC). The connection valve between the elemental analyzer and the isotope ratio mass spectrometer (VG Optima, Micromass, Villeurbanne, France) was opened when the $CO_2$ peak emerged from the elemental analyzer. Carbon isotope analysis of carbohydrates and bulk organic matter was conducted using the same elemental analyzer and IRMS. Carbon isotope compositions were calculated as the deviation of the carbon isotope ratio ($^{13}C/^{12}C$, called $R$) from international standards (Pee Dee Belemnite): $\delta^{13}C = 10^3\ [(R_{sample} - R_{standard})/R_{standard}]$. A laboratory standard (glutamic acid) was measured every 12 samples in order to correct for the drift of the IRMS. The precision of the $\delta^{13}C$ measurements was ±0.2‰.

Experiments in continuous darkness started after a 10 h photoperiod. Before measurements, roots were carefully cleaned with tap water in order to remove vermiculite, rinsed with distilled water, and blotted dry. Gas exchange measurements were thus performed on clean intact whole root systems. The respiratory quotient (RQ) was calculated as the ratio of carbon production $v(CO_2)$ to oxygen consumption $v(O_2)$: $RQ = v(CO_2)/v(O_2)$. The $CO_2$ production in darkness was measured with the infrared gas analyzer described earlier in this section. Subsequently detached, $O_2$ consumption of the same root system was measured with a gas-phase oxygen electrode (Hansatech, King's Lynn, UK) using a specially designed chamber (ø, 3.57 cm; *h*, 6 cm) wherein the whole root could fit. The time elapsed between $CO_2$ and $O_2$ measurements was *c.* 25 min. The 25 min time period before $O_2$ measurement had no effect on the $O_2$ consumption rate, so that, starting with $O_2$ measurement, prior $CO_2$ production measurement did not influence the RQ value at all (data not shown).

## $^{13}C$-labeling procedures

For the experiments, plants were taken either from the glasshouse after a 10 h light period (control treatment) or after four consecutive days of darkness (starvation treatment), and roots were carefully cleaned with tap water to remove vermiculite. Each data point required nine plants, that is, three $^{13}C$-labeled roots, three untreated roots and three water control roots. In other words, the following experimental procedure was performed three times (three replicates, $n = 3$).

Two cleaned roots (still attached to stem and leaves) were immersed for 2 h in either distilled water or a 10 mM solution of the studied labeled compound ($\delta^{13}C = 200‰$ for glucose, $\delta^{13}C = 600‰$ for pyruvate). Solutions were continuously bubbled with air in order to avoid hypoxia. Roots of the third plant were blotted dry, and directly placed into the respiration cuvette, still attached to their aerial part, and isotopic composition of dark-respired $CO_2$ was analyzed online (as described previously) to obtain a $\delta^{13}C$ value before the immersion treatment. After the 2 h immersion treatment, root-respired $CO_2$ (respiration and $\delta^{13}C$) of the plant dipped into water was analyzed in the same way. After labeling, a portion of *c.* 2.5 g was taken from the root system and immediately frozen with liquid nitrogen for metabolite analysis. The remaining roots were placed in the respiration chamber, and both respiration and isotopic composition of respired $CO_2$ were measured online every 30–40 min during 120–160 min in order to follow the kinetic of the label in respired $CO_2$. Decreasing exponential curves were fitted to the obtained values (data not shown), which allowed us to extrapolate the $\delta^{13}C$ and flux values of respired $CO_2$ at $t = 0$, that is, the time at which roots were removed from the solution of labeled substrate. After gas exchange measurements, roots were systematically frozen with liquid nitrogen, freeze-dried, and powdered for metabolite analysis (content and/or $\delta^{13}C$). This analysis provided values for root metabolites at the end of the chase period that could be compared with those obtained in the 2.5 g portion taken from the root just after labeling.

## Metabolite extraction

Soluble sugars were extracted and purified by HPLC following the procedure of Tcherkez *et al.* (2003). Starch was extracted with cold precipitation in methanol, following the procedure of Duranceau *et al.* (1999). The lipid extraction and purification procedure was as described in Tcherkez *et al.* (2003).

## Calculation of respiratory contributions

All the following calculations used the $^{13}C$ percentage denoted as λ, which can be deduced from the isotopic composition ($\delta^{13}C$) and the isotope ratio ($R = {}^{13}C/{}^{12}C$) with the relationship:

$$\lambda = \frac{{}^{13}C}{{}^{12}C + {}^{13}C} = \frac{{}^{13}C/{}^{12}C}{1 + {}^{13}C/{}^{12}C} = \frac{R}{1+R} \qquad \text{Eqn 1}$$

As $\delta = \frac{R - R_{st}}{R_{st}}$, where $R_{st}$ is the isotope ratio of the Pee Dee Bee international standard ($R_{st} = 0.0112372$), we have:

$$\lambda = 1/\left[1 + \frac{1}{R_{st}(\delta + 1)}\right] \qquad \text{Eqn 2}$$

Feeding roots with positionally enriched compounds enabled us to calculate contributions of the different decarboxylating steps to respiration, using the method previously described by Tcherkez *et al.* (2005). It is assumed that the labeled substrate fed to roots leads to additional decarboxylations through the

pyruvate dehydrogenase (PDH, EC 1.2.1.51) and the Krebs cycle, which are denoted as $r_{PDH}$ and $r_k$, respectively, and can be estimated from the label found in respired $CO_2$. For example, when roots are supplied with [1-$^{13}$C]Pyr, which is decarboxylated by the PDH, the isotope mass-balance equation is as follows:

$$\boldsymbol{R}_1\lambda_1^{obs} = (\boldsymbol{R}_1 - r_{PDH} - r_k)\lambda_1^{control} + r_{PDH}\lambda_1 + r_k\lambda_c \qquad \text{Eqn 3}$$

Similarly, when [3-$^{13}$C]Pyr, which is decarboxylated by the Krebs cycle, is supplied to roots, the isotope mass balance equation is then:

$$\boldsymbol{R}_2\lambda_2^{obs} = (\boldsymbol{R}_2 - r_{PDH} - r_k)\lambda_2^{control} + r_{PDH}\lambda_c + r_k\frac{\lambda_c + \lambda_3}{2} \qquad \text{Eqn 4}$$

where $\boldsymbol{R}_1$ and $\boldsymbol{R}_2$ are total respiration fluxes of roots fed with [1-$^{13}$C]Pyr and [3-$^{13}$C]Pyr, respectively. $\lambda_1^{obs}$ and $\lambda_2^{obs}$ are the $^{13}$C percentages in respired $CO_2$ of roots fed with [1-$^{13}$C]Pyr and [3-$^{13}$C]Pyr, respectively. $\lambda_1^{control}$ and $\lambda_2^{control}$ are the $^{13}$C percentage in respired $CO_2$ of control roots. $\lambda_1$ and $\lambda_3$ are the $^{13}$C percentage in the C-1 and C-3 positions of labeled Pyr, respectively, and $\lambda_c$ is the $^{13}$C percentage of the other unlabeled positions.

Rearranging the equations gives:

$$\boldsymbol{R}_1(\lambda_1^{obs} - \lambda_1^{control}) = r_{PDH}(\lambda_1 - \lambda_1^{control}) + r_k(\lambda_c - \lambda_1^{control}) \qquad \text{Eqn 5}$$

$$\boldsymbol{R}_2(\lambda_2^{obs} - \lambda_2^{control}) = r_{PDH}(\lambda_c - \lambda_2^{control}) + r_k\left(\frac{\lambda_c + \lambda_3}{2} - \lambda_2^{control}\right) \qquad \text{Eqn 6}$$

In order to avoid bias resulting from any slight variation in the respiration rate ($\boldsymbol{R}$) between experiments or labeling conditions, equations were normalized by dividing by $\boldsymbol{R}$, and the ratios $r_{PDH}/\boldsymbol{R}$ and $r_k/\boldsymbol{R}$ were denoted as $\rho_{PDH}$ and $\rho_k$, respectively. $\rho$ values represented the proportion of respired $CO_2$ coming from the step considered (PDH or Krebs cycle). They were assumed to be constant in the feeding experiments that used a given substrate, whatever its positional $^{13}$C labeling. Thus we have:

$$(\lambda_1^{obs} - \lambda_1^{control}) = \rho_{PDH}(\lambda_1 - \lambda_1^{control}) + \rho_k(\lambda_c - \lambda_1^{control}) \qquad \text{Eqn 7}$$

$$(\lambda_2^{obs} - \lambda_2^{control}) = \rho_{PDH}(\lambda_c - \lambda_2^{control}) + \rho_k\left(\frac{\lambda_c + \lambda_3}{2} - \lambda_2^{control}\right) \qquad \text{Eqn 8}$$

The system can be solved by a substitution procedure, leading to the following solutions:

$$\rho_k = \frac{(\lambda_2^{obs} - \lambda_2^{control})(\lambda_1 - \lambda_1^{control}) - (\lambda_1^{obs} - \lambda_1^{control})(\lambda_c - \lambda_2^{control})}{\left(\frac{\lambda_c + \lambda_3}{2} - \lambda_2^{control}\right)(\lambda_1 - \lambda_1^{control}) - (\lambda_c - \lambda_2^{control})(\lambda_c - \lambda_1^{control})} \qquad \text{Eqn 9}$$

$$\rho_{PDH} = \frac{(\lambda_2^{obs} - \lambda_1^{control}) - \rho_k(\lambda_c - \lambda_1^{control})}{\lambda_1 - \lambda_1^{control}} \qquad \text{Eqn 10}$$

The method was similar for feeding experiments with $^{13}$C glucose. Note that, in this case, the C-3 position is decarboxylated by the PDH and C-1 (or C-2) position by the Krebs cycle.

## Nuclear magnetic resonance

The labeling procedure was similar to that described previously for gas exchange experiments, but with 10 mM solutions of labeled substrates exhibiting a positional enrichment of 99%. Two replicates were done for each experimental condition. Perchloric acid (PCA) extracts were prepared from 5 g of frozen root material as described by Aubert *et al.* (1996) for phloem cells. Spectra were obtained on a spectrometer (AMX 400) equipped with a 10 mm multinuclear probe tuned at 100.6 MHz for $^{13}$C-NMR. The deuterium resonance of $^2H_2O$ (100 µl added per ml of extract) was used as a lock signal.

Conditions for $^{13}$C-NMR acquisition utilized 19 µs pulses (90°) at 6 s intervals and a sweep width of 20 kHz. Broadband decoupling at 2.5 W during acquisition and 0.5 W during the delay was applied using the Waltz sequence; the signal was digitized using 32 000 data points zero-filled to 64 000 and processed with a 0.2 Hz line broadening. $^{13}$C-NMR spectra are referenced to hexamethyldisiloxane at 2.7 ppm. $Mn^{2+}$ ions were chelated by the addition of 1 mmol $l^{-1}$ 1,2-cyclohexylenedinitrilotetraacetic acid. The assignments of resonance of $^{13}$C peaks were carried out according to Gout *et al.* (1993). Identified compounds were quantified from the height of their resonance peaks using fully relaxed conditions for spectra acquisition (pulses at 20 s intervals). Peak intensities were normalized to a known amount of the reference compound (maleate) that is added to the sample (internal standard). A carbon atom is here considered to be labeled when its estimated positional $^{13}$C proportion $^{13}C/(^{13}C + ^{12}C)$ is > 2% (the natural abundance is 1.1%).

## Statistical analysis

Linear regression and *t*-test for comparison (with a 5% threshold) were carried out with the R.2.6.1 software (The R Project for Statistical Computing, R Development Core Team)

The $^{13}$C-NMR data are presented as an isotopomics array using the method described in Tcherkez *et al.* (2007). In this case, the logarithm of the positional $^{13}$C-abundances was used to build the array because of the strong enrichment in certain

positions (up to 80%) relative to natural abundances (1.1%). The intensity of the red colour represents the strength of the enrichment in the carbon atom position considered. Both the drawing of the array and the clustering analysis were done with MeV 4.1 software (Saeed *et al.*, 2003). The clustering was based on the Pearson correlation method.

## Results

### Variation in respiration rate, RQ, and respired $\delta^{13}CO_2$ of roots under continuous darkness

Plants were subjected to six consecutive days of darkness in order to modify root respiratory regime. Gas exchanges as well as isotopic measurements were made on each day, and the results are shown in Fig. 1. Root respiratory flux stayed constant during the first day and then continuously decreased, reaching *c.* 30% of its initial value after 6 d. The decrease of the Suc content in roots (from 24 to 8 mg $g^{-1}$ DW, data not shown) followed a very similar pattern so that there was a positive correlation between respiration and Suc concentration ($R^2 = 0.9024$, $P = 0.0024$). The RQ remained slightly above 1 (between 1.025 and 1.17) for the first 3 d of darkness, suggesting either the use of some organic acids in the respiratory substrate mix or the occurrence of reduction processes other than $H_2O$ production in the mitochondria electron transport chain. Indeed, the exclusive oxidation of carbohydrates through respiration would theoretically lead to a RQ of 1 (see the Discussion section). It then fell below 1 from day 4–6 (0.82–0.91), which might indicate a switch toward a greater use of less oxygenized substrates (such as lipids or proteins).

Remarkably, almost no variation was seen in the isotope signature of root-respired $CO_2$ during the dark-mediated starvation. It stayed constant (at *c.* −27.5‰), as did the isotope composition of Suc (−26.5‰), starch (−26.5 to −27‰), proteins (−27.5‰) and lipids (*c.* −31‰). Thus, in contrast to leaves (see Tcherkez *et al.*, 2003), there was no relationship between $\delta^{13}C$ of root-respired $CO_2$ and RQ, and the respiratory fractionation was constantly *c.* +1‰ against $^{13}C$ (considering Suc as the respiratory source material). It can be seen from Fig. 1 that the respiration rate, the RQ and the $\delta^{13}CO_2$ stayed constant from day 4, and so in the following, experiments were carried out on days 0 and 4.

### Labeling patterns in respired $CO_2$ and metabolites and metabolic fluxes

Roots were fed with positionally labeled substrates (Glc and Pyr $^{13}C$-enriched in position 1, 2, or 3) and the obtained kinetics of $\delta^{13}C$ in root-respired $CO_2$ (Fig. 2) were used to approximate relative fluxes in the main decarboxylating pathways, that is, glycolysis, Krebs cycle, and the pentose phosphate pathway (Fig. 3). Qualitatively, feeding roots for

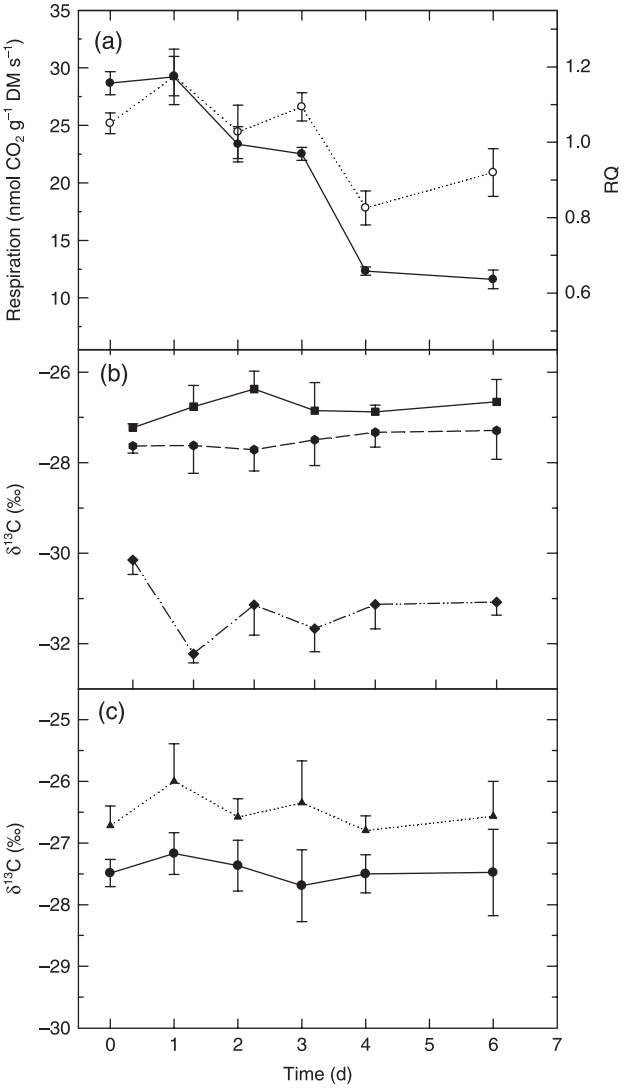


**Fig. 1** Evolution of respiration (a, closed circles), respiratory quotient (RQ; a, open circles) and the isotopic composition of starch (b, squares), soluble proteins (b, hexagons), lipids (b, diamonds), sucrose (c, triangles) and respired $CO_2$ (c, closed circles) in roots of French bean (*Phaseolus vulgaris*) exposed to six consecutive days of darkness. Data represent means ± SE of three plants. $CO_2$ was collected in a closed system for respiration and isotope analysis on an intact washed root system (still attached to shoots). At day 0, plants were taken after a 10 h photoperiod.

2 h with [1-$^{13}C$], [2-$^{13}C$] or [3-$^{13}C$]Glc ($\delta^{13}C = 200$‰) resulted in a $^{13}C$ enrichment in Suc, indicating that Glc entered the roots where it was metabolized. It was more pronounced in starved plants (*c.* 11‰ relative to unlabeled roots) than in plants taken after illumination (*c.* 5‰ relative to unlabeled roots) and stayed constant during the 2 h chase period in both conditions (data not shown). In other words, Suc was a major sink for exogenous applied Glc, so that the $^{12}C$ dilution effect of endogenous Suc on $^{13}CO_2$ abundance after labeling was very small. A significant label was also measured in soluble proteins ($P < 0.01$). The $^{13}C$ enrichment reached

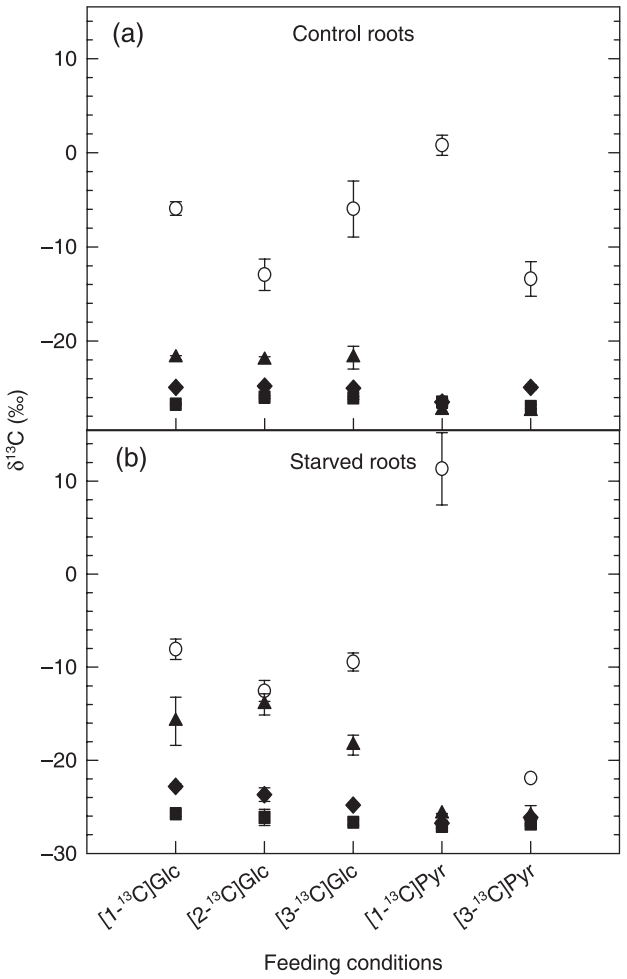


**Fig. 2** Isotope composition of respired $CO_2$ (circles), sucrose (triangles), starch (squares) and soluble proteins (diamonds) in control (a) and starved (b) French bean (*Phaseolus vulgaris*) roots fed with positionally $^{13}C$-enriched Glc (position 1, 2 or 3) and Pyr (position 1 or 3). Values are means ± SE of three independent measurements. Control plants were taken after a 10 h photoperiod, and starved plants after four consecutive days of darkness. Intact washed roots (still attached to shoots) were labeled for 2 h in 10 mM solutions of $^{13}C$-labeled substrates bubbled with air. $CO_2$ was collected in a closed system for isotope analysis. The $\delta^{13}C$ of respired $CO_2$ for nonlabeled roots (water controls) was –26.62 ± 1.14‰ for control roots, and –25.67 ± 1‰ for starved roots.

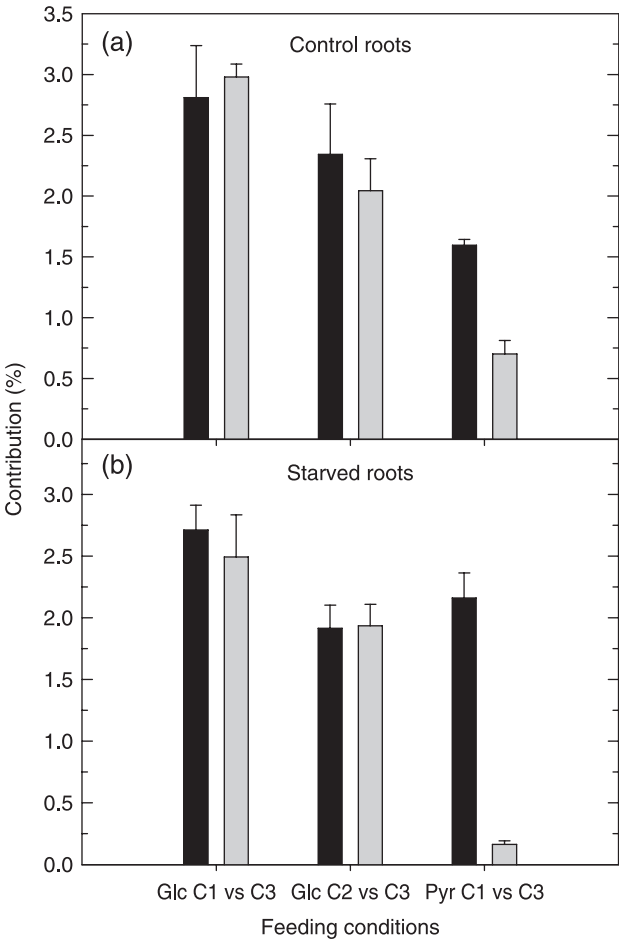


**Fig. 3** Relative fluxes (in % of the $CO_2$ evolution flux) in glycolysis ($R_{PDH}$, black bars) and the Krebs cycle ($R_k$, gray bars) calculated from respired $CO_2$ data of Fig. 2 for control (a) and starved (b) French bean (*Phaseolus vulgaris*) roots. See the Materials and Methods section for the calculation procedure. Error bars represent the distance between values obtained for calculation with either upper or lower values. The $R_k$ value represents the flux through citrate synthase so that it has to be multiplied by 2 to obtain the equivalent $CO_2$ contribution of the Krebs cycle, that is $\rho_k = 2 \times R_k$ (see the Materials and Methods section for the definition of $\rho_k$).

*c.* 3‰ in both control and starved roots just after the feeding period, and then almost fully disappeared during the 2 h chase (data not shown).

$\delta^{13}C$ of respired $CO_2$ was very similar for roots fed with [1-$^{13}$C]Glc or [3-$^{13}$C]Glc, although it was slightly higher in plants from illuminated (control) conditions (–6 vs –8.5‰) (Fig. 2). Remarkably, roots fed with [2-$^{13}$C]Glc exhibited lower $\delta^{13}CO_2$*r* values, *c.* –12.5‰ in both treatments (Fig. 2). Unsurprisingly then, calculated relative contributions of glycolysis ($R_{PDH}$) and Krebs cycle ($R_k$) were quite similar in both illuminated and starved plants. In addition, lower absolute values were obtained while using position C-2 as a tracer instead of position C-1 (Fig. 3). The overestimation of $R_k$ when using data from [1-$^{13}$C]Glc and [3-$^{13}$C]Glc feeding experiments suggests that position 1 is partly decarboxylated in metabolic pathways other than the Krebs cycle (see the Discussion section).

When roots were given labeled Pyr as a substrate, contrasting patterns were observed depending on the enriched position considered (i.e. position 1 or 3). After a 12 h photoperiod, roots fed with [1-$^{13}$C]-Pyr, which was expected to be rapidly decarboxylated in the mitochondria by PDH, showed a substantial enrichment in respired $CO_2$ just after the labeling ($\delta^{13}C = 0.8 \pm 1.07$‰), while no significant differences were measured in the isotopic composition of either Suc or soluble proteins as compared with unlabeled roots. By contrast, in roots fed with [3-$^{13}$C]Pyr, respired $CO_2$ was much less

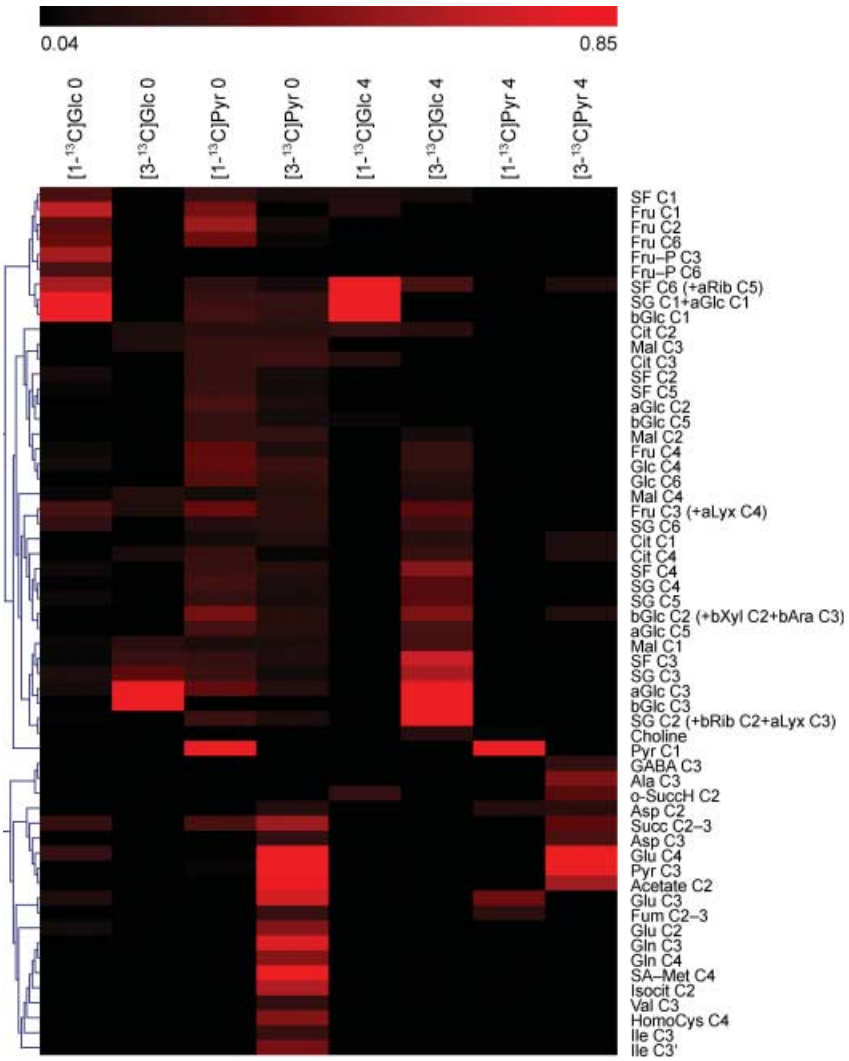


**Fig. 4** Isotopomic array representation of the logarithm of $^{13}C$ abundance in the carbon atom positions of metabolites detected by nuclear magnetic resonance on perchloric acid extracts from French bean (*Phaseolus vulgaris*) roots. Roots were supplied with $^{13}C$-enriched substrates (Glc or Pyr) for 2 h at 21°C after a 10 h photoperiod (feeding conditions denoted '0') or following 4 d of darkness (feeding conditions denoted '4'). Each column represents the mean of two experiments for a given feeding condition. bAra, b-arabinose; Cit, citrate; Fum, fumarate; GABA, γ-amino-butyric acid; Homocys, homocystein; Isocit, isocitrate; Isoleu, isoleucine; aLyx, a-lyxose; Mal, malate; o-SuccH, o-succinyl-homo-Ser; Pyr, pyruvate; bRib, b-ribose; SA-Met, S-adenosyl-Met; SF and SG, fructosyl and glucosyl moieties of sucrose; Succ, succinate; a and b stand for a and b. Black cells indicate positions very close to natural abundance (i.e. 1.1% $^{13}C$), and the intensity of the red colour represents the intensity enrichment in the carbon atom position considered.

enriched ($\delta^{13}C = -13.4 \pm 1.84$‰), and soluble proteins were slightly labeled (3‰ enrichment) (Fig. 2).

Such a contrast was more pronounced in starved plants: the isotope composition of respired $CO_2$ reached $+11.3 \pm 3.9$‰ in [1-$^{13}$C]Pyr fed roots, and $-21.9 \pm 0.13$‰ in [3-$^{13}$C]Pyr fed roots (Fig. 2b). However, neither Suc nor soluble proteins were found to be significantly affected in both feeding conditions ([1-$^{13}$C]Pyr and [3-$^{13}$C]Pyr) for starved roots. The observed contrast in $\delta^{13}C$ of respired $CO_2$ between the two feeding conditions resulted in a substantially higher calculated $R_{PDH}$ compared with $R_k$, especially for starved roots (Fig. 3).

Starch and lipids were never found significantly labeled relative to water-soaked roots in any of the experimental conditions.

## Distribution of the $^{13}C$ in metabolites of control and starved roots

Labeling experiments were also carried out with fully positionally $^{13}C$-enriched substrates (99% $^{13}C$ positional label) so as to trace carbon atoms in the metabolic pathways with NMR analysis. Results are presented as a clustered isotopomics array (Fig. 4) according to the method described in Tcherkez *et al.* (2007).

**After illumination (10 h photoperiod)** In roots fed with [$^{13}$C]Glc, the label detected with NMR was mainly recovered in Glc, Fru and in both moieties of Suc, suggesting that Glc, after entering the root, was metabolized through glycolysis and used for Suc synthesis. The redistributing effect of aldolase and triosephosphate isomerase proceeding at equilibrium could be observed in Fru and Suc fructosyl moiety (SF), which were both found to be $^{13}C$-enriched in positions C-1 and C-6 so that Glc, Fru, SF-C-1 and SF-C-6 clustered. Besides, a slight redistribution of the label in positions 2 and 3 of Fru was observed under [1-$^{13}$C]Glc feeding conditions, potentially reflecting the activity of the nonoxidative branch of the pentose phosphate pathway, with transaldolase and transketolase at equilibrium. The small $^{13}C$ enrichment in

Mal C-1 found in roots supplied with [3-$^{13}$C]Glc suggests that part of Glc-derived PEP was carboxylated by PEPc to form oxaloacetate and subsequently malate. Glc oxidized through glycolysis also participated in fueling the Krebs cycle with Pyr, as evidenced by the small $^{13}$C enrichment in glutamate and succinate under [1-$^{13}$C]Glc feeding conditions. The weakness of the signal probably stems from the diluting effect of internal Glc and of the other competing biosynthetic pathways.

$^{13}$C supplied to roots with [3-$^{13}$C]Pyr was distributed into some organic acids (isocitrate and succinate) and in several organic acid-derived amino acids (glutamate, aspartate, homocystein and S-adenosyl-methionin), unequivocally demonstrating Pyr commitment to the Krebs cycle. This view is supported by the $^{13}$C enrichment measured in C-3-Glu and Gln and C-2-Glu (found in the same cluster), which is an expected consequence of organic acid cycling into the Krebs cycle. Val and Ile, two amino acids that belong to a biosynthetic pathway fueled by Pyr, were also found to be labeled.

**After 4 d of darkness** Similarly, roots fed with $^{13}$C-Glc were essentially labeled in Glc, Fru and Suc (both moieties), indicating that first steps of glycolysis and Suc synthesis were still active in starved roots. Redistribution by aldolase and triosphosphate isomerase was observed in SF-C-6 ([1-$^{13}$C]Glc feeding conditions), SF-C-4 and SG-C-4 ([3-$^{13}$C]Glc feeding conditions). An important redistribution of the label was also measured in SG-C-2, Glc-C-2, and SF-C-6 for roots supplied with [3-$^{13}$C]Glc. Remarkably, these three positions exhibit similar chemical shifts as several pentoses, that is, α-ribose C-2 (or α-lyxose C-3), β-xylose C-2 (or β-arabinose C-3), and α-ribose C-5, respectively. Thus, the observed enrichments could result from both the direct labeling of the pentoses via the PPP and the subsequent redistributing effect in hexoses. Again, the slight label observed in Mal-C-1 of roots supplied with [3-$^{13}$C]Glc indicates PEPc fixation activity in starved roots.

In roots fed with [3-$^{13}$C]Pyr, the sole labeled organic acid was succinate. Among TCA-derived amino acids, only glutamate (in C-4), aspartate (C-2 and C-3) and o-succinylhomoserine, an intermediary of Met biosynthesis pathway, were found to be $^{13}$C-enriched, thus suggesting that the Krebs cycle was significantly slackened. Ala-C-3 was substantially labeled, indicating that part of the Pyr was directly aminated.

## Discussion

### Relationships between respiration, RQ, and $\delta^{13}$C of root-respired $CO_2$

As expected, there was a general depletion of root metabolism when plants were maintained under continuous darkness, as revealed by the decrease of the respiration rate (Fig. 1) and Suc content. Such a response has already been observed in leaves (Tcherkez *et al.*, 2003) and in different types of plant cells subjected to sugar starvation (Brouquisse *et al.*, 1991 and references therein). RQ values decreased as well, from nearly 1.1 to 0.9 after 6 d in darkness, indicating an involvement of less oxygenized respiratory substrates, such as lipids or proteins, together with carbohydrates; proteins represent a good candidate as a respiratory substrate because their $\delta^{13}$C is very similar to that of evolved $CO_2$. The decline of both the respiration rate and RQ was much slower (3–4 d) here than that reported for excised maize roots tips (10 h) (Saglio & Pradet, 1980; Dieuaide-Noubhani *et al.*, 1997). This is the consequence of Suc-export from leaf starch, which fueled root metabolism for several days in darkness (Devaux *et al.*, 2003).

The $\delta^{13}$C value of root-respired $CO_2$ did not vary throughout the whole experiment, (*c.* −27.5‰) and this contrasts with what has been observed in leaves (Tcherkez *et al.*, 2003). As a consequence, the $^{13}$C abundance of root-respired $CO_2$ did not correlate with the respiration rate or the respiratory quotient (Fig. 1). This effect cannot originate from a variation of the $\delta^{13}$C value of root metabolites that might compensate for the switch of respiratory substrate: all the major root metabolites had invariant $^{13}$C abundance (Fig. 1). Thus the adaptation of root respiration to starvation clearly involved metabolic changes that nevertheless resulted in respired $CO_2$ of similar $^{13}$C abundance.

### Respiratory metabolic pathway in roots after illumination

$CO_2$ production by roots involves the three major decarboxylating processes (PDH, the Krebs cycle and the PPP), the rates of which were assessed with $^{13}$C labeling. It is clear that the PDH-catalyzed decarboxylation dominates under our experimental conditions: in roots fed with [1-$^{13}$C]Pyr, respired $CO_2$ was significantly more $^{13}$C-enriched than in those fed with [3-$^{13}$C]Pyr (Fig. 2). Accordingly, the relative flux through PDH was calculated to be slightly more than twice that through citrate synthase (Krebs cycle) (Fig. 3, right-hand side). This imbalance stems from the different biosynthetic pathways that consumed some of the $^{13}$C label from [3-$^{13}$C]Pyr: fatty acid biosynthesis from acetyl-CoA, (as demonstrated in maize root tips by Dieuaide-Noubhani *et al.*, (1995)); the use of Krebs cycle intermediates for N and S assimilation, as evidenced by the $^{13}$C enrichment found by NMR in Glu, Asp, S-adenosyl-Met and homo-Cys (Fig. 4).

While the contribution values are always larger with [$^{13}$C]Glc labeling than with [$^{13}$C]Pyr labeling (Glc was more easily imported into root cells, as they have Glc transporters in their membrane, Farrar & Jones, 2000) (Fig. 3), the contributions of the Krebs cycle- and PDH-catalyzed decarboxylations appear strikingly similar when roots are labeled with [$^{13}$C]Glc (Figs 2, 3). This is because several metabolic steps that may use Glc and its glycolytic products as substrates may decarboxylate the C-1 or C-2 atoms of Glc. The PPP has an important role

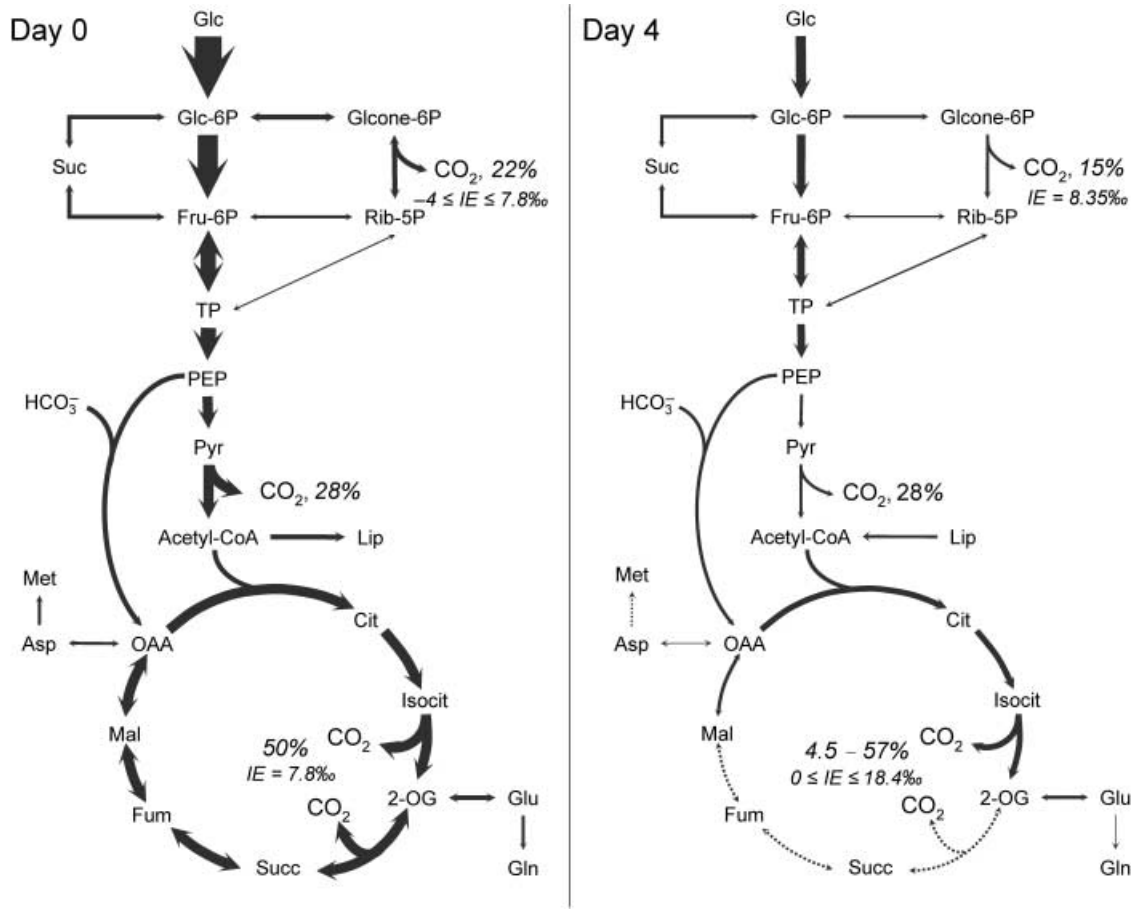


**Fig. 5** Schematic overview of the metabolic fluxes in roots after 10 h of light (day 0) or four consecutive days of darkness (day 4). The thickness of the arrows is related to the fluxes estimated from values shown in Fig. 3. For each decarboxylating step, both the relative contribution to the total $CO_2$ efflux (in %) and the putative isotope effect (IE, used for $\delta^{13}CO_2 r$ calculations (see the Discussion section)) are reported. At day 4, extreme values are presented for the Krebs cycle $CO_2$ flux and isotope effect, which correspond to those obtained for the [$^{13}$C]Pyr and [$^{13}$C]Glc feeding conditions, respectively.

in this regard. In fact, after [1-$^{13}$C]Glc labeling, the $R_k$ value is quite large (Fig. 3, left-hand side), demonstrating that the $^{13}CO_2$ production by the PPP, which decarboxylates the C-1 atom of Glc through the 6-phosphogluconate decarboxylase-catalyzed reaction, did indeed occur. The prevalence of the PPP can be simply deduced from the difference between the [1-$^{13}$C]Glc and [2-$^{13}$C]Glc labeling conditions (for a recent example, see Kruger *et al.*, 2007). This gives a contribution of *c.* 22% of the total net $CO_2$ efflux (Fig. 5). This value is within the same range as previously reported for maize root tips using steady-state labeling (24%; Dieuaide-Noubhani *et al.*, 1995). The involvement of the PPP agrees with the observed $^{13}$C-enrichment patterns after [1-$^{13}$C]Glc feeding and NMR analysis: the labeling in C-3-Fru and C-3-Fru-6-phosphate indicates that the synthesis of pentose phosphates from Fru-6-phosphate occurred through the reversible action of transaldolase and transketolase (Fig. 4). Such a contribution of the PPP agrees with RQ values slightly above 1 (near 1.17 after 1 d in darkness) because the PPP does not consume $O_2$ but produces $CO_2$ (Fig. 1). This scenario is also consistent with the need for reducing power (PPP-derived NADPH) for nitrate reduction (Redinbaugh & Campbell, 1998; Bowsher *et al.*, 2007).

Nevertheless, we recognize that the use of [2-$^{13}$C]Glc instead of [1-$^{13}$C]Glc did not completely mimic the $R_k$-to-$R_{PDH}$ ratio obtained with labeled Pyr (Fig. 3). This difference is probably caused by:

- The interconversion between the C-1 and C-2 atom positions of Glc by the interplay of the PPP (as already observed in maize root tips (Dieuaide-Noubhani *et al.*, 1995)). This effect induces an overestimation of $R_k$, and thus a slight underestimation of the PPP flux given earlier.
- Some anaplerotic PEPc activity that ordinarily occurs in roots (Jacobson, 1955; Jackson & Coleman, 1959; Bryce & ap Rees, 1985; Chang & Roberts, 1992; Dieuaide-Noubhani *et al.*, 1995, 1997; Edwards *et al.*, 1998; Sriram *et al.*, 2007) and is thought to compensate for the consumption of Krebs cycle intermediates by N assimilation (Britto & Kronzucker, 2005).

In addition to acetyl-CoA, [2-$^{13}$C]Glc did label phospho*enol* pyruvate (PEP) molecules that were in turn consumed by the

PEPc to provide oxaloacetate to the Krebs cycle. The resulting $^{13}$C-enriched oxaloacetate further enriched in $^{13}$C both Krebs cycle intermediates and decarboxylated $CO_2$. Such an effect occurred with [1-$^{13}$C]Glc as well, although to a much lower extent (because of the PPP-catalyzed $^{13}CO_2$-loss).

In other words, the difference between the $R_k/R_{PDH}$ ratio values obtained with Glc ([2-$^{13}$C]Glc vs [3-$^{13}$C]Glc) and Pyr ([1-$^{13}$C]Pyr vs [3-$^{13}$C]Pyr) was mainly caused by the input of PEP into the Krebs cycle via the anaplerotic pathway. Let us denote this difference as $R_{PEP}/R_{PDH}$. The maximum value is 0.43, which is consistent with the value of 0.4 obtained for the PEPc-to-PDH flux ratio in maize root tips (Dieuaide-Noubhani *et al.*, 1995). The involvement of PEPc is also supported by both the much stronger enrichment found in Glu and the dilution of the label in C-2 and C-3 of Glu as compared with C-4, when roots are labeled with [3-$^{13}$C]Pyr (Fig. 4).

## Metabolic changes induced upon darkness

After four consecutive days of darkness, root respiratory metabolism was significantly reduced, as evidenced by the decrease of *c*. 60% in the $CO_2$ evolution rate (Fig. 1). Such a decrease of the $CO_2$ efflux is primarily caused by the diminution of the Krebs cycle activity, which is demonstrated by the very weak $^{13}$C-decarboxylation rate of [3-$^{13}$C]Pyr (Fig. 3). This view is consistent with the decrease in the number of mitochondria observed under starvation in heterotrophic cambial cells (Aubert *et al.*, 1996). The nature of respiratory substrates also changed to proteins (Devaux *et al.*, 2003; Brouquisse *et al.*, 2007) and lipids (Dieuaide-Noubhani *et al.*, 1993, 1997), leading to both the slight decrease of the RQ (Fig. 1) and the detection of choline by NMR (data not shown). Parenthetically, we note that acetyl-CoA molecules produced from [3-$^{13}$C]Pyr may have been isotopically diluted by the endogenous acetyl-CoA sources, such as lipid oxidation, inducing an underestimation of Krebs cycle decarboxylations in Fig. 3. PPP-mediated decarboxylations occurred at (slightly) smaller relative rate than at day 0 (near 15% of the $CO_2$ efflux, Fig. 5), as suggested by the $R_k$ excess under [1-$^{13}$C]Glc labeling conditions as compared with [2-$^{13}$C]Glc conditions (Fig. 3).

Again, the large discrepancy between $R_k$ values obtained after [$^{13}$C]Glc and [$^{13}$C]Pyr labeling (Fig. 3) is partly explained by the anaplerotic PEPc activity that enriched in $^{13}$C the Krebs cycle intermediates by the interplay of PEP consumption. The involvement of the PEPc anaplerotic activity is supported by the similar, if not larger, malate-to-citrate concentration ratio in starved roots (5.6) as compared with control roots (4.2) (data not shown). This view also accords with the strong $^{13}$C labeling of the C-4 atom position in Glu, which indicates that N assimilation was maintained after 4 d (Fig. 4). It is thus plausible that the Krebs cycle did not keep its cyclic nature in starved roots, a substantial part of 2OG molecules being consumed for N assimilation to Glu, while PEPc activity maintained high concentrations of malate and fumarate through the backward reactions of the reversible enzymes malate dehydrogenase and fumarase (Fig. 5, right panel).

## Plausible relationships between respiratory metabolism and the $\delta^{13}$C of root-respired $CO_2$

The metabolic origin of the natural $\delta^{13}$C value of root-respired $CO_2$ may be solved in the light of the metabolic fluxes found in the present study. Again, root-respired $CO_2$ is constant and $^{13}$C-depleted while important metabolic changes occurred upon starvation (see earlier discussion). Just after illumination (day 0), the PDH flux accounted for nearly one-third of the total $CO_2$ efflux ($R_{PDH}$ on Fig. 3a, and see Fig. 5 left panel). This reaction decarboxylates carbon atoms that originate from C-3 and C-4 atom positions of Glc which are $^{13}$C-enriched (Rossmann *et al.*, 1991). However, such a $^{13}$C enrichment is compensated by the $^{12}$C/$^{13}$C isotope fractionation (of 23.8‰ *in vitro*) associated with the PDH-catalyzed decarboxylation (DeNiro & Epstein, 1977; Melzer & Schmidt, 1987). That said, the isotope effect is likely to be very small *in vivo* because of the large commitment of the PDH reaction to Pyr consumption (it is assumed to be zero in Fig. 5).

By contrast, both the PPP and the Krebs cycle produce $^{13}$C-depleted $CO_2$. The PPP decarboxylates a carbon atom that originates from the C-1 atom position of Glc, which is $^{13}$C-depleted (Rossmann *et al.*, 1991). In addition, 6-phosphogluconate dehydrogenase fractionates against $^{13}$C during $CO_2$ production by 9.6‰ (kinetic isotope effect, Rendina *et al.*, 1984) or against $^{12}$C by 4‰ (equilibrium isotope effect, Rendina *et al.*, 1984). The Krebs cycle decarboxylates the $^{13}$C-depleted carbon atom positions inherited from Glc C-1, C-2, C-5 and C-6. In addition, several enzymes associated with the Krebs cycle fractionate against $^{13}$C (citrate synthase (*c.* 20‰) and, plausibly, decarboxylases (*c.* 20‰), Tcherkez & Farquhar, 2005), so that Krebs cycle-derived $CO_2$ is clearly $^{13}$C-depleted. Nevertheless, we recognize that the anaplerotic $CO_2$ fixation of PEPc may influence the isotope composition of Krebs cycle intermediates, unless all the PEPc-derived oxaloacetate molecules are subsequently decarboxylated (in which case the isotopic contribution of PEPc-fixation would be zero because of isotopic mass-balance; Edwards *et al.*, 1998). In roots, a study analyzing the isotope composition of the α-carboxyl carbon of amino acids suggested that this pool was dominated by $^{13}$C-depleted respiratory carbon (Savidge & Blair, 2004). In addition, the $^{12}$C/$^{13}$C fractionation associated with PEPc is low (typically 2–4‰, O'Leary *et al.*, 1981), and so the isotope composition of refixed respired $CO_2$ is probably close to that of endogenous Krebs cycle intermediates. We thus assume that the isotopic effect of PEPc fixation on the $\delta^{13}$C value of root-respired $CO_2$ is small.

### Towards a metabolic model of root-respired $\delta^{13}CO_2$?

Assuming contribution values of 28, 50 and 22% to total respired $CO_2$ of the PDH, Krebs cycle and the PPP, respectively (Fig. 5 left panel and earlier discussion), we may calculate roughly what the $\delta^{13}C$ value of root-respired $CO_2$ should be, with the $^{12}C/^{13}C$ fractionations (shown in Fig. 5 and recalled earlier) and $\delta^{13}C$ values within Glc from Rossmann *et al.* (1991). The isotope composition of PDH-derived $CO_2$ is thus near –20.9‰ (average value of C-3 and C-4 in Glc, and no fractionation because of the large commitment of the reaction). That of PPP-derived $CO_2$ is near –26.4‰ ($\delta^{13}C$ value of C-1 in Glc) minus the kinetic fractionation associated with decarboxylation (denoted as $\Delta$) corrected for the commitment to decarboxylation (denoted as $c$). The actual fractionation (denoted as $\Delta_{act}$) is as follows (O'Leary, 1980):

$$\Delta_{act} = \Delta/(1 + c) \qquad \text{Eqn 11}$$

Here, we assume that $c$ is equal to the fraction of Glc molecules committed to PPP decarboxylation, that is, 0.22. Applying Eqn 11, the actual PPP fractionation is thus: 9.6/(1 + 0.22) = 7.8‰. The expected value of the PPP-derived $CO_2$ is thus –26.4–7.8 = –34.2‰. If the equilibrium fractionation were to apply (–4‰, Rendina *et al.*, 1984), that would give a $\delta^{13}CO_2$ value of –26.4 + 4 = –22.4‰. The PPP-derived $CO_2$ is thus between –34.2 and –22.4‰.

The $\delta^{13}C$ value of the Krebs cycle-derived $CO_2$ is that of inherited Glc positions (average $\delta^{13}C$ value of *c.* –27.2‰) minus the fractionation (*c.* 20‰, Tcherkez & Farquhar, 2005) corrected for the commitment to the Krebs cycle. The latter can be assumed to be equal to the Krebs cycle flux ($R_k$ = 0.7 %) relative to the flux through other acetyl-CoA consuming reactions (that is, $R_{PDH} - R_k = 1.6 - 0.7\%$, see Fig. 3); in other words, the commitment to the Krebs cycle is 0.7/(1.6–0.7) = 0.8. Applying Eqn 11, the Krebs cycle-derived $CO_2$ then has a $\delta^{13}C$ value of –27.2 – 20/(1 + 0.8) = –38.4‰.

Under these assumptions, we find that the calculated $\delta^{13}C$ value of total respired $CO_2$ is within –27.1 and –30.6‰. This is in agreement with the observed values for root-respired $CO_2$ in the present paper (Fig. 1) and in the references quoted in the Introduction.

Upon starvation, a similar calculation may be attempted, with the following assumptions: (i) the actual Krebs cycle flux, and thus the commitment of acetyl-CoA, lies somewhere in between those obtained for Pyr and Glc (C-1 vs C-2) feeding conditions (Fig. 5, right panel); (ii) the Krebs cycle is mainly fueled by $^{13}C$-depleted substrates (such as lipids, near –31‰) (RQ, Fig. 1); (iii) the commitment of Glc molecules to the PPP pathway is reduced to 0.15, so that the kinetic isotope fractionation by the 6-phospho-gluconate dehydrogenase is 9.6/(1 + 0.15) = 8.35‰ with Eqn 11 (Fig. 5, upper right). Such assumptions give a $\delta^{13}C$ value that should be within –27.9 and –28.7‰. Unless other prevailing processes occur (such as heterogeneous $^{13}C$ distribution within acetyl-CoA molecules, the involvement of $^{13}C$-enriched metabolite remobilization, etc.), these values are within the range calculated at day 0. The natural isotope composition of root-respired $CO_2$ is not much influenced by the metabolic changes induced by starvation and evidenced by $^{13}C$-NMR tracing; this feature is reproduced by our rough calculations, suggesting that the assumed *in vivo* commitment values are realistic.

### Conclusion

The $^{13}C$ depletion in root-respired $CO_2$ is a critical element to interpret variations of $\delta^{13}C$ in ecosystem-respired $CO_2$. Roots do contribute to soil $CO_2$ efflux, which in turn represents more than half the whole ecosystem respiration. In contrast to leaves (Tcherkez *et al.*, 2003), our results indicate that the root $\delta^{13}C$ signal does not depend on the availability of root respiratory substrates (Fig. 1). It is likely then that, unless the $^{13}C$ photosynthetic fractionation varies at the leaf level, root $\delta^{13}C$ signal does not change under natural environmental conditions throughout a circadian day : night cycle, while photosynthetic sugar input from leaves may vary, for example, with light conditions. We nevertheless recognize that several unknowns remain, such as: possible variations of the $\delta^{13}C$ value of root-respired $CO_2$ with other environmental parameters such as temperature; secondary $^{12}C/^{13}C$ isotope effects by glycolytic and Krebs cycle enzymes; or changes in several metabolite concentrations involved in key processes such as N assimilation (e.g. 2OG, Glu) and known to be potential metabolic regulators (Forde & Lea, 2007). The latter aspect will be addressed in a subsequent study.

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