Root and Shoot Respiration of Perennial Ryegrass Are Supplied by the Same Substrate Pools: Assessment by Dynamic 13C Labeling and Compartmental Analysis of Tracer Kinetics1[OA]

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The substrate supply system for respiration of the shoot and root of perennial ryegrass (Lolium perenne) was characterized in terms of component pools and the pools’ functional properties: size, half-life, and contribution to respiration of the root and shoot. These investigations were performed with perennial ryegrass growing in constant conditions with continuous light. Plants were labeled with 13CO2/12CO2 for periods ranging from 1 to 600 h, followed by measurements of the rates and 13C/12C ratios of CO2 respired by shoots and roots in the dark. Label appearance in roots was delayed by approximately 1 h relative to shoots; otherwise, the tracer time course was very similar in both organs. Compartmental analysis of respiratory tracer kinetics indicated that, in both organs, three pools supplied 95% of all respired carbon (a very slow pool whose kinetics could not be characterized provided the remaining 5%). The pools’ half-lives and relative sizes were also nearly identical in shoot and root (half-life < 15 min, approximately 3 h, and 33 h). An important role of short-term storage in supplying respiration was apparent in both organs: only 43% of respiration was supplied by current photosynthate (fixed carbon transferred directly to centers of respiration via the two fastest pools). The residence time of carbon in the respiratory supply system was practically the same in shoot and root. From this and other evidence, we argue that both organs were supplied by the same pools and that the residence time was controlled by the shoot via current photosynthate and storage deposition/mobilization fluxes.

This article deals with the substrate supply system of respiration in roots and shoots of intact plants of perennial ryegrass (Lolium perenne). This system is an integral part of the total pool of available substrates for growth and maintenance processes in the root and shoot and a major sink for carbon fixed in photosynthesis (Amthor, 1989). In the narrow sense, respired carbon mainly derives from a few compounds: malate, pyruvate, isocitrate, α-ketoglutarate, or gluconate-6-P (Heldt, 2005), which together account for only a small fraction of total plant biomass. Conversely, in the broad sense, all respired carbon derives from photosynthesis, and, ultimately, most of the carbon fixed in photosynthesis is returned back to the atmosphere by way of respiration (Schimel, 1995; Trumbore, 2006). Before being respired, carbon may visit various biochemical compounds in different organs. In principle, the physical and biochemical paths taken by carbon before being used as a substrate in respiration can be intricate, reflecting the physical and biochemical complexity of plant metabolic networks (ap Rees, 1980; Plaxton and Podestá, 2006).

The intermediary fate (or allocation history) of carbon controls its residence time inside the plant (i.e. the lapse of time between fixation and respiration). Thus, for instance, if carbon fixed in photosynthesis is transferred directly to centers of respiration, then the residence time in the plant is short (seconds to minutes). In contrast, if carbon is first deposited in long-lived molecules (such as proteins or storage carbohydrates), then the residence time is long (days to months). Respired carbon, therefore, originates from a heterogeneous mixture of molecules that cycle more or less extensively through a network of biochemical compounds and physical compartments. So the residence time of respired carbon reveals functional properties of the supply system feeding respiration and can be used to shed light on structural-functional differences between supply systems feeding different plant parts, such as roots and shoots. We are not aware of any comparative studies of the residence time of carbon feeding shoot and root respiration.

The residence time of carbon can be characterized by quantitative tracer techniques (Ryle et al., 1976; Kouchi et al., 1985; Schnyder et al., 2003; Lötchers and
Gayler, 2005). Studies at the level of whole plants (Schnyder et al., 2003) or with root systems (Kouchi et al., 1985, 1986; Lötscher and Gayler, 2005) have revealed two distinct phases in the kinetics of tracer appearance in respired CO₂: a phase with fast label appearance, which indicated a supply component that was closely connected with current photosynthetic activity; and a phase with slow label appearance, which indicated the participation of one (or more) store(s) in supplying respiration. Several types of compounds, including starch, vacuolar Suc, and fructan, as well as proteins have been suggested as stores supplying substrates for respiration (ap Rees, 1980; Farrar, 1980). It is unknown if the contribution of stores and products of current assimilation to respiration is the same or different in shoots and roots.

Whereas most of the interpretations of label appearance (in dynamic labeling), or label disappearance (in pulse-chase labeling), in respired CO₂ have been qualitative, the tracer kinetics can also be quantitatively and mechanistically interpreted in terms of the number, size, kinetic properties (half-life, turnover rate), and contribution of the pools that constitute the supply system of respiration. This is best done using the mathematical methodology of compartmental analysis (Atkins, 1969; Jacquez, 1996), which has been applied to various problems of the assimilation, transport, and metabolism of carbon in plants (Moorby and Jarman, 1975; Prosser and Farrar, 1981; Rocher and Prioul, 1987; Bürkle et al., 1998; Lattanzi et al., 2005). A pool is defined here as a set of compounds that exhibit the same proportion of labeled carbon atoms; that is, a pool represents a “space” in which the isotopic composition is uniform (Rescigno, 2001). 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. Most importantly, however, by characterizing the pool on the basis of respiratory tracer release, the pool is identified by its function: supplying respiration with substrate.

Here, we use compartmental analysis to provide a quantitative description and comparison of the compartmental structure and kinetic properties of the supply system feeding root and shoot respiration. Specifically, we address the following questions: What are the kinetics and sizes of the major respiratory pools supplying carbon to respiration of ryegrass? How are these pools connected? How do shoot and root differ in terms of carbon supply by those pools? And what are the contributions of current assimilation and stores to respiration?

One basic difficulty in the characterization of carbon pools supplying respiration is a sufficient range of tracer application (or chase) times. Putative substrates for respiration have turnover times in the range of less than 1 h to many days (Simpson et al., 1981; Dungey and Davies, 1982; Farrar and Farrar, 1986; Rocher and Prioul, 1987; Schnyder et al., 2003) or possibly weeks, meaning that labeling (or chase) times must vary by about 4 orders of magnitude if all components of the respiratory supply system are to be characterized. Typically, however, the range of tracer exposure (or chase) times has been much narrower, thus capturing only fast or slow pools. In this study, we aimed to characterize all major components of the respiratory supply system by using labeling times ranging from 1 to 600 h. To this end, we labeled all carbon assimilated by individual plants with a known constant 13C/12C ratio in CO₂ over a period of up to 25 d, when respired CO₂ had reached 95% label saturation (this labeling method is termed “steady-state labeling” in “classical” plant physiology literature [Geiger and Swanson, 1965; Geiger et al., 1969] but is now referred to as “dynamic labeling” [Ratcliffe and Shachar-Hill, 2006]). The 13C/12C ratio of respiratory CO₂ produced in the root and shoot was measured at various times, and the time course of tracer in respired CO₂ was evaluated with compartmental analysis.

RESULTS

Meeting the Steady-State Conditions of Compartmental Analysis: Constant Specific Growth and Respiration Rates

Inferring the number and kinetics of mixing pools by compartmental analysis relies on several assumptions (presented in full, and their validity discussed, in “Materials and Methods”). A major one is that the system under consideration shows no change in time except for tracer content (referred to as “metabolic steady state” by Ratcliffe and Shachar-Hill, 2006). By performing this study in controlled environments, constant growth conditions were provided: plants grew with continuous illumination, and temperature, relative humidity, and CO₂ concentration were maintained at constant values throughout the experiment. Water and nutrients were supplied frequently.

During the experiment, shoots and roots exhibited constant specific growth rates (shoot, 0.085 g carbon [C] g⁻¹ shoot C d⁻¹ ± 0.011, CI0.95: root, 0.072 g C g⁻¹ root C d⁻¹ ± 0.014, CI0.95). Moreover, specific respiration rates were steady throughout the labeling period (P > 0.05; Fig. 1), with shoot respiration (0.97 mg C g⁻¹ plant C h⁻¹ ± 0.13 SD; n = 60) being nearly twice as high as root respiration (0.53 mg C g⁻¹ plant C h⁻¹ ± 0.09 SD; n = 60). Furthermore, due to the similarity of shoot- and root-specific growth rates, the shoot to root ratio (3.8 g C g⁻¹ C) was nearly constant. No differences in the rates of growth and respiration were observed between growth chambers (P > 0.05). These results indicate that plants were growing nearly exponentially, with constant specific demands on respiration; thus, the system was virtually in a steady state.

Water-Soluble Carbohydrate Concentration in Root and Shoot Biomass

Water-soluble carbohydrates accounted for 0.337 g C g⁻¹ total shoot C (±0.035 SD; n = 6). This was more
than four times higher than the concentration in the roots (0.079 g C g⁻¹ root C ± 0.006 sd; n = 6).

Labeling Kinetics of Respired CO₂ in the Shoot and Root

The time courses of tracer incorporation into shoot- and root-respired CO₂ were strikingly similar (Fig. 2), except that first label incorporation into respiratory CO₂ of roots occurred with a delay of approximately 1 h and that the degree of labeling of root-respired CO₂ was about 5% less than that of shoots during the first week of labeling.

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Figure 1. Specific respiration rates of shoots (black symbols) and roots (white symbols) of perennial ryegrass, labeled for different time intervals, and of nonlabeled controls (C; at left). Each value is the mean of four to 10 replicate plants (± se). Dashed lines indicate average values (see “Results”). Note the logarithmic scaling of the x axis.

Figure 2. Evolution of the fraction of unlabeled carbon (f_unlabeled) in CO₂ respired by shoots (A) and roots (B) of perennial ryegrass during labeling. Each value is the mean of four to six replicate plants (± se). Lines denote model predictions (Fig. 3). Insets expand the first 48 h.

Compartmental Model of Substrate Pools for Respiration

The labeling kinetics reflected the operation of a substrate pool system supplying respiration. The structure of this system (number of pools, links between pools, delays, and sites of tracer entry and outlet) was determined by analysis of the tracer kinetics of respiratory CO₂ (Fig. 2), including multieponential curve fitting to the tracer kinetics (similar to that described by Moorby and Jarman, 1975) and consideration of established compartmental concepts of respiratory carbon metabolism (Farrar, 1990; Dewar et al., 1998) while respecting the (reductionist) principle of parsimony (“all other things being equal, the simplest solution is the best”). (1) The fast initial labeling of respired CO₂ (phase 1) revealed a respiratory activity fed by a substrate pool very close to photosynthetic metabolism and hence rapidly renewed by assimilated tracer. This pool was named Q₁, and its respiratory activity was named F₁₁. (2) Further respiratory tracer release occurred only after a delay of several hours (phase 2), revealing the existence of a second respiratory activity (F₂) fed via another pool. (3) Fitting of a dual-exponential (instead of a monoexponential) decay function to the tracer kinetics beyond 4 h of labeling increased the goodness of fit and gave a better distribution of residuals. More exponential terms, however, improved neither the fit nor the distribution of residuals. This indicated the existence of (at least) two additional respiratory substrate pools with distinct turnover times (phases 3 and 4). These pools were named Q₂ and Q₃. (4) A small residual respiratory activity (approximately 5%; phase 5) could not be characterized in terms of pool size and half-life because it released no tracer during the duration of...
Respiratory Carbon Pools and Fluxes

This model was translated into a set of differential equations (similar to Lattanzi et al., 2005), which described the system in terms of fluxes between pools and the environment, and implemented in a custom-made program using the free software R (R Development Core Team, 2007). This program systematically tested millions of preset values for pool sizes, fluxes between pools, and delays to find the lowest root mean squared error (RMSE). This extensive evaluation (1) ensured that the absolute minimum RMSE was identified rather than a “local” minimum, and (2) revealed its sensitivity to changes in parameter values (Fig. 4). These procedures were performed independently for the shoot and root data, thus generating independent estimates of system properties for the shoot and root.

Pool Sizes, Half-Lives, and Contributions to Respiration

Pool half-lives were derived from fitted pool sizes and fluxes. The contribution of each pool to respiration was determined as the probability of carbon cycling through that pool before being respired. Pools Q1, Q2, and Q3 differed greatly in size and half-life (Table I). The relative sizes of the three pools were similar in the shoot and root, but root pools were 30% to 50% smaller than shoot pools, because root respiration rate was half that of the shoot (Fig. 1).

Q1 was a very small, rapidly turned-over pool. Both in the shoot and in the root, it was equivalent to 0.02% of total plant carbon, and its half-life was on the order of 0.1 to 0.2 h. Q2 of the shoot represented approximately 1% and Q3 of the root represented approximately 0.7% of total plant carbon, and both had half-lives of approximately 3 h (Table I). Q3 was the largest: its shoot component constituted 7% and the root component constituted 4.5% of total plant carbon. The half-life of Q3 was virtually identical in both organs: 33 h. In total, 13.2% of all plant carbon formed part of respiratory substrate pools.

Although Q1 was a very small pool, it served a significant role in respiration: 16% of shoot-respired carbon and 13% of root-respired carbon cycled only through Q1 (Table I). The bulk, 79% of shoot respiration and 82% of root respiration, was supplied by Q2. Respiration via Q2 was supplied by direct transfer of current photosynthate via Q1 and by carbon that first cycled through Q3 (Fig. 3). Direct transfer accounted for 28% of shoot respiration and 27% of root respiration. This meant that two pools whose carbon was renewed very rapidly by current photosynthetic assimilation supplied 44% of shoot respiration and 40% of root respiration. On the contrary, Q3, with a half-life of 33 h, played a (short-term) storage role and was the main source of substrates for respiration: 51% of all carbon respired in the shoot and 55% of that respired in the root cycled through this pool at least once before being respired (Table I). In both organs, 5% of respired carbon derived from a pool that could not be characterized in terms of size and half-life. Sensitivity analyses showed that estimates of pool size, half-life, and

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Figure 3. Three-pool model of the substrate supply system of dark respiration of the shoot of perennial ryegrass. Carbon fixed in photosynthesis enters the respiratory system via pool Q1, where it is either respired (respiratory flux F10) or transferred to pool Q2. In Q2, carbon is either respired directly (F20) or first cycled through Q3 before being respired via Q3. Respiratory tracer release from Q3 is associated with a delay. Functional characteristics of the pools (size, half-life, and contribution to shoot and root respiration; Table I) were estimated by translating the model into a set of differential equations and fitting the model to the tracer kinetics of shoot respiration. The same model also fitted the tracer kinetics of dark respiration of the root but included an additional delay of 0.8 h for tracer release in F10 and F20. Arrows and boxes are scaled to indicate the magnitude of fluxes and pool sizes.

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contributions to respiration were well constrained by the data (Fig. 4).

**DISCUSSION**

**The Identity of Respiratory Substrate Pools**

This work indicates the existence of three pools supplying 95% of all substrate for respiration in intact plants of perennial ryegrass. A most distinctive difference between these pools was the speed of carbon exchange by current assimilate: half-lives differed by almost 4 orders of magnitude between the fastest (Q1) and the slowest (Q3) pool (Table I; Fig. 4). Each of these pools likely did not represent a single biochemical compound with a specific spatial location; rather, they were probably mixtures of substrates distributed in different tissues and organs throughout the plant. Heterogeneous as they may be, these mixtures nonetheless shared a common pattern of tracer incorporation/release that compartmental analysis recognized. Hence, derived half-lives can be compared with known half-lives of putative substrates for respiration with the aim of attributing functional-biochemical identities to Q1, Q2, and Q3.

Q1 very quickly incorporated and released tracer. Thus, it was intimately connected with both CO2 fixation and respiration. Its rapid turnover rate is consistent with the speed of labeling of primary photosynthetic products that are also involved in decarboxylation, including organic acids (Calvin and Bassham, 1962). Q1 also contributed to root respiration, indicating a phloem-translocated component. Malate could have been a major constituent of Q1; it is rapidly labeled in leaves (Heber and Willenbrink, 1964), it is translocated to roots (Imsande and Touraine, 1994), and it is thought to be decarboxylated there (Imsande and Touraine, 1994; Stitt et al., 2002). Malate concentration is high in plants growing on nitrate (Leport et al., 1996) and may serve as a control and substrate for the nitrate uptake system (Imsande and Touraine, 1994). If this conclusion is true, then the relevance of Q1 would depend on nitrogen source. We know of no other comparative studies of whole shoot and root respiratory tracer kinetics that would allow an assessment of the generality of our

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<th>Pool</th>
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<td>size (mg C g⁻¹ plant C)</td>
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<tr>
<td>Q1</td>
<td>≤0.2</td>
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<td>Q2</td>
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<td>Q2</td>
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<td>Q3</td>
<td>33.0</td>
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<td>Q2</td>
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<td>Q3</td>
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<td>delay 1 (h)</td>
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<td>Q1</td>
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<td>Q2</td>
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<td>Q3</td>
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findings. Nogués et al. (2004) reported a rapid incorporation of tracer in CO₂ respired by *Phaseolus vulgaris* leaves, consistent with tracer kinetics in Q₁. But no study of tracer incorporation in root respiration has reported the existence of Q₃. Perhaps the participation of Q₃ in respiration is not a ubiquitous feature. An essential factor in identifying Q₃ was the existence of a lag of a few hours between tracer incorporation and release from Q₂ (see below). Had there been no lag, then tracer release from Q₂ would have overlain that of Q₁, rendering it unnoticeable.

The half-life of Q₂ (3 h) was close to, but longer than, the half-life often ascribed to a pool of “transport Suc” (1–2 h; Moorby and Jarman, 1975; Bell and Incoll, 1982; Farrar and Farrar, 1986). This pool is composed of Suc in the cytoplasm, apoplast, and sieve tubes and companion cells of the phloem in actively photosynthesizing and exporting C₃ leaves (Geiger et al., 1983). Our study derived respiratory pool kinetics from measurements at the scale of whole shoots and roots; that is, from tissues of very different developmental status: growing (sink), mature, and senescing. We know of no studies of the kinetics of the transport pool in vegetative sink tissues. In the leaf growth zone of *Festuca arundinacea*, the turnover rate of total tissue Suc varied between less than 1 h and approximately 4 h, depending mainly on growth rate and related Suc import and use (Schnyder and Nelson, 1987). Suc turnover in metabolically active sink tissue, it seems, would be similar to that in actively photosynthesizing leaves. Therefore, an interpretation of the half-life of 3 h of Q₂ is that it represents the (activity-weighted) mean of the kinetics of the transport pool extending over both source and sink tissues in the plant.

There was a substantial delay between tracer uptake and respiratory tracer release from Q₂ (delay 1; Figs. 2 and 3; Table I). This effect was observed in both shoot and root; therefore, it must have been related to metabolism and not to transport. Results of others suggest some delay between the arrival of Suc in sink tissue and its use in respiration: in a study with *F. arundinacea*, Allard and Nelson (1991) found that 90% of the tracer imported into leaf growth zones was still present in the water-soluble carbohydrate fraction at 2 h after labeling source leaves, and hardly any label was present in structural material. In the work of Kouchi et al. (1985) and Lötscher and Gayler (2005), respiratory tracer release from roots of legumes did not start until approximately 2 h or longer after the beginning of labeling. But Dilkes et al. (2004) observed a very rapid labeling of root exudates in wheat (*Triticum aestivum*) and no evidence for a marked delay between tracer release via exudates and respiration. A similar lag was observed in other experiments with perennial ryegrass growing with a limited supply of nitrogen (C.A. Lehmeier, F.A. Lattanzi, R. Schäufele, and H. Schnyder, unpublished data), showing that our observation was not a singular result. We cannot rule out the possibility that growth in continuous light was a factor. However, we cannot envisage the physiological mechanism of such an effect.

The half-life of 33 h and the large size of Q₃ suggest a storage pool. Nonstructural carbohydrates are generally considered as the main source of respired carbon (ap Rees, 1980; Thcherkez et al., 2003), and some of them are used as temporary stores (Smith and Stitt, 2007). In C₃ grasses, such as perennial ryegrass, carbohydrate storage occurs mainly in vacuoles in the form of Suc or fructan (Farrar and Farrar, 1986; Pollock and Cairns, 1991; Vijn and Smeekeens, 1999). Starch was only a trace component of biomass in this study (<1% of plant dry weight; data not shown), but water-soluble carbohydrates were present at high concentration, particularly in the shoot. A storage pool with a half-life in the range of 12 to 24 h is often found in C₃ plants and ascribed to vacuolar Suc (Moorby and Jarman, 1975; Bell and Incoll, 1982; Farrar and Farrar, 1986). The dynamics of fructan turnover are less clear. Its half-life was found to be in the range of 2 to 5 h in leaf blades of *Hordeum distichum* and two *Poa* species (Borland and Farrar, 1988; Farrar, 1989) and 14 to 18 h in leaf sheaths of *Poa* (similar to a 9- to 15-h half-life of vacuolar Suc; Borland and Farrar, 1988). Fructan stored in wheat stems did not turn over during the storage phase (Winzeler et al., 1990).

Proteins constitute another large plant fraction in which turnover is closely connected with respiratory pathways (Lea and Ireland, 1999). Half-lives of soluble proteins are on the order of 3.5 to 8 d (Simpson et al., 1981; Dungey and Davies, 1982), much longer than the half-life of Q₃. This indicates that if proteins contributed to Q₃, then this contribution must have been relatively small. Forcing the model to split Q₃ into two storage pools gave tentative support to this conclusion, as it yielded one pool with a half-life of 20 h contributing approximately 40% of total respiration and the other pool with a half-life of approximately 4 d contributing approximately 10% of total respiration.

The Size of the Respiratory Substrate Pool System and Carbon Use Efficiency

Collectively, the respiratory substrate pool system constituted 13.2% of the total carbon mass of plants, and most of this (approximately 87%) was contained in Q₃, the storage pool. In comparison, water-soluble carbohydrates accounted for 28% of total plant carbon, meaning that it contained much more carbon than all respiratory pools combined. This is expected because stores supply not only respiration but also carbon skeletons for new biomass. Assuming that water-soluble carbohydrates were the exclusive substrate for respiration (thus neglecting any contribution of other putative substrates, such as malate or proteins), then 47% of the water-soluble carbohydrate carbon was allocated to respiratory CO₂. In that case, the remainder (53%) must have been allocated to new (structural) biomass. This corresponds to a carbon use efficiency (CUE) of 53% for water-soluble carbohydrates. This is a conservative (i.e. low) estimate of the CUE of water-soluble carbohydrates, as it ignores possible contributions to
respiration by other substrates. Yet, this efficiency is close to empirical and theoretical estimates of photosynthetic CUE in young herbaceous plants (van Iersel, 2003).

Are Shoot and Root Respiration Supplied by the Same Pools?

The most striking result of this work was the great similarity of root and shoot respiratory tracer kinetics. This meant that the same compartmental model fitted the root and shoot data equally well (Table I; Figs. 2 and 4): number of pools, their half-lives and relative sizes, and their relative contributions to respired carbon were practically the same in both organs. The only notable difference was that tracer appearance in root respiration was delayed by approximately 0.8 h (delay 2; Table I), a time entirely in agreement with phloem transport velocity (Windt et al., 2006). These features are consistent with a single three-pool system feeding shoot and root respiration.

If the supply system for root and shoot respiration consisted of only three pools, where were they located? Q1 and Q2 supplied respiration directly and were active in the root and shoot (Fig. 3), so both must have had shoot and root compartments connected via the phloem. Conversely, a large part of Q3 must have been located in the shoot. This is because the “root component” of Q3 would have been equivalent to greater than 30% of the carbon mass of the root system (calculated by multiplying Q3 root of 45 mg C g⁻¹ plant C with the shoot to root ratio of 3.8 and dividing by the estimated CUE of 0.53), a value much greater than the total mass of nonstructural carbon in the roots (water-soluble carbohydrates, 7.9% of root carbon; protein, 10% of root carbon, estimated from nitrogen content and a 3.1 carbon to nitrogen ratio). So, only a fraction of the respiratory CO2 of roots could have come from stores located in the root. Accordingly, most of the Q3-derived respiratory CO2 of roots must have come from the shoot store(s). Indeed, as is typical in grasses (Sullivan and Sprague, 1943; Davidson and Milthorpe, 1966a), the bulk of nonstructural carbohydrates and protein (94% and 83% of plant total, respectively) were contained in the shoot.

The Role of Stores and Current Photosynthesis in Supplying Respiration

More than half of respired carbon cycled, at least once, through a storage pool before being respired. Clearly, stores were a central part of respiratory carbon metabolism. That a significant fraction of respiration is supplied by stores has been suggested before (Kouchi et al., 1985, 1986; Dilkes et al., 2004; Lötscher and Gayler, 2005), although the kinetic properties of pools were not determined in these studies, nor were the localization and operating controls discussed. This study revealed that these carbon stores were quite short lived and, therefore, might have a limited capacity to sustain current carbon use rates over extended periods.

Yet, carbon stores used in respiration showed a longer half-life (this study) than those supplying leaf growth (Lattanzi et al., 2005). Thus, leaf growth seems to be much more dependent on continued assimilation of carbon, which agrees well with results on carbon allocation shortly after severe defoliation: most carbon used for leaf growth was new (Avice et al., 1996; Schnyder and de Visser, 1999), while that sustaining root respiration was largely old (Avice et al., 1996).

Indeed, rapid and drastic decrease of root respiration following defoliation (Davidson and Milthorpe, 1966b) may be due to the fact that substrate for root respiration is essentially derived from current CO2 fixation and stores in the shoot. Thus, the localization of most of the respiratory substrate in the shoot indicates that the control of root activity by the shoot would occur via the control of both current photosynthesis and storage mobilization.

In conclusion, this work revealed a tight plant-level integration of respiratory substrate pools and fluxes. Incidentally, the results of this work suggest that the tracer kinetics of root respiration can be inferred from that of the shoot (which was nearly identical to that of the root), which is useful information for partitioning of autotrophic and heterotrophic respiration in ecosystem-scale studies. Future work should address the possible variability and controls of substrate pool properties (half-life and size) and their contributions to root and shoot respiration.

Figure 5. Time course of the fraction of unlabeled carbon in CO2 respired by shoots (black symbols) and roots (white symbols) of perennial ryegrass plants during respiration measurements, for plants that were previously labeled for 1 h (triangles) and 24 h (circles). Error bars denote se (n = 4). The dashed line denotes the linear regression for shoots labeled for 1 h (y = 0.83 + 0.48 x, r² = 0.74; see “Materials and Methods”). The regression for the other labeling times was nonsignificant (data not shown).
MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of perennial ryegrass (Lolium perenne ‘Aceroto’) were sown individually in plastic pots (350 mm height, 50 mm diameter) filled with 800 g of washed quartz sand (0.3–0.8 mm grain size). The bottom of every pot had a drainage hole (7 mm diameter) covered with a fine nylon net. Pots were arranged in plastic containers (760 × 560 × 320 mm) at a density of 378 plants m⁻². Two containers were placed in each of two growth chambers (Conviron E15; Conviron). Plants were grown in continuous light supplied by cool-white fluorescent tubes. Irradiance was maintained at 275 mol m⁻² s⁻¹ photosynthetic photon flux density at the top of the canopy. Temperature was controlled at 20°C, and relative humidity was kept near 85%. The stands were irrigated by flooding the boxes every 3 h briefly with modified Hoagland solution (2.5 mM Ca(NO₃)₂, 2.5 mM KNO₃, 1.0 mM MgSO₄, 0.18 mM KH₂PO₄, 0.21 mM KH₂PO₄, 0.5 mM NaCl, 0.4 mM KCl, 0.4 mM CaCl₂, 0.125 mM iron as EDTA, and micronutrients). Stands were periodically flushed with demineralized water to prevent salt accumulation.

CO₂ Control in the Growth Chambers

The two growth chambers formed part of the ¹³CO₂/¹²CO₂ gas exchange and labeling system described by Schnyder et al. (2003). Air supply to the chambers was performed by mixing CO₂-free air and CO₂ with known carbon isotope composition (δ¹³C, with δ¹³C = ([¹³C/¹²C]sample/ [¹³C/¹²C](international VPDB standard)) - 1) using mass flow controllers. Control was facilitated by measuring concentration and δ¹³C of CO₂ online every 20 to 30 min by an infrared (gas analyzer IRGA; Li-6262, Li-Cor) and a continuous-flow isotope-ratio mass spectrometer (CF-IRMS; Delta Plus; Finnigan). One chamber received ¹³C-depleted CO₂ (δ¹³C, ~28.8‰), and the other received ¹³C-enriched CO₂ (δ¹³C, ~1.7‰; both from Linde AG). The δ¹³C and concentration of CO₂ (360 µL L⁻¹) inside the chambers were kept nearly constant by periodically adjusting air flow and CO₂ concentration in the inlet air of each chamber. The rate of CO₂ supply to the chambers exceeded the CO₂ exchange of the plant stands by a factor of 9. This minimized the effects of photosynthesis and respiration on δ¹³C and the concentration of CO₂ in the chambers and suppressed the recycling of respiratory CO₂.

Chamber doors were equipped with custom-made transparent air locks that had small ports through which plants could be handled and sampled. These air locks ensured minimal disturbance of the δ¹³C and concentration of CO₂ in the chamber atmosphere when chambers had to be opened during the experiment. Empty chamber tests of air locks demonstrated that with doors opened for 20 min, the CO₂ concentration in the chambers changed by only 4 µL L⁻¹ and δ¹³C changed by about 1‰. Twenty minutes after closing the chambers, CO₂ concentration and δ¹³C in the chambers had returned to setpoint values.

¹³C Labeling

From 3 weeks after imbibition of seeds, when plants had three tillers, individual plants were labeled by swapping randomly selected plants between chambers. Thus, plants growing in the chamber with ¹³C-enriched CO₂ were transferred to the chamber with ¹³C-depleted CO₂, and vice versa. Plants were kept in the presence of the “new” CO₂ for 1, 2, 4, 8, or 16 h or for 1, 2, 4, 8, 12, 17, or 25 d. At the end of the given labeling intervals, plants were removed from the stands and transferred to a root/shoot respiration measurement system. This was done for at least four replicate plants for each labeling interval. To minimize possible size- and development-related effects on respiration, labeling periods were scheduled in such a way that labeling duration and plant age at sampling were not correlated.

Respiration Measurements

Shoot and root respiration rates as well as the δ¹³C of shoot- and root-respired CO₂ of individual plants were measured in the gas-exchange system described and used by Lötscher et al. (2004) and Klumpp et al. (2005). This system included four single-plant cuvettes interfaced to an IRGA and CF-IRMS via Teflon tubes. The cuvettes were kept in a temperature-controlled cabinet held at the same temperature as the two growth chambers. Each cuvette consisted of an open cylinder (200 mm height, 153 mm diameter) and a top and bottom plate (all made of polyvinylchloride), which could be opened and closed quickly to insert a pot. The bottom plate contained a duct that matched exactly the cross-sectional area of the pot. A similar system was used to seal the bottom of the pot. Rubber seals and vacuum grease ascertainment that cuvettes were air tight. Air with known constant δ¹³C (~5‰) and concentration of CO₂ (223 ± 1 µL L⁻¹) was supplied to cuvettes at a rate of 0.75 L min⁻¹ after passage of a humidifier. Air flow was controlled by mass flow controllers. Each cuvette had two outlets: one in the shoot section on the opposite side of the inlet, and the other at the bottom of the pot that enclosed the root compartment. Air in the shoot compartment was ventilated by a fan. Part of the air stream feeding the shoot compartment (0.25 L min⁻¹) was drawn through the root compartment with a gas-tight Teflon-lined peristaltic membrane pump. The air was then dried and the flow to a multiway valve block (sample air selector [SAS]) was controlled by a mass flow controller. The remaining air from the shoot compartment was directly conveyed to the SAS. A reference air line (0.9 L min⁻¹) was also connected to the SAS. The SAS sequentially sampled the reference air line and the eight sample air lines and fed the air to the IRGA and CF-IRMS as described by Schnyder et al. (2003).

Prior to measurements, just after removal from the growth chambers, the pots were rinsed with demineralized water, which was previously aerated with CO₂-free air for 1 d. Plants were then enclosed in the respiration cuvettes and the cuvettes flushed with CO₂-free air. After excess water had drained off the bottom section of the cuvette, all measuring air lines were installed and air flow rates were adjusted, as described above. These procedures aimed at removing all extraneous air from shoot and root compartments as quickly as possible.

A full measurement cycle of all four cuvettes was completed in approximately 45 min and included three replicate measurements of δ¹³C and concentration of CO₂ in the air exiting the shoot and root compartment of each cuvette plus one reference air measurement. Dark respiration of shoot and root was recorded for about 5 h, thus yielding six full measurements for each plant (compare with Fig. 5). First reliable measurements of the rates and δ¹³C of shoot respiration were obtained approximately 30 min after removing plants from the stands, but it took up to 1.5 h to purge the root system free of all extraneous CO₂ (compare with Lötscher et al., 2004). Therefore, it can be ruled out that photosynthetic CO₂ release has contributed to the measured isotopic signal, since the time to purge the cuvettes previous to measurements was much longer than the duration of the (photosynthetic) postillumination burst. Each δ¹³C sample was measured against a working gas standard, which was previously calibrated against a VPDB-gauged laboratory CO₂ standard. The so of repeated single measurements was 0.10‰ for δ¹³C and 0.34 µL L⁻¹ for the concentration of CO₂ on average of all measurements. The respiration rate of roots decreased slightly (approximately 6%) during the 5-h measurement period, while that of shoots was constant. Average rates were used to calculate specific respiration rates on a carbon basis.

Plant Harvest and Elemental Analysis

Immediately after the termination of 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. All samples were freeze dried for 72 h, weighed again, and ground to flour mesh quality in a ball mill. Aliquots of 0.05 ± 0.05 mg of each sample were weighed into tin cups (IVA Analysentechnik) and combusted in an elemental analyzer (Carlo Erba NA 1110; Carlo Erba Instruments), interfaced to the CF-IRMS, to determine carbon and nitrogen contents.

Analysis of Water-Soluble Carbohydrates

Water-soluble carbohydrates were extracted and quantified as described by Schnyder and de Visser (1999).

Data Analysis

The proportion of carbon in shoot- and root-respired CO₂ that was assimilated before (unlabeled) and during labeling, f labeling-C and f labeling-C (where labeling-C = 1 − f labeling-C), was calculated as by Schnyder and de Visser (1999):

\[
f_{\text{labeling-C}} = \frac{\delta^{13}C_{\text{O}_2} - \delta^{13}C_{\text{air}}}{\delta^{13}C_{\text{air}} - \delta^{13}C_{\text{new}}}
\]
the chamber of origin (old) or in the labeling chamber (new). $\delta^{13}C_{o}$, $\delta^{13}C_{\text{old}}$, and $\delta^{13}C_{\text{new}}$ of shoots were obtained as:

$$\delta^{13}C_{x} = (\delta^{13}C_{o} \times F_{x} - \delta^{13}C_{\text{old}} \times F_{\text{in,x}})/(F_{\text{in}} - F_{\text{out}})$$  (2)

where X stands for sample, new, or old (as appropriate) and $\delta^{13}C_{x}$, $\delta^{13}C_{\text{old}}$, $\delta^{13}C_{\text{new}}$, $F_{x}$, and $F_{\text{out}}$ are the isotopic signatures and the flow rates of the CO$_{2}$ entering and leaving the shoot cuvettes. Calculations for the root compartment were done in the same way in considering that the concentration and $\delta^{13}C$ of the CO$_{2}$ entering the root compartment was equal to that in the shoot compartment (compare with Klumpp et al., 2005). The $\delta^{13}C$ of shoot-respired CO$_{2}$ of individual control plants as well as that of labeled plants did not change during the 5 h of respiration measurements ($P > 0.05$). From 1.5 h after transfer, the $\delta^{13}C$ of root respiration was also stable (Fig. 5). Barbour et al. (2007) observed rapid and pronounced changes in $\delta^{13}C$ of respired CO$_{2}$ during the first few minutes following light-to-dark transition in Ricinus communis. We did not observe such an effect, probably because our first measurements started 30 min after removal from the chamber. Thus, $\delta^{13}C$ of respiratory CO$_{2}$ of the shoot or root of one plant was taken as the mean of all measurements of that plant. This was true for all measurements, except for shoots labeled for only 1 h: in these, $f_{\text{unlabeled}}$ increased markedly during the measurement (Fig. 5), suggesting depletion of a rapidly labeled carbon pool.

Carbon isotope discrimination, $\Delta^{13}C$ (defined as $\Delta^{13}C = (\delta^{13}C_{\text{CO}_2} - \delta^{13}C_{\text{respiratory}})/(1 + \delta^{13}C_{\text{respiratory}})$), was determined for nonlabeled plants from both chambers. Chambers did not differ ($P > 0.05$), as would be expected from the fact that growth conditions were the same. However, there was a difference in $\Delta^{13}C$ of shoots and roots (23.2% ± 1.0 s.d versus 25.8% ± 0.7 s.d), consistent with the observations of Klumpp et al. (2005). This effect was accounted for in the labeling data evaluation using shoot- and root-specific $\delta^{13}C_{o}$ and $\delta^{13}C_{\text{old}}$ values in Equation 1 (see also Schnyder and de Visser, 1999).

Refixation of respiratory CO$_{2}$ was considered unimportant in this work. Principally, there are two aspects of refixation that are potentially relevant: one relates to refixation of respiratory CO$_{2}$ that has been released into the chamber atmosphere, the other concerns (internal) refixation within the photosynthetic tissue. Refixation of respiratory CO$_{2}$ from the chamber atmosphere was insignificant in this open, rapidly turned-over system, in which the rate of CO$_{2}$ supply to the chambers exceeded the stand CO$_{2}$ exchange rate by a factor of 9. The carbon isotope composition of CO$_{2}$ in the chamber air was measured nearly continuously, and these measurements were taken as the actual source CO$_{2}$ isotope composition. Moreover, the small number of labeling plants present in a chamber at any moment had no measurable effect on the isotopic composition of CO$_{2}$ in chamber air. Internal refixation was estimated using knowledge of $^{13}C$ discrimination in shoot biomass, assumptions about the fractional contribution of leaf respiration to stand respiration, and the ratio of respiration to photosynthesis. With a $^{13}C$ discrimination of 23.0‰, the ratio of leaf internal to atmospheric CO$_{2}$ concentration was near 0.82 (Farquhar et al., 1989); thus, the probability for refixation of leaf-respired CO$_{2}$ was approximately 18%. Assuming that leaf-respired carbon accounted for about one-third of plant respiration and that total plant respiration in light was one-third of the photosynthetic flux, the contribution of refixation to total photosynthetic CO$_{2}$ fixation was approximately 1.6% (0.18 × 0.3 × 0.3 = 0.016 = 1.6%). This effect was considered insignificant.

Compartmental Modeling of TRacer Time Course in Respired CO$_{2}$

The model shown in Figure 3 was described mathematically assuming that the system was in steady state, an assumption supported by constant specific growth and respiration rates of shoots and roots. Estimated turnover rates and half-lives assume first-order kinetics.

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

$$f_{\text{unlabeled-C}} = (Q_1 \times f_{\text{unlabeled-C}} + F_2 \times f_{\text{unlabeled-C}} - F_2 \times f_{\text{unlabeled-C}} - F_3 \times f_{\text{unlabeled-C}})/(Q_1 + F_3)$$  (3a)

$$f_{\text{unlabeled-C}} = (Q_1 \times f_{\text{unlabeled-C}} + F_2 \times f_{\text{unlabeled-C}} - F_2 \times f_{\text{unlabeled-C}} - F_3 \times f_{\text{unlabeled-C}})/(Q_1 + F_3)$$  (3b)

$$f_{\text{unlabeled-C}} = (F_3 \times f_{\text{unlabeled-C}} - F_3 \times f_{\text{unlabeled-C}})/(F_3 + F_4)$$  (3d)

where $Q_1$, $Q_3$, and $Q_4$ are the pool sizes and $F_3$ is the flux of photosynthetically assimilated carbon (tracer) that enters the respiratory system. Since the system is in steady state and $F_3$ equals the specific respiration rate, $F_3 = F_{\text{res}}$. The initial condition, $F_{\text{res}} = F_{\text{res,old}} = F_{\text{F12}} = F_{\text{F13}} = F_{\text{F32}}$ for $t = 0$, $F_{\text{F32,old}}$ indicates indices to donor and receptor pools, respectively. Index 0 represents the environment. The fraction of unlabeled carbon in shoot- or root-respired CO$_{2}$ is $f_{\text{unlabeled-C}}$. This is the measured parameter against which the model prediction is compared. $f_{\text{unlabeled-C}}$ is the fraction of unlabeled carbon in the pool $Q_3$ and $f_{\text{unlabeled-C}}$ is the constant fraction of fully labeled carbon entering the system after the start of labeling.

In order to fit the initial part of the tracer time course observed in root respiration, delay 2 was inserted between the beginning of labeling and the start of tracer incorporation in $Q_3$. In other words, tracer entered $Q_3$ in the root model a little later than in the shoot model, which would account for phloem transport time from shoot to root. Delay 2 was not necessary to simulate the tracer time course observed in shoot respirations.

To model the stable degree of labeling in the first hours (Fig. 2, insets), delay 1 between tracer acquisition in pool $Q_3$ and its efflux in $Q_4$ (Fig. 3) was required in both shoot and root simulations. Mathematically, $f_{\text{unlabeled-C}}$ in Equation 3d was forced to lag temporally behind $f_{\text{unlabeled-C}}$ in Equations 3b and 3c for the numerical value of delay 1. Since delay 1 operated only on the release side of $Q_3$ (i.e., $F_{\text{F32}}$), it had no effect on the estimation of the half-lives of $Q_3$ and $Q_4$. Considering the steady state of the system, it is important to note that delay 1 and delay 2 only apply to tracer content in respired CO$_{2}$ and not to the rate of respiration itself.

These equations were 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 delays were inserted, and the set of numerical equations (Eqs. 3a–3d) was solved. In that way, a tracer time course across the entire labeling period (600 h) was generated. The goodness of the fit was expressed as the RMSE:

$$\text{RMSE} = \sqrt{\sum_{i=1}^{n}(x(t_i) - \hat{x}(t_i))^2/n}$$  (4)

with $x$ and $\hat{x}$ the observed and model-predicted $f_{\text{unlabeled-C}}$ at labeling time $t_i$ and $n$ the number of labeling times.

This procedure was followed many times by stepwise and systematic variation of pool sizes, fluxes, and delays to identify the combination of values yielding the minimum RMSE (Table I, Fig. 4).

Optimized pool sizes and fluxes served to calculate the half-life ($t_{1/2}$) of a pool of size $Q_i$.

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

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

Based upon optimized fluxes, the contribution of a pool Q, ($C_Q$) to respiratory carbon release was derived, which is defined here as the probability of tracer moving in a certain flux of the respiratory system (compare with Fig. 3):

$$C_Q = F_3/(F_3 + F_4)$$  (6a)

$$C_Q = (1 - F_3/(F_3 + F_4)) \times F_3/(F_3 + F_4)$$  (6b)

$$C_Q = (1 - F_3/(F_3 + F_4)) \times F_3/(F_3 + F_4)$$  (6c)

$$C_Q + C_Q + C_Q = 0.95$$  (6)

$C_Q$ is thus the probability that tracer enters the system and leaves it in $F_3$ without visiting any other pool. $C_Q$ implies that tracer enters $Q_3$ via $Q_4$ and is

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respired in $F_{29}$ without moving through $Q_5$. $C_{13}$ is the probability of tracer cycling through the storage pool at least once.

**Validity of Model Assumptions**

As is the general case for compartmental analyses (Farrar, 1990; Lattanzi et al., 2005), ours was based on the assumptions that (1) the system is in a steady state, (2) fluxes obey first-order kinetics, and (3) pools are homogeneous and well mixed (Farrar, 1990; Lattanzi et al., 2005). Assumption 1 was well satisfied in the experiment: specific growth and respiration rates of shoots and roots were constant (see "Results" and "Discussion"). Also, the carbon to nitrogen ratio of biomass (24:1) did not change ($P > 0.05$; data not shown). Growing plants in continuous light ensured that short-term changes of pool sizes and fluxes (which are common to plants growing in day/night cycles) did not occur. Assumption 2 is probably false in a strict sense, but its practical validity seems supported (see Farrar, 1990, for discussion).

Assumption 3 is perhaps the most drastic simplification in the model. Probably, the different pools are not truly homogeneous but may constitute several biochemical compounds located in different spatial compartments, such as protein and fructan pools in different leaves. However, further compartmentalization did not improve goodness of fit, indicating that the kinetic properties of the components of a pool were similar. The observed lags for tracer arrival in the root and respiratory carbon release from $Q_5$ represent exemptions from the well-mixing assumption, which were explicitly accounted for by inserting (and optimizing) appropriate delays. Tracer studies normally assume that isotopic discrimination in pool exchange processes can be neglected. In our study, any effects of carbon isotope fractionation during photosynthesis, transport, and metabolism on carbon isotope composition of respired $CO_2$ were accounted for in the evaluation of labeling data by assessing (and correcting for) isotopic discrimination in unlabeled plants (de Visser et al., 1997).

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**LITERATURE CITED**


Heber U, Willenbrink J (1964) Sites of synthesis and transport of photosynthetic products within the leaf cell. Biochim Biophys Acta 82: 313–324


Kouchi H, Nakaji K, Yoneyama T, Ishizuka J (1985) Dynamics of carbon photosynthetically assimilated in nodulated soybean plants under steady-state conditions. 3. Time-course study on $^{13}C$ incorporation into soluble metabolites and respiratory evolution of $^{13}CO_2$ from roots and nodules. Ann Bot (Lond) 56: 333–346


Ryle GJA, Cobby JM, Powell CE (1976) Synthetic and maintenance respiratory losses of $^{13}$CO$_2$ in unculm barley and maize. Ann Bot (Lond) 40: 571–586

Lehmeier et al.