Plant, Cell & Environment

*Plant, Cell and Environment* (2010) **33**, 76–87 doi: 10.1111/j.1365-3040.2009.02058.x

# Nitrogen deficiency increases the residence time of respiratory carbon in the respiratory substrate supply system of perennial ryegrass

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## ABSTRACT

**Plant respiration draws on substrate pools of different functional/biochemical identity. Little is known about the effect of nitrogen deficiency on those pools' sizes, half-lives and relative contribution to respiration, and consequently, of carbon residence time in respiratory metabolism. Here we studied how nitrogen fertilization affects the respiratory carbon supply system of shoots and roots of *Lolium perenne*, a perennial grass. Plants grown at two nitrogen supply levels in continuous light were labelled with $^{13}CO_2$/$^{12}CO_2$ for intervals ranging from 1 h to 1 month. The rate and isotopic composition of shoot, root and plant respiration were measured, and the time-courses of tracer incorporation into respired $CO_2$ were analysed by compartmental modelling. Nitrogen deficiency reduced specific respiration rate by 30%, but increased the size of the respiratory supply system by 30%. In consequence, mean residence time of respiratory carbon increased with nitrogen deficiency (4.6 d at high nitrogen and 9.2 d at low nitrogen supply). To a large extent, this was due to a greater involvement of stores with a long half-life in respiratory carbon metabolism of nitrogen-deficient plants. At both nitrogen supply levels, stores supplying root respiration were primarily located in the shoot, probably in the form of fructans.**

*Key-words*: *Lolium perenne*; $^{13}C$ labelling; allocation; compartmental analysis; dynamic labelling; half-life; mean residence time; respiration; tracer kinetics.

## INTRODUCTION

Knowledge about the factors governing the mean residence time of respiratory carbon in plants ($\tau$) is of fundamental importance for understanding processes from plant allocation patterns to ecosystem carbon fluxes and global carbon balance (Trumbore 2006). In principle, $\tau$ – the average time carbon atoms reside in the respiratory supply system – is determined by the size of the respiratory supply system and by the flux of carbon through this system. However, the substrates serving plant respiration are heterogeneous, including carbon from current photosynthesis and stores located in different plant parts (Ryle, Cobby & Powell 1976; Flanagan *et al.* 1996; Schnyder *et al.* 2003; Lötscher & Gayler 2005; Carbone & Trumbore 2007; Mortazavi *et al.* 2009), generating uncertainty and complicating predictions of $\tau$.

In a dynamic labelling experiment with $^{13}CO_2$, Lehmeier *et al.* (2008) followed the time course of tracer incorporation in respiratory $CO_2$ in intact shoots and roots of perennial ryegrass (*Lolium perenne* L.), a cool-season C3 grass. This work revealed three major carbon pools with contrasting rates of turnover supplying substrate to both shoot and root respiration (Fig. 1). Two of these pools included current assimilate (i.e. non-stored substrate) and supplied half of the carbon respired in the shoot and root. The other half of respired carbon was provided by a short-term storage pool. The store supplying root respiration was much larger than the amount of non-structural carbohydrates and proteins in the root. This supported the view that stores in the shoot supplied both shoot and root respiration (Lehmeier *et al.* 2008).

Information about the variability of this allocation pattern, and consequently, of $\tau$ in the plant, is scarce. Nitrogen nutrition, in particular, may have strong effects as it affects the balance between growth and carbon storage (Robson & Deacon 1978; Evans 1983; Morvan-Bertrand, Boucaud & Prud'homme 1999). Stores (in the form of carbohydrate pools) are considered an important substrate for respiration (ap Rees 1980; Tcherkez *et al.* 2003). Also, respiration rate correlates positively with plant nitrogen concentration (Makino & Osmond 1991; Reich *et al.* 2006). As nitrogen deficiency causes slower respiration and larger carbohydrate stores, one may expect that $\tau$ increases with decreasing nitrogen supply. Knowledge about these relationships may help in better understanding plant carbon and nitrogen dynamics (e.g. Gastal & Lemaire 2002; Jeuffroy, Ney & Ourry 2002) and interpretation of auto- and heterotrophic carbon fluxes at the ecosystem scale (Carbone & Trumbore 2007; Gamnitzer, Schäufele & Schnyder 2009). Effects of nitrogen deficiency on $\tau$ may be the norm rather than the exception in natural conditions (Vitousek & Howarth 1991), but these relationships have not been tested experimentally.

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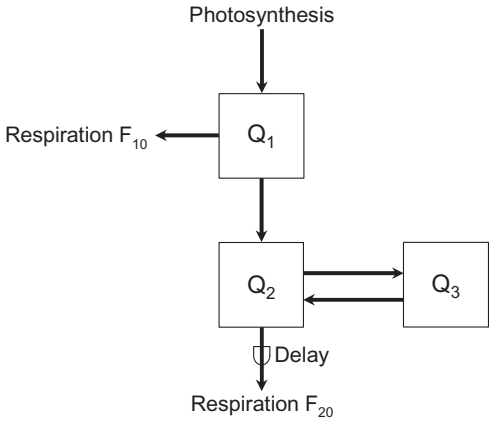


**Figure 1.** Compartmental model respiratory supply system of intact perennial ryegrass plants. Tracer enters the plant during photosynthesis and is respired via $Q_1$ ($F_{10}$) or $Q_2$ ($F_{20}$). 'Delay' means a lag between tracer acquisition by $Q_2$ and its release in respiration. Functional characteristics of the pools were estimated by translating the model into a set of differential equations, and fitting the model to the tracer kinetics.

The aim of this study was to assess nitrogen deficiency effects on kinetic and functional properties of respiratory carbon pools in perennial ryegrass. Plants were grown with either a low or a high supply of nitrogen, labelled dynamically with $^{13}CO_2/^{12}CO_2$ for periods ranging from 1 h to almost 1 month, and the rate and isotopic composition of respired $CO_2$ in the root, shoot and whole plant were measured. The time course of tracer incorporation into respired $CO_2$ was evaluated with compartmental analysis to infer the number and kinetics of the substrate pools supplying respiration. Specifically, we addressed the following questions: Does the size of the respiratory supply system increase with nitrogen deficiency? Does nitrogen deficiency increase the importance of stores as carbon source for respiration? Which characteristics of the respiratory supply system exert the main influence on $\tau$?

## MATERIALS AND METHODS

### Plant material and growth conditions

Seeds of perennial ryegrass (*L. perenne* L., cv. Acento) were sown individually in plastic pots filled with washed quartz sand and arranged in plastic containers at a density of 378 plants $m^{-2}$. Two containers were placed in each of four growth chambers (Conviron E15, Conviron, Winnipeg, Canada). Plants were grown in continuous light, supplied by cool white fluorescent tubes. Irradiance was maintained at 275 $\mu$mol $m^{-2}$ $s^{-1}$ photosynthetic photon flux density at plant height. Temperature was controlled at 20 °C and relative humidity near 85%. Water and nutrients were supplied by briefly flooding the containers every 3 h.

Half the stands received a modified Hoagland solution containing 1 mM $NO_3^-$ (low-nitrogen plants), with 1 mM $KNO_3$, 1 mM $MgSO_4$, 0.18 mM $KH_2PO_4$, 0.21 mM $K_2HPO_4$, 0.5 mM NaCl, 0.7 mM $K_2SO_4$ and 2 mM $CaCl_2$; micronutrients: 125 $\mu$M Fe-ethylenediaminetetraacetic acid, 46 $\mu$M $H_3BO_3$, 9 $\mu$M $MnSO_4$, 1 $\mu$M $ZnSO_4$, 0.3 $\mu$M $CuSO_4$ and 0.1 $\mu$M $Na_2MoO_4$. The other stands received a nutrient solution containing 7.5 mM $NO_3^-$ (high-nitrogen plants) with 2.5 mM $Ca(NO_3)_2$, 2.5 mM $KNO_3$, 1.0 mM $MgSO_4$, 0.18 mM $KH_2PO_4$, 0.21 mM $K_2HPO_4$, 0.5 mM NaCl, 0.4 mM KCl, 0.4 mM $CaCl_2$, and the same levels of micronutrients. All stands were periodically flushed with demineralized water to prevent salt accumulation.

### $CO_2$ control in the growth chambers and $^{13}C$ labelling

The labelling system described by Schnyder *et al.* (2003) with air-locks as detailed by Lehmeier *et al.* (2008), was used. In brief, air supply to the growth chambers was performed by mixing $CO_2$-free air and $CO_2$ of known carbon isotope composition ($\delta$, with $\delta = [^{13}C/^{12}C_{sample}/^{13}C/^{12}C_{VPDB\ standard}] - 1$). Both $\delta^{13}C$ and concentration of $CO_2$ (360 $\mu$L $L^{-1}$) inside the chambers were constantly monitored by an infrared gas analyser (Li-6262, Li-Cor Inc., Lincoln, NE, USA) and a continuous-flow isotope-ratio mass spectrometer (CF-IRMS, Delta Plus, Finnigan MAT, Bremen, Germany). At high nitrogen supply, half the stands grew with $^{13}C$-depleted $CO_2$ ($\delta^{13}C$ –28.8‰ ± 0.2 SD), while the other half grew with $^{13}C$-enriched $CO_2$ ($\delta^{13}C$ –1.7‰ ± 0.2). For stands of low nitrogen supply, $\delta^{13}C$ was –3.6‰ (±0.2) and –30.9‰ (±0.3), respectively ($CO_2$ from Linde AG, Höllriegelskreuth, Germany). The stability of the isotopic composition and concentration of $CO_2$ (±3 $\mu$L $L^{-1}$ SD on average over all measurements in one chamber) inside the chambers was provided by periodic adjustments of airflows and $CO_2$ concentrations in chamber inlets.

When plants had three tillers (about 3 and 6 weeks after sowing at high and low nitrogen supply, respectively), labelling was initiated by swapping randomly selected individual plants between chambers of the same nitrogen supply level (i.e. $^{13}C$-enriched $CO_2 \rightarrow$ $^{13}C$-depleted $CO_2$ and vice versa). This ensured that high- and low-nitrogen plants were compared at a similar size, so that possible size-related effects on the respiratory supply system were minimized. Plants at high nitrogen supply were kept in the labelling chamber for 1, 2, 4, 8 or 16 h; or for 1, 2, 4, 8, 12, 17 or 25 d. The durations of labelling at low nitrogen supply were 1, 2, 4, 8 or 16 h; or 1, 2, 4, 8 or 29 d. Within one nitrogen supply level, labelling was scheduled in such a way that respiration measurements occurred at the same mean plant age (and size).

### Respiration measurements

Respiration of labelled and non-labelled (control) plants was measured in the system described by Lötscher, Klumpp & Schnyder (2004) and Klumpp *et al.* (2005). Briefly, four plants were removed from the stands, rapidly installed in individual gas exchange cuvettes and placed in a growth cabinet held at the same temperature as the growth chambers. Three replicate measurements of $CO_2$ and $\delta^{13}C$ entering and leaving the shoot and root compartments of each cuvette were taken every 45 min during the following 5 h. Each $\delta^{13}C$ measurement was compared against a working

standard gas, which was previously calibrated against a VPDB-gauged laboratory $CO_2$ standard. The average standard deviation of repeated single measurements was 0.08‰ for $\delta^{13}C$ and 0.33 $\mu$L $L^{-1}$ for $[CO_2]$.

Thus, this system permitted repeated sequential measurements of the rates and isotopic composition of dark respiration by shoots and roots of individual plants, as detailed in Lehmeier *et al.* (2008). Rates and $\delta^{13}C$ of shoot respiration reached constant values ~30 min after removing plants form the stands. However, it took ~1.5 h to completely purge the root compartment from extraneous $CO_2$ (cf. Lötscher *et al.* 2004). During the 5 h measurements, dark respiration rates of roots decreased by about 3% and 6% for plants grown at low and high nitrogen supply, respectively, while that of shoots was constant in both treatments. Average rates were taken to calculate specific respiration rates (for the stability of $\delta^{13}C$ in respired $CO_2$ see further discussion).

## Plant harvest and elemental analysis

Immediately after respiration measurements, plants were removed from the pots, washed free of sand, dissected into shoot and root, weighed, frozen in liquid nitrogen and stored at –30 °C in chest freezers. All samples were freeze-dried for 72 h, weighed again and ground to flour mesh quality in a ball mill. Aliquots of 0.75 mg ± 0.05 mg of each sample were weighed into tin cups (IVA Analysentechnik e.K., Meerbusch, Germany) and combusted in an elemental analyser (Carlo Erba NA 1110, Carlo Erba Instruments, Milan, Italy), interfaced to the CF-IRMS, to determine carbon and nitrogen contents.

## Analysis of water-soluble carbohydrates

Water-soluble carbohydrates in plant biomass were analysed using a similar procedure as Thome & Kühbauch (1985). In short, 60 and 80 mg of freeze-dried ground material of shoot and root samples, respectively, were weighed in Eppendorf tubes and extracted with 2 mL $H_2O$ for 10 min in a water bath at 93 °C. Afterwards, samples were transferred to a rotating Heidolph shaker for 45 min at room temperature and then centrifuged at 20.000 *g* for 15 min. A 0.2 mL aliquot of the supernatant was transferred to a preparative HPLC system for separation of water-soluble carbohydrate fractions. Separation occurred in a Shodex KS 2002 chromatographic column (Showa Denko, Tokyo, Japan) held at 50 °C and a system pressure of 17 bar and at an elution rate of 0.9 mL $min^{-1}$ using HPLC-grade water (Baker, Deventer, The Netherlands) as the eluent.

Samples eluting from the HPLC-system were immediately conveyed to a continuous-flow system. There, 1.25% (v/v) sulphuric acid was added at a rate of 0.9 mL $min^{-1}$, and di- and oligosaccharides were hydrolysed during the ~15 min passage through a sample loop of 12.5 m length which was kept in a water bath at 95 °C. Carbohydrates were quantified by measuring the reducing power of the hydrolysed carbohydrates by way of the oxidation-reduction reaction with potassium ferricyanide (Suzuki 1971) and detection of the reduced potassium ferricyanide solution at a wave length of 425 nm in a spectral photometer (K-2500/A4080, Knauer, Berlin, Germany). Analytical grade fructose (D(-)-Fructose, Merck, Darmstadt, Germany) served as the routine standard for carbohydrate quantification. Periodic verification with glucose, sucrose and fructan standards demonstrated relative response factors which were reasonably close to theoretical expectations: glucose, 0.94; fructose, 1.0; sucrose, 1.06; fructan, 1.04. The concentration of carbohydrates in total plant tissue was calculated from the carbohydrate contents of shoot and root samples and the shoot- and root-mass fractions of the individual plants.

## Data analysis

The proportion of carbon in shoot- and root-respired $CO_2$ assimilated before (unlabelled) and during labelling, $f_{unlabelled\text{-}C}$ and $f_{labelled\text{-}C}$ (where $f_{labelled\text{-}C} = 1 - f_{unlabelled\text{-}C}$), was obtained by means of an isotopic mass balance:

$$f_{unlabelled\text{-}C} = (\delta^{13}C_S - \delta^{13}C_{new})/(\delta^{13}C_{old} - \delta^{13}C_{new}) \quad (1)$$

with $\delta^{13}C_S$ is the $\delta^{13}C$ of respiratory $CO_2$ produced by the labelled sample plant, and $\delta^{13}C_{old}$ and $\delta^{13}C_{new}$ the $\delta^{13}C$ of respiratory $CO_2$ produced by non-labelled plants growing continuously in the chamber of origin ('old') or in the labelling chamber ('new').

For shoots, $\delta^{13}C_S$, $\delta^{13}C_{old}$ and $\delta^{13}C_{new}$ were obtained as:

$$\delta^{13}C_X = (\delta^{13}C_{in}F_{in} - \delta^{13}C_{out}F_{out})/(F_{in} - F_{out}) \quad (2)$$

where X stands for 'sample', 'new' or 'old' (as appropriate), and $\delta^{13}C_{in}$, $\delta^{13}C_{out}$, $F_{in}$ and $F_{out}$ are the isotopic compositions and the flow rates of the $CO_2$ entering and leaving the shoot compartment, respectively. The same procedure was followed for roots. In this case, $\delta^{13}C_{in}$ and $F_{in}$ represented the $\delta^{13}C$ and the flow rates of the $CO_2$ leaving the shoot compartment (cf. Klumpp *et al.* 2005).

The $\delta^{13}C$ of respired $CO_2$ of both labelled and non-labelled (control) plants did not show a trend during the respiration measurements (which stabilized 30 min after the insertion of shoots and 1.5 h after insertion of roots in the gas-exchange cuvettes). Thus, we used the mean of all 5 h measurements of one plant to estimate $f_{unlabelled\text{-}C}$. This was true, except for high-nitrogen plants labelled for 1 h, where $f_{unlabelled\text{-}C}$ of the shoot increased significantly during the 5 h measurements. In that case, $f_{unlabelled\text{-}C}$ was taken as the y-intercept at 0 h of a linear regression of $f_{unlabelled\text{-}C}$ (y) versus time during the 5 h measurement (cf. Fig. 5 in Lehmeier *et al.* 2008).

The fraction of unlabelled carbon in $CO_2$ respired by a plant was calculated as the flux-weighted mean of shoot and root respiration:

$$f_{unlabelled\text{-}C} = (f_{unlabelled\text{-}C\,shoot} \times R_{shoot} + f_{unlabelled\text{-}C\,root} \times R_{root})/(R_{shoot} + R_{root}) \quad (3)$$

where $R_{shoot}$ and $R_{root}$ are the absolute respiration rates (in g C $h^{-1}$) of shoot and root, respectively.

## Compartmental analysis of tracer time courses in respired $CO_2$

The labelling kinetics of $CO_2$ respired by shoots, roots and whole plants of both nitrogen treatments show that tracer incorporation occurred in distinct phases, which reflected the operation of substrate pools supplying carbon to respiration. The fitting of exponential decay functions to the tracer kinetics (similar to Moorby & Jarman 1975) indicated that the respiratory supply system included three pools in each nitrogen treatment. Yet, we also considered other compartmental concepts of respiratory carbon metabolism (e.g. Farrar 1990; Dewar, Medlyn & McMurtrie 1998) and designed several compartmental models which differed in the number of pools (two-, three- and four-pool models) or in the way the pools were interconnected and exchanged carbon with the environment (i.e. photosynthetic and respiratory fluxes).

Each model was translated into a set of equations, which described a given respiratory carbon supply system in terms of fluxes between pools and the environment using the assumption, that pool sizes were steady and fluxes obeyed first-order kinetics. Each model was tested for its ability to predict the tracer kinetics as detailed in the following for the three-pool model shown in Fig. 1. On the basis of this comparison (and respecting the principle of parsimony) the three-pool model of Fig. 1 emerged as the one closest to the real properties of the respiratory supply systems of the plants of both nitrogen supply levels. To account for a stable degree of labelling from about 2–4 h of labelling duration a delay was inserted in the model that implied that tracer release via $Q_1$ occurred immediately after the start of labelling, while tracer release via $Q_2$ only occurred after the delay.

The fraction of tracer in each compartment with respect to time was given by:

$$f_{\text{labelled-C-Q1}}(t) = [Q_1 \times f_{\text{labelled-C-Q1}}(t-\Delta t) + F_{In} \times f_{\text{labelled-C}}(t) - F_{10} \times f_{\text{labelled-C-Q1}}(t-\Delta t) - F_{12} \times f_{\text{labelled-C-Q1}}(t-\Delta t)]/Q_1 \quad (4a)$$

$$f_{\text{labelled-C-Q2}}(t) = [Q_2 \times f_{\text{labelled-C-Q2}}(t-\Delta t) + F_{12} \times f_{\text{labelled-C-Q1}}(t-\Delta t) + F_{32} \times f_{\text{labelled-C-Q3}}(t-\Delta t) - F_{23} \times f_{\text{labelled-C-Q2}}(t-\Delta t) - F_{20} \times f_{\text{labelled-C-Q2}}(t-\Delta t)]/Q_2 \quad (4b)$$

$$f_{\text{labelled-C-Q3}}(t) = [Q_3 \times f_{\text{labelled-C-Q3}}(t-\Delta t) + F_{23} \times f_{\text{labelled-C-Q2}}(t-\Delta t) - F_{32} \times f_{\text{labelled-C-Q3}}(t-\Delta t)]/Q_3 \quad (4c)$$

$$f_{\text{labelled-C}}(t) = [F_{10} \times f_{\text{labelled-C-Q1}}(t) + F_{20} \times f_{\text{labelled-C-Q2}}(t_0)]/(F_{10} + F_{20}) \text{ for } t \le \text{delay}; \quad (4d)$$

$$f_{\text{labelled-C}}(t) = [F_{10} \times f_{\text{labelled-C-Q1}}(t) + F_{20} \times f_{\text{labelled-C-Q2}}(t\text{-delay})]/(F_{10} + F_{20}) \text{ for } t > \text{delay}; \quad (4e)$$

$$f_{\text{unlabelled-C}}(t) = 1 - f_{\text{labelled-C}}(t) \quad (4f)$$

where $Q_1$, $Q_2$ and $Q_3$ are pool sizes and $F_{In}$ is the flux of assimilated carbon (tracer) that enters the respiratory system. As the system was considered steady, $F_{In}$ equalled the specific respiration rate (cf. Table 1), and $F_{In} = F_{Out}$, $F_{Out} = F_{10} + F_{20}$, $F_{12} = F_{20}$ and $F_{23} = F_{32}$ (indices refer to donor and receptor pools, respectively, index 0 represents the environment; Fig. 1). The measured parameter against which the model prediction was compared is $f_{\text{unlabelled-C}}$. $f_{\text{unlabelled-C-Qi}}$ is the fraction of unlabeled carbon in pool $Q_i$. $f_{\text{labelled-C}}$ is the constant fraction of fully labelled carbon entering the system after the start of labelling. Subscript t denotes time after the onset of labelling (i.e. labelling duration), $t_0$ refers to time just before the onset of labelling. $\Delta t$ is the time step with which the model was run in the calculation and was set to 6 min. Thus, the time step was small even in comparison with the minimum time resolution of labelling, which was 1 h.

The set of equations (4) was implemented in a custom-made program using the free software 'R' (R Development Core Team 2007). Initial values for pool sizes, fluxes between pools and the delay were inserted, and the equations were solved. In that way, a tracer time course across the entire labelling period (600 and 696 h for plants grown at high and low nitrogen supply, respectively) was generated. The quality of the fit was expressed as the root mean squared error (RMSE).

This procedure was executed millions of times by stepwise and systematic variation of preset values for pool sizes, fluxes between pools and the delay. In doing so, the combinations of pool sizes, fluxes and the delay giving the best fits, i.e. the lowest RMSEs, were taken as the ones closest to the real properties of the respiratory supply systems. This extensive scanning procedure aimed to detect the global minimum RMSE rather than a local minimum, an aspect, compartmental analyses must cope with. This procedure also revealed the sensitivity of the fits to changes in parameter values. The minimum RMSE of each RMSE response curve corresponds to the optimum of pool half-lives, sizes and contributions. A high sensitivity of the goodness of fit to changes in one parameter is revealed by steep increases of the RMSE response curve on either side of the optimum.

Optimized pool sizes and fluxes served to calculate the half-life of a pool of size $Q_i$:

$$t_{1/2}(Q_i) = \ln(2)/(F_i/Q_i) \quad (5)$$

with $F_i$ the sum of all fluxes leaving the pool $Q_i$.

The quantitative contribution of a pool $Q_i$ ($C_{Qi}$) to respiratory carbon release was derived based on optimized fluxes. It is defined here as the probability of tracer moving in a certain flux of the respiratory system (Fig. 1):

$$C_{Q1} = F_{10}/(F_{10} + F_{12}) \quad (6a)$$

$$C_{Q2} = [1 - F_{10}/(F_{10} + F_{12})] \times F_{20}/(F_{20} + F_{23}) \quad (6b)$$

$$C_{Q3} = [1 - F_{10}/(F_{10} + F_{12})] \times F_{23}/(F_{20} + F_{23}) \quad (6c)$$

**Table 1.** Growth parameters of perennial ryegrass grown with either a low (1.0 mM) or a high (7.5 mM) supply of nitrate-nitrogen

| Parameter | Low nitrogen | High nitrogen |
|---|---|---|
| Specific respiration rate | | |
| plant, mg plant-respired C $g^{-1}$ plant-C $h^{-1}$ | 0.99 ± 0.03 | 1.50 ± 0.02 |
| shoot, mg shoot-respired C $g^{-1}$ plant-C $h^{-1}$ | 0.62 ± 0.02 | 0.97 ± 0.02 |
| root, mg root-respired C $g^{-1}$ plant-C $h^{-1}$ | 0.37 ± 0.01 | 0.53 ± 0.01 |
| Specific growth rate, mg C $g^{-1}$ C $h^{-1}$ | 1.58 ± 0.31 | 3.23 ± 0.21 |
| Specific nitrogen uptake rate, mg N $g^{-1}$ C $h^{-1}$ | 0.035 ± 0.007 | 0.124 ± 0.010 |
| Shoot : root ratio | 2.96 ± 0.10 | 3.84 ± 0.14 |
| C : N ratio (w/w) | 48.8 ± 1.3 | 24.1 ± 0.5 |

Values are means ± 1SE; 56 and 60 replicate plants were used for calculations for low and high nitrogen, respectively. $P < 0.001$ for differences in all parameters between treatments. Specific respiration rates of the shoot and root at high nitrogen supply are from Lehmeier *et al.* (2008).

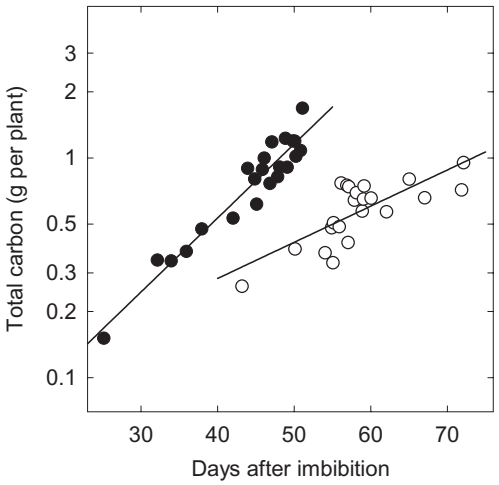


**Figure 2.** Total carbon mass of perennial ryegrass grown with a nitrogen supply of either 1.0 mM nitrate (open symbols) or 7.5 mM nitrate (closed symbols). Each value is the mean of 3–6 replicate plants. Lines denote linear regression ($P < 0.05$; see also Table 1). Note the logarithmic scaling of the *y* axis.

$C_{Q1}$ is the probability that tracer enters the system and leaves it in $F_{10}$ without visiting any other pool. $C_{Q2}$ implies, that tracer enters $Q_2$ via $Q_1$ and is respired in $F_{20}$ without moving through $Q_3$. $C_{Q3}$ is the probability of tracer cycling through $Q_3$ at least once.

The mean residence time of carbon in the respiratory supply system ($\tau$) was calculated as

$$\tau\,(\mathrm{h}) = Q_{\mathrm{total}}/r_{\mathrm{plant}} \quad (7)$$

with $Q_{\mathrm{total}}$ the total size of all respiratory substrate pools, i.e. the sum of $Q_1$, $Q_2$ and $Q_3$ in mg C $g^{-1}$ plant-C (Table 2) and $r_{\mathrm{plant}}$ the specific respiration rate of the whole plant (Table 1) in mg C $g^{-1}$ plant-C $h^{-1}$.

The analysis of tracer time courses conducted in the present study holds the assumptions generally made in compartmental modelling, namely: (1) the system is in a steady-state, i.e. pool sizes and fluxes in the system are constant and only $f_{\mathrm{unlabelled\text{-}C}}$ in respired $CO_2$ changes with time; (2) fluxes obey first-order kinetics; and (3) pools are homogeneous and well mixed. Support for the validity of assumption (1) is obtained by the constancy of plant specific growth and respiration rates (Figs 2 & 3). Plant growth in continuous light eliminated short-term changes in pool sizes and fluxes which would have complicated the analysis in day/night cycles. Assumption (2) is probably false in a strict sense, but support for its practical validity has been found repeatedly (see Farrar 1990 for a discussion). Assumption (3) is a simplification, particularly for studies at the whole organ and plant level, in the sense that different pools are probably not biochemically homogeneous and distributed in different tissues. Yet, in the context of labelling, a pool is defined as a set of compounds which exhibit the same proportion of labelled carbon atoms. So, in principle, one pool can include several populations of anatomical (physical) features and biochemical species on the condition that they exhibit the same proportion of label (Atkins 1969; Rescigno 2001).

**Table 2.** Optimized parameters of the model shown in Fig. 1 as fitted to tracer time courses of $CO_2$ respired by perennial ryegrass plants (Fig. 4c) grown with either a low (1.0 mM) or a high (7.5 mM) supply of nitrate-nitrogen

| | Low nitrogen | High nitrogen |
|---|---|---|
| | Size (mg C $g^{-1}$ plant-C) | |
| $Q_1$ | 1 | 1 |
| $Q_2$ | 40 | 19 |
| $Q_3$ | 178 | 146 |
| total | 219 | 166 |
| | Half-life (h) | |
| $Q_1$ | 0.4 | 0.5 |
| $Q_2$ | 20 | 3.4 |
| $Q_3$ | 263 | 40 |
| | Contribution (%) | |
| $Q_1$ | 9 | 15 |
| $Q_2$ | 60 | 28 |
| $Q_3$ | 31 | 57 |
| | Flux (mg C $g^{-1}$ plant-C $h^{-1}$) | |
| $F_{10}$ | 0.09 | 0.23 |
| $F_{12}$, $F_{20}$ | 0.90 | 1.28 |
| $F_{23}$, $F_{32}$ | 0.47 | 2.53 |
| | Delay (h) | |
| – | 3.2 | 3.5 |
| | RMSE | |
| – | 0.008 | 0.020 |

The goodness of the fits is expressed as the root mean squared error (RMSE).

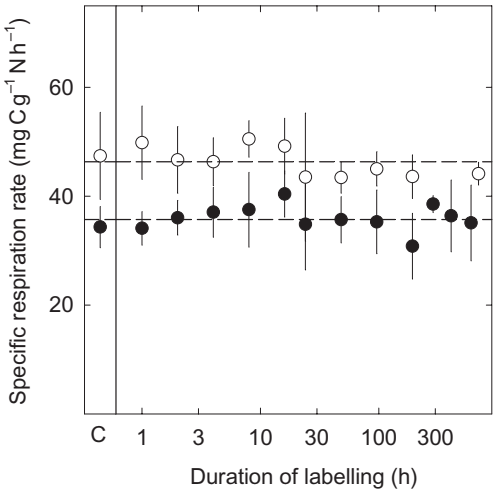


**Figure 3.** Specific respiration rates of perennial ryegrass grown with a nitrogen supply of either 1.0 mM nitrate (open symbols) or 7.5 mM nitrate (closed symbols), labelled for different time intervals, and of non-labelled controls (C; at left). Each value is the mean of 3–12 replicate plants (±1SE). Average rates were 46.3 ± 0.8 (n = 56) and 35.7 ± 0.7 (n = 60) for low- and high-nitrogen plants, respectively (dashed lines). Regression analysis yielded no significant trends ($P > 0.05$). Note the logarithmic scaling of the *x* axis.

## RESULTS

### Growth, respiration and photosynthetic carbon use efficiency

Nitrogen supply had large effects on several plant growth parameters. Plants grew at constant specific rates at both nitrogen levels (Fig. 2). Linear regression of ln-transformed carbon mass data yielded average increments of 1.6 mg C g$^{-1}$ C h$^{-1}$ at low nitrogen and 3.2 mg C g$^{-1}$ C h$^{-1}$ at high nitrogen supply (Table 1). Within one nitrogen treatment, specific growth rates of shoot and root were similar, resulting in near constant shoot to root ratios that averaged 3.0 at low nitrogen and 3.8 g C g$^{-1}$ C at high nitrogen supply (Table 1).

The specific respiration rate of plants at high nitrogen was 1.50 mg C g$^{-1}$ C h$^{-1}$, about 50% higher than that at low nitrogen supply (Table 1). This corresponded to photosynthetic carbon use efficiencies [CUE, with CUE = growth rate/(growth rate + respiration rate)] of 0.68 at high nitrogen supply and 0.61 at low nitrogen. The nitrogen uptake rate of plants was calculated by multiplying the slope of a linear regression of plant nitrogen mass per unit plant carbon mass times the specific carbon growth rate. Nitrogen uptake rate in low-nitrogen plants was 30% that of high-nitrogen plants, while the carbon to nitrogen ratio in biomass was twice that at high nitrogen supply (Table 1). Rates of growth and respiration did not differ between growth chambers within one nitrogen supply level ($P > 0.05$).

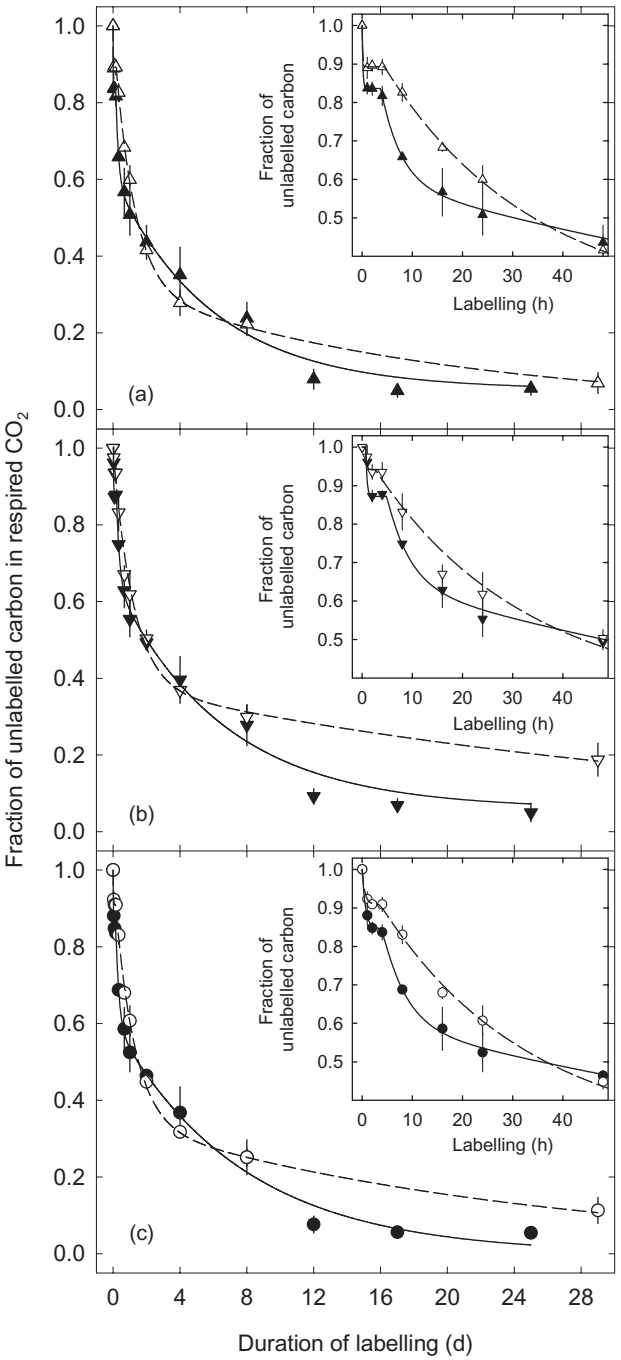


**Figure 4.** The fraction of unlabelled carbon in $CO_2$ respired by shoots (a), roots (b) or whole plants (c) of perennial ryegrass grown with either 1.0 mM (open symbols) or 7.5 mM nitrate-nitrogen supply (closed symbols) during labelling. Each value is the mean of 3–6 replicate plants (±1SE). Insets expand the first 49 h. The data points at 8 d labelling duration in panel c overlap. Solid and dashed lines denote model predictions of high and low nitrogen supply, respectively. Data for shoot and root of high-nitrogen plants are taken from Lehmeier *et al.* (2008).

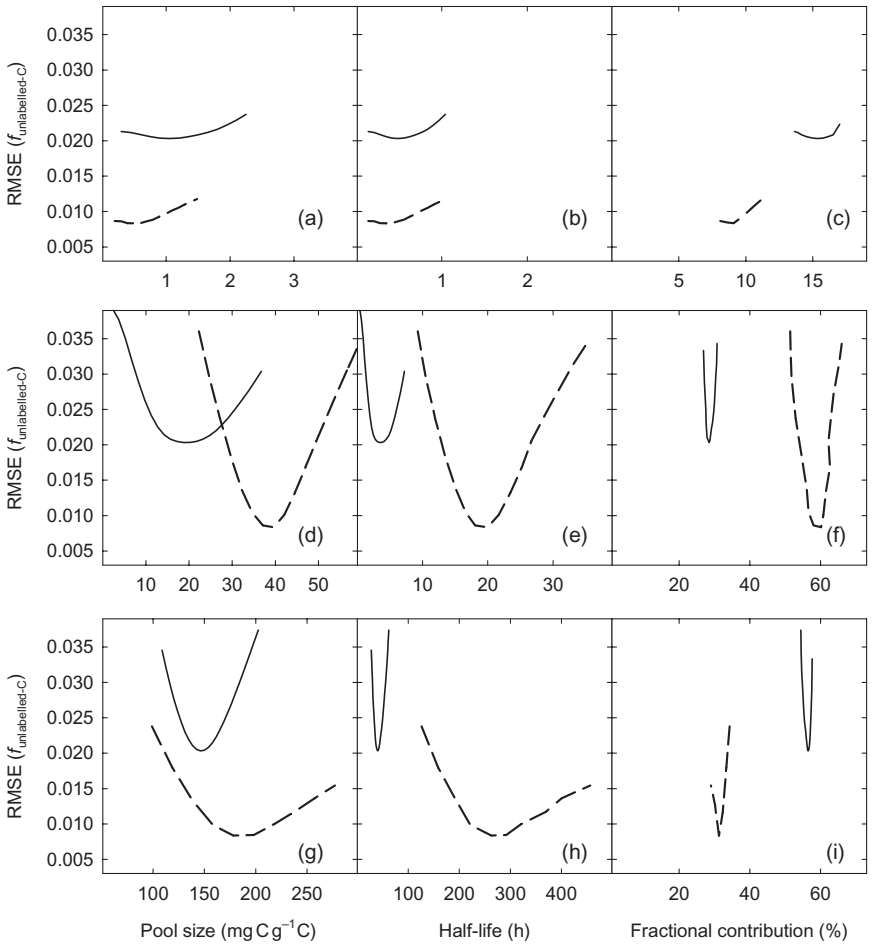


**Figure 5.** Sensitivity of the goodness of model fits for perennial ryegrass plants grown with a nitrogen supply of either 1.0 mM nitrate (dashed lines) or 7.5 mM nitrate (solid lines) to departures from optimized values of pool size, half-life and contribution to respiration for the pools $Q_1$ (a, b, c), $Q_2$ (d, e, f) and $Q_3$ (g, h, i). Sensitivity is expressed as the root mean squared error (RMSE) of the fit (minimum value indicates the optimum value of a model parameter).

## Concentration of water-soluble carbohydrates

Nitrogen-deficient plants had a high concentration (369 mg C g$^{-1}$ plant-C) of water-soluble carbohydrates (WSC) (Table 3). Of this, 331 mg C g$^{-1}$ plant-C was located in the shoot and 38 mg C g$^{-1}$ plant C in the root. High-nitrogen plants contained less WSC (291 mg C g$^{-1}$ plant-C), and 95% of this was located in the shoot.

In both treatments, more than 80% of total WSC were stored in the form of fructans. The sucrose concentration was the same in both treatments, while the concentrations of glucose and fructose were slightly lower at low nitrogen supply. At both nitrogen supply levels, sucrose and hexoses together accounted for less than 5% of total plant carbon (Table 3).

**Table 3.** Content of water-soluble carbohydrate fractions of perennial ryegrass grown with either a low (1.0 mM) or a high (7.5 mM) supply of nitrogen

| | Low nitrogen | High nitrogen | |
|---|---|---|---|
| | mg C g$^{-1}$ plant-C | | |
| Fructan | 332 ± 22 | 242 ± 20 | * |
| Sucrose | 20 ± 1 | 23 ± 1 | NS |
| Glucose | 7 ± 0.3 | 12 ± 1 | * |
| Fructose | 10 ± 1 | 14 ± 1 | * |

Values are means of six replicate plants ± 1SE, along with the significance of the difference based on a t-test.
*$P \leq 0.05$.
NS, not significant, $P > 0.05$.

## Labelling kinetics of respired $CO_2$

The labelling kinetics of shoot- and root-respired $CO_2$ were similar within a nitrogen treatment (Fig. 4a,b), except for a delay of approx. 1 h for the arrival of respiratory carbon in the root relative to that in the shoot, and a 5 to 10% lesser labelling of root-respired $CO_2$ at a given time. In both organs, a first phase of fast initial labelling (first 1–2 h) was observed (Fig. 4a,b, insets), which was followed by a period in which the degree of labelling remained virtually constant (phase 2). Between ~4 h and ~2–4 d, the fraction of unlabelled carbon decreased again very rapidly in both organs (phase 3). Thereafter (phase 4), the fraction of unlabelled carbon decreased at slower rates.

Nitrogen supply affected the time course of tracer incorporation in respiration of the shoot, root and whole plant. In the first phase of rapid labelling, respired $CO_2$ was more strongly labelled (+7%) in plants growing with high nitrogen supply (Fig. 4c, inset), and also at the scale of shoot and

root respiration (Fig. 4a,b, insets). Also, after the initial lag (phase 2), the decrease in the fraction of unlabelled carbon was faster at high nitrogen supply and was completed faster (~1 d at high nitrogen and 2 d to 4 d at low nitrogen supply; phase 3). Similarly, the last phase (phase 4) exhibited a more rapid tracer incorporation in respiratory $CO_2$ when plants were grown at high nitrogen supply. Thus, after 12 d, unlabelled respiratory $CO_2$ accounted for only about 5% of total respiration at high nitrogen supply. Conversely, in low-nitrogen plants, about 11% of plant-respired $CO_2$ was still unlabelled after 29 d (Fig. 4c).

### The respiratory carbon supply systems

Tracer appearance in different phases indicated the involvement of substrate pools exhibiting distinct rates of turnover in the different nitrogen treatments. Yet, the same three-pool model structure (Fig. 1) adequately described the observed time courses of tracer incorporation into shoot-, root- and plant-respired $CO_2$ at both nitrogen supply levels (Figs. 4 & 5). At the same time, this model was the simplest that accounted for all features of tracer kinetics: compartmental models with less than three pools exhibited a significant lack of fit to the data and, hence, were not able to fit the tracer kinetics with a low RMSE (results not shown). On the other hand, more complex models did not improve the fits.

The three-pool model was defined as follows: tracer fixed in photosynthesis entered the system via pool $Q_1$, from which it was either respired directly via $F_{10}$ or entered $Q_2$. Once in $Q_2$, carbon either first cycled through pool $Q_3$ or left the system directly in the respiratory flux $F_{20}$ without entering $Q_3$. In all time courses, tracer appearance in $F_{20}$ only occurred after a delay of 3–4 h after the onset of labelling (cf. Fig. 4, insets; Table 2).

### Nitrogen effects on substrate pools supplying plant respiration

Pools' sizes, half-lives and contribution to respiration differed greatly between the two nitrogen treatments (Table 2). Nitrogen limitation caused a 30% increase in the specific size of the respiratory supply system: the system comprised 166 mg C $g^{-1}$ plant-C in high-nitrogen plants and 219 mg C $g^{-1}$ plant-C in low-nitrogen plants. This derived from large increases in the sizes of $Q_2$ (which doubled), and $Q_3$ (which increased by about 20%). In both nitrogen treatments $Q_3$ represented more than 80% of all respiratory carbon in the plant.

$Q_1$, the smallest and fastest pool, had a half-life less than 30 min. Because of its rapid turnover, the assessment of the pool's half-life was less precise than for the other two pools. This may have been the reason why a nitrogen effect on $Q_1$ turnover was not detected. Conversely, the half-lives of the pools $Q_2$ and $Q_3$ showed pronounced responses to nitrogen supply, since both increased by a factor of 6 with nitrogen limitation (Table 2). Despite its small size, $Q_1$ supplied 15% of total respired carbon in high-nitrogen plants. This proportion decreased to 9% in low-nitrogen plants (Table 2).

Almost 60% of all carbon respired by plants at high nitrogen supply first cycled through $Q_3$, whereas only 28% of the carbon was respired directly via $Q_2$. These relationships were effectively reversed in plants with low nitrogen supply: the contribution of $Q_3$ was 31%, whereas that of $Q_2$ was 60%, more than twice that at high nitrogen supply (Table 2). As a result, $\tau$ increased from 4.6 d in plants at high nitrogen supply to 9.2 d with nitrogen limitation.

## DISCUSSION

Plant responses to nitrogen deficiency were here in good agreement with typical responses, including lower growth and respiration rates (Makino & Osmond 1991; Reich *et al.* 2006), lower shoot to root biomass ratio (Mooney, Fichtner & Schulze 1995; Poorter & Nagel 2000) and higher concentration of WSC (Evans 1983; Morvan-Bertrand *et al.* 1999). Further, photosynthetic CUEs were in the range of values observed in young herbaceous plants (Van Iersel 2003). The CUE of low-nitrogen plants was lower, and at least part of this difference was probably caused by their lower shoot to root ratio and a higher contribution of root respiration to total respiration (Table 1; Van der Werf *et al.* 1993a). These observations lend support to the idea that responses of the respiratory carbon supply system in this experiment were actually related to differences in nitrogen status.

In the following, we present the effect of nitrogen supply on the respiratory supply system mainly at the whole plant level, rather than at the level of roots and shoots, because nitrogen effects on the whole plant tracer kinetics were, in principle, the same as at the level of shoots and roots (Fig. 4). We have already discussed the striking similarity of shoot and root tracer kinetics at high nitrogen supply and shown evidence that the stores supplying (shoot and) root respiration are primarily located in the shoot (Lehmeier *et al.* 2008). Essentially, the low nitrogen data support the same conclusion: Compartmental analysis of the tracer kinetics showed that the size of the store supplying root respiration was much larger than the mass of water-soluble carbohydrates and proteins in the root. Thus, the carbohydrates and proteins in roots could not have sustained the storage-derived respiratory carbon fluxes in the roots.

### Rapidly labelled photosynthetic products are a quantitatively less important source of respired carbon under nitrogen deficiency

At both nitrogen supply rates, a significant amount of plant-respired $CO_2$ derived from the small and rapidly turned over pool $Q_1$ (Table 2). This pool was closely connected with photosynthesis and it directly released $CO_2$ in both shoot and root. This led us to suggest that it included mainly organic acids (Lehmeier *et al.* 2008). The presence and relevance of a comparable rapidly labelled respiratory carbon

pool has been reported for mature blades of *Phaseolus vulgaris* (Nogués *et al.* 2004). Gessler *et al.* (2009) reported a 22% contribution of malate decarboxylation to total leaf respiration shortly after transfer to darkness.

Malate in particular plays a prominent role in nitrate uptake: it is thought to be transported to roots where a carboxyl group is released in exchange for a nitrate ion (Imsande & Touraine 1994; Stitt *et al.* 2002). Thus, the fact that $Q_1$ routed proportionally less carbon to respiration in plants with a lower nitrate uptake rate (9% in low versus 15% in high nitrogen supply; Table 2) agrees well with a smaller need for organic acids in the nitrate uptake system of these plants. Assuming that $Q_1$ was formed by malate, and $F_{10}$ (the respiratory flux from malate) was associated with nitrate uptake, we can estimate the specific costs for nitrate uptake (in g C respired per g nitrate-N uptake). For this, we multiply the specific respiration rates of roots (Table 1) times the contribution of $Q_1$ to total root respiration. This yields 6% of root respiration at low and 12% at high nitrogen supply (data not shown). Division by the specific nitrogen uptake rate (Table 1) yields the specific respiratory cost of nitrate uptake, which was 0.64 g C $g^{-1}$ N for low-nitrogen plants, and 0.51 g C $g^{-1}$ N for high-nitrogen plants. These values are well within the range of theoretical and empirical estimates (0.4 to 0.9 g C $g^{-1}$ N: Johnson 1990; Bouma, Broekhuysen & Veen 1996; Scheurwater *et al.* 1998; Amthor 2000; Cannell & Thornley 2000), and agree with the expected higher specific costs of nitrate uptake under nitrogen deficiency (Van der Werf *et al.* 1993b).

## Does nitrogen deficiency affect the importance of stores as substrate for respiration?

In both nitrogen supply levels, $Q_2$ exhibited a central role in the respiratory system, exchanging large amounts of carbon with the store $Q_3$ and releasing more than 85% of all respired $CO_2$. In high-nitrogen plants, the half-life of $Q_2$ (3.4 h) was in the range reported for cytosolic and apoplastic sucrose in the mesophyll and phloem of source leaves. Sucrose in these compartments is commonly referred to as 'transport sucrose' (Moorby & Jarman 1975; Bell & Incoll 1982; review by Farrar 1989), but hexoses associated with sucrose metabolism in source leaves may also be part of this pool. In contrast, under nitrogen deficiency the half-life of $Q_2$ was almost six-fold greater (20 h).

The size estimates of respiratory carbon pools (Table 2) are inferred purely from their function that is supplying carbon to respiration. If a given compound, such as sucrose or fructan, serves respiration plus other sinks like growth, its total quantity in biomass (Table 3) must be greater than the fraction supplying respiration. Accounting for this issue, a comparison of estimated $Q_2$ size with measured levels of mono- and disaccharides revealed further differences between nitrogen treatments. At high nitrogen supply the total pool of mono- and disaccharides was larger than $Q_2$ (49 versus 19 mg C $g^{-1}$ plant-C, respectively), consistent with the expectation that $Q_2$ included mono- and disaccharides and that these compounds served other sinks as well. However, at low nitrogen supply the total pool of mono- and disaccharides was smaller than $Q_2$ (37 versus 40 mg C $g^{-1}$ plant-C, respectively), demonstrating that other substrates (perhaps fructans) must have contributed to $Q_2$ (Tables 2 & 3). This comparison suggests a substantial difference in the identity of $Q_2$ in high- and low-nitrogen plants.

At low nitrogen supply $Q_2$ must have included compounds other than simple sugars in cytosolic and transport compartments. Information about nitrogen effects on turnover of specific compounds is scarce. However, the half-life of $Q_2$ in nitrogen-deficient plants probably represented a mixture of cytoplasmic/apoplastic transport and vacuolar short-term storage pools, where both compartments exhibited a degree of labelling too similar to be resolved by compartmental analysis. Indeed, involvement of short-term storage may have been substantial, as the 20 h half-life of $Q_2$ was close to that of vacuolar stores (Moorby & Jarman 1975; Bell & Incoll 1982; Farrar 1989). Clearly, nitrogen-deficient plants depended to a lesser degree on recently fixed carbon that was respired within 12 h after its assimilation (Fig. 6).

At both nitrogen supply levels, more than 80% of the total respiratory supply system was accounted for by $Q_3$ (Table 2). Considering the pool sizes of putative substrates, fructans were probably the main source of carbon for $Q_3$ (Table 3). The concentration of other non-structural carbohydrates was much smaller than $Q_3$, as was the concentration of proteins. Proteins accounted for 13% of total plant carbon at high nitrogen and 6% at low nitrogen supply [as estimated from plant nitrogen content and a 3.1 carbon to nitrogen (w/w) ratio]. It is known that the turnover of proteins is intimately connected with respiratory pathways (Lea & Ireland 1999). For plants with high nitrogen supply,

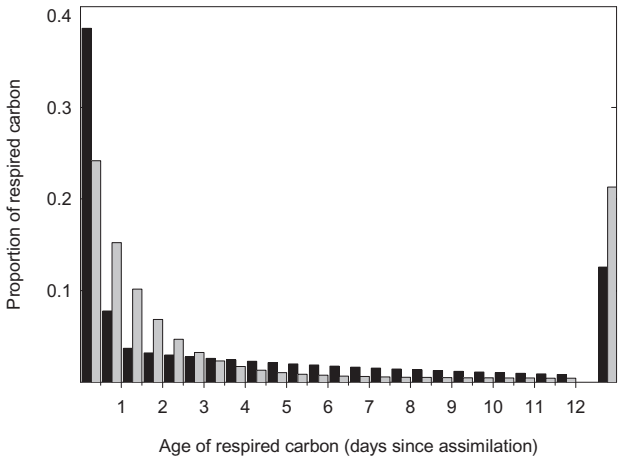


**Figure 6.** Contribution to total respiration of carbon molecules of different age in perennial ryegrass plants grown with a nitrogen supply of either 1.0 mM nitrate (grey bars) or 7.5 mM nitrate (dark bars). Carbon age is shown as the time elapsed between its assimilation and its respiratory release as $CO_2$, in 12-h intervals.

we suggested that the contribution of protein-C to total respired carbon was about 10% (Lehmeier *et al.* 2008), but protein turnover (and recycling) is probably higher in nitrogen limited conditions (Vierstra 1993).

The discrepancy in $Q_3$ half-life between 1.7 d at high and 10 d at low nitrogen supply (Table 2) highlights a fundamental difference in the pool's functional identity in the two nitrogen treatments. In nitrogen-deficient plants, $Q_3$ was a long-term store. Conversely, the 1.7 d half-life at high nitrogen supply indicates a short-lived store. Clearly, long-term stores did not contribute any significant amount of carbon to respiration at high nitrogen supply (Fig. 6). The 30% contribution of long-term stores to total respiration of nitrogen-deficient plants indicates that (at least part of) the photoassimilate surplus, if stored in slowly turning over compartments, was subject to continuous turnover and acted as a constitutive part of the supply system. In basal shoot parts of perennial C3 grasses like *L. perenne*, fructans become an important carbohydrate store in conditions when photosynthetic carbon supply exceeds sink demands (Pollock & Cairns 1991). This was obviously the case at low nitrogen supply where growth rate was reduced and fructan store enhanced (Fig. 2, Tables 1 & 3). We suggest that this store became a substantial source of respired carbon in nitrogen-deficient plants.

### Nitrogen deficiency increases the mean residence time of respiratory carbon ($\tau$) in grass plants

The most distinctive difference between nitrogen treatments was the doubling of $\tau$ in nitrogen-deficient relative to nitrogen-sufficient conditions (9.2 d versus 4.6 d). The finding that $\tau$ in high-nitrogen plants was more than two times longer than the half-life of its slowest pool $Q_3$ (1.7 d) was related to the cycling of carbon between $Q_2$ and $Q_3$. ($F_{23}$ & $F_{32}$; Table 2). That is, tracer entered the storage compartment more than once before it was respired. At low nitrogen supply, the storage deposition/mobilization fluxes $F_{23}$ and $F_{32}$ were smaller while $Q_3$ was bigger, indicating that carbon stayed longer in the storage pool (Fig. 6).

The characterization of a complex system with the single parameter $\tau$ alone may be of limited physiological significance (Atkins 1969). But when information about the main components of the system is available, then we can explore the controls of $\tau$. We did this by performing a sensitivity analysis of the compartmental model. Starting with the model-optimization results for low-nitrogen plants (Table 2), we modified individual model parameters, one by one, to quantify their influence on $\tau$. This demonstrated that increasing the contribution of $Q_1$ from 9% to 15% of total respiration reduced $\tau$ only slightly (9.2 $\rightarrow$ 8.5 d). Also, forcing $Q_2$ half-life from 20 h to 3.4 h reduced $\tau$ only slightly (9.2 $\rightarrow$ 8.5 d). The largest effect resulted from the turnover rate of $Q_3$: Forcing $Q_3$ half-life from 10 d to 40 h reduced $\tau$ from 9.2 to 2.8 d. Thus, the involvement of a store with a very long half-life had the largest influence on the long residence time of respiratory carbon in nitrogen-deficient plants.

## CONCLUDING COMMENTS

One substrate supply system, including three pools with greatly differing turnover rates and two distinct respiratory activities in root and shoot, adequately described the labelling kinetics of respiratory $CO_2$ in the shoot and root of perennial ryegrass growing with contrasting supplies of nitrogen fertilizer. In both nitrogen treatments, the bulk of the respiratory substrate resided in the shoot supporting the view that the shoot controlled the substrate supply for root respiration via both storage- and current assimilation-derived substrate supply independently of the nitrogen status of the plant. Certainly, the aggregation of respiratory carbon metabolism into a few major compartments -as done here- is a great simplification of the real complexity of the system. However, it did permit the identification of key determinants of respiration which may improve physiology-based crop models (Jeuffroy *et al.* 2002). The present model may serve as a basis to describe the respiratory substrate supply system of grass plants in a wider range of environmental conditions, and so broaden its relevance for the understanding of carbon and nitrogen dynamics in plants (Gastal & Lemaire 2002).

Nitrogen deficiency enhanced the participation of long-term stores as substrate for respiration, and this was effectively responsible for the longer mean residence time of respiratory carbon under nitrogen deficiency. Residence times as assessed here compared well with the range of carbon residence times in above- and below-ground autotrophic respiration in natural ecosystems (Carbone & Trumbore 2007; Högberg *et al.* 2008; Gamnitzer *et al.* 2009). We expect that the longer mean residence time of (autotrophic) respiratory carbon in nitrogen-deficient plants will also be observed in field studies comparing nitrogen-limited versus nitrogen-rich grasslands. This would also support the view that current growth conditions can have a large effect on the 'speed of link' between assimilation and respiration carbon isotope signatures observed in ecosystem studies (Ekblad & Högberg 2001; Bowling *et al.* 2002).

## ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 607). Ulrike Gamnitzer is thanked for helpful discussions; Anja Schmidt provided expert technical assistance in carbohydrate analysis. Two anonymous referees are thanked for constructive comments.

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*Received 24 July 2009; received in revised form 10 September 2009; accepted for publication 6 October 2009*