Photoassimilate allocation and dynamics of hotspots in roots visualized by ^{14}C phosphor imaging

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Abstract

Understanding photoassimilate allocation into the roots and the release of organic substances from the roots into the rhizosphere is an important prerequisite for characterizing the belowground C input, the spatial and temporal distribution of C, and the interactions between plants and soil microorganisms. Based on ^{14}C phosphor imaging, we visualized the allocation of assimilates into *Lolium perenne* roots and estimated the life time of hotspots at the root tips.

Lolium shoots were labeled in a $^{14}CO_2$ atmosphere, and herbariums of roots and shoots were prepared 6 h, 2 d, and 11 d after the ^{14}C pulse. The ^{14}C distribution in roots and leaves revealed that pulse labeling does not yield homogeneously labeled plant material. The spatial distribution of assimilate allocation was evaluated based on the ^{14}C specific activity expressed as digital light units (DLU mm^{-2}) of the imaging plates. Areas with high relative ^{14}C activity were classified as hotspots. Strong ^{14}C hotspots were detected mainly at the root tips already 6 h after the ^{14}C assimilation, and they remained active for at least 2 d. Eleven days after the ^{14}C assimilation, the hotspots at the root tips disappeared and the ^{14}C distribution was much more even than after 6 h or after 2 d.

^{14}C phosphor imaging proved to be a promising tool to visualize the allocation of photoassimilates into the roots and the rhizosphere and can be used to identify hotspots and their dynamics.

Key words: rhizodeposition / ^{14}C labeling / hotspots / autoradiography / *Lolium perenne* / rhizosphere

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1 Introduction

Although the volume of the rhizosphere is typically < 1% of the total soil volume, the importance of the rhizosphere within the global C cycle is enormous (*Hinsinger* et al., 2005). The rhizosphere is a complex habitat in which conditions differ from those in the bulk soil. Root-derived C forms a localized source of easily available energy for microbial activity. This leads to a unique biological niche within the soil environment characterized by a high abundance, activity, and diversity of microorganisms. Understanding the allocation of photoassimilates into the roots and the rhizosphere is crucial to provide insight into the complex interactions between soil, microorganisms, and plants. However, investigating root-derived C including rhizodeposition is complicated because roots release organic compounds similar to those already present in the soil and because rhizosphere microorganisms rapidly decompose rhizodeposits. To overcome these problems, most methods for analyzing the distribution of C released by roots in various soil pools are based on applying the C isotopes ^{14}C and ^{13}C and on quantifying the total ^{14}C radioactivity or ^{13}C content in the soil surrounding the roots. This enables distinguishing the root-derived C from the native soil organic compounds (*Whipps*, 1990; *Buyanovsky* et al., 1994). A very few studies also applied the short-lived ^{11}C to show the translocation of photoassimilates (*Farrar* et al., 1994; *Keutgen* et al., 1995). For isotope applications, continuous labeling (*e.g., Johnen* and *Sauerbeck*, 1977; *Whipps*, 1987; *Meharg*, 1994) or pulse labeling (*e.g., Warembourg* and *Billes*, 1979; *Meharg* and *Killham*, 1990; *Cheng* et al., 1993; *Swinnen* et al., 1994; *Nguyen* et al., 1999; *Kuzyakov* et al., 1999, 2001) was used. The advantages and disadvantages of these labeling approaches were reviewed in several publications (*Whipps*, 1990; *Kuzyakov* and *Domanski*, 2000; *Nguyen*, 2003; *Kuzyakov* and *Schneckenberger*, 2004; *Werth* and *Kuzyakov*, 2008).

Based on these labeling techniques, total rhizodeposition was estimated for various plants, although most studies focused on agricultural cereals. Therefore, much less is known about pasture plants. The absolute C input is similar for pasture plants and for agricultural cereals (1500 kg C ha^{-1} y^{-1}) when the same growth period is considered (*Jensen*, 1993; *Swinnen* et al., 1995b). Nevertheless, the relative C translocation into soil is higher for pasture plants than for agricultural cereals. In order to determine the allocation of assimilates, ^{14}C or ^{13}C pulse labeling of *Lolium perenne* was used (*Meharg*, 1994; *Swinnen* et al., 1994; *Tinker* et al., 1994). Studies based on the application of C isotopes can help evaluate: (1) the total belowground **budget** of assimilated C, (2) the **dynamics** of assimilated C in various belowground pools, and (3) the **localization** of C allocation. Most of the previous studies focused on the first two items—budget and dynamics—while the present study concentrates on the allocation of ^{14}C assimilates and their dynamics within the root system.

^{14}C can be detected using autoradiography. In recent years, the traditional autoradiographic technique using X-ray films has been replaced by the so-called phosphor-imaging approach,

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which is based on photoinduced chemiluminescence. Compared to traditional autoradiography, phosphor imaging is about two orders more sensitive to β- and γ-rays, it has a wider radioactivity range for imaging, a wider linear dynamic range between the intensity of the image and the activity of the isotope, it avoids handling chemicals necessary for film development, and it reduces the exposure time (*Hamaoka*, 1990).

The present study was designed to prove the suitability of phosphor imaging for visualizing the allocation of ^{14}C-labeled assimilates. As a first step towards more detailed information about the complex interactions between plants and soil, we investigated the allocation of C assimilates in roots of ryegrass (*Lolium perenne* L., ssp. Gremie) and identified the ^{14}C hotspots in these roots.

2 Materials and methods

We studied the distribution pattern of the radiotracer ^{14}C in ryegrass at three different time intervals: 6 h, 2 d, and 11 d after start of assimilation of $^{14}CO_2$ *via* the shoots of the plants. The ^{14}C-distribution pattern was visualized using phosphor imaging of leaves and roots. The main focus of this study was on the ^{14}C distribution in roots.

2.1 Soil properties and plant growth conditions

The experiments were conducted with *Lolium perenne* grown on a fine loamy gleyic Cambisol. The soil samples were taken from the Ah horizon (top 10 cm) of a long-term pasture in Allgäu (S Germany). Basic characteristics of the soil are shown in Tab. 1. The wet soil samples were air-dried, homogenized, and passed through a 2 mm sieve to separate large roots and stones. An amount of 1.6 kg of dried soil with a final density of 1.2 g cm^{-3} was filled into each pot (height 10 cm, inner diameter 14 cm). One prevernalized seedling of ryegrass was grown per pot. The plants were grown at temperatures of 26°C–28°C (day) and at 22°C–23°C (night) with a day length of 14 h and light intensity of ≈ 800 μmol m^{-2} s^{-1}. The soil water content of each pot was measured gravimetrically and was adjusted daily to ≈ 60% of the available field capacity.

Table 1: Basic characteristics of the soil sampled from the Ah horizon of a fine loamy gleyic Cambisol from a pasture in the Allgäu (S Germany) (FC, field capacity [pF = 1.8]; AWC, available water capacity [pF 1.8–4.2]) (*Kleber*, 1997).

Parameter	
pH ($CaCl_2$)	5.2
C_{org} / %	4.7
N_{total} / %	0.46
C : N	10
Clay (< 2 μm) / %	28.4
Silt (2 to <63 μm) / %	47.1
Sand (63 to 2000 μm) / %	24.5
FC / %	50
AWC / %	23
$CaCO_3$ / %	0

2.2 Labeling of plants in a $^{14}CO_2$ atmosphere

For ^{14}C labeling, a perspex chamber previously described by *Kuzyakov* et al. (1999) was used. The airtight chamber consisted of two compartments. The lower compartment (height 200 mm, inner diameter 138 mm) contained the soil, and the upper compartment (height 300 mm, inner diameter 138 mm) was used for the tracer application to the leaves. Both compartments were separated from each other by a perspex lid with drilled holes (inner diameter 8 mm) for the plants. The day before labeling, the holes were sealed at the base using silicone paste (NG 3170, Thauer & Co., Dresden) (*Gregory* et al., 1991; *Swinnen* et al., 1995a). The seals were tested for air leaks. All plants were labeled simultaneously. 381 kBq of ^{14}C as $Na_2{}^{14}CO_3$ solution were put in a 2 cm^3 Eppendorf micro–test tube placed in the upper compartment of the chamber. Then the chamber was closed and 1 mL of 5 M H_2SO_4 was added to the solution through a pipe. Assimilation took place within 2 h after the $^{14}CO_2$ pulsing, but most of the $^{14}CO_2$ was assimilated within the first 30 min. After labeling, the CO_2 from the upper compartment was trapped to remove the remaining nonassimilated $^{14}CO_2$. Finally, the top of the chamber was removed, and the plants were grown under normal conditions. After opening the labeling chamber, the CO_2 evolving from the lower compartment was trapped in a 20 mL solution of 0.5 M NaOH by continuous pumping (100 cm^3 min^{-1}) with a membrane pump. This removes the $^{14}CO_2$ respired by roots and microorganisms and avoids possible re-uptake of ^{14}C from the soil solution by roots.

The plants were harvested at three different times after start of labeling: 6 h, 2 d, and 11 d. This was done by cutting the plants at the base and opening the bottom compartment of the chamber. Finally, the soil was pulled out. Roots were carefully separated from the soil by handpicking. All picked roots were gently washed in 400 mL of de-ionized water to remove the soil adhering to the roots. The leaf material and the roots were distributed on a white paper, prepared as a herbarium and dried at 60°C.

2.3 Tracer detection by phosphor imaging

The distribution pattern of the ^{14}C within leaves and roots was determined by CyclonePlus Storage Phosphor System (Perkin Elmer, Germany). Each herbarium (6 h, 2 d, 11 d) with roots or shoots was exposed to a sensitive imaging plate in the dark for 1 or 3 weeks. The plate was then scanned by CyclonePlus (Perkin Elmer, Germany) and digitalized by OptiQuant software (Perkin Elmer, Germany). We used two approaches to demonstrate the distribution pattern of assimilated ^{14}C within the roots: (1) evaluation of the evenness of the ^{14}C distribution within the roots and identification of ^{14}C hotspots and (2) visualization of the longitudinal allocation in individual roots.

2.3.1 Evenness of the ^{14}C distribution within the roots and identification of ^{14}C hotspots

To verify the visual findings of the image-plate pictures, the evenness of the ^{14}C distribution within the roots was calculated and hotspots were identified by applying a grid to each image (241 columns, 122 rows, square width 1 mm, square length 1 mm, center-to-center spacing: columns 1 mm, rows 1 mm). The ^{14}C activities of the single squares were added up and set as the total activity of the grid. The activity per square expressed as digital light units (DLU) per mm^2 was then put in reference to the total activity. The resulting relative activities were categorized into 39 size ranges with the statistical package Statistica 7 for Windows. The smallest range with a relative activity of 0.006 (> 82% of the image area) was set up as background and excluded from the evaluation.

2.3.2 Longitudinal ^{14}C allocation in individual roots

Within each image, 10 roots were selected and squares (1 mm × 1 mm) were applied to the individual roots in longitudinal direction up to 19 mm. The squares along each root were numbered starting at the root tip. Subsequently, the mean out of all squares of #1, #2, *etc.* per image was calculated and the data were normalized with reference to the square with the maximum DLU value out of all images. In this approach, we used another reference to normalize the data because, despite the same root length, the area around the roots differs and thus cannot be normalized as described under section 2.3.1.

3 Results

3.1 Distribution of ^{14}C in the shoots

The evenness of the ^{14}C distribution within the shoots (and roots) was estimated based on the number of DLU per area of the phosphor image, with the red color indicating the highest ^{14}C activity (Fig. 1). Hotspots of ^{14}C activity within the image of the shoot 6 h after labeling (Fig. 1 a) are located at the growing parts at the leaf base and in the highly photosynthetically active tissue at the tips. Two days and eleven days after labeling, the redistribution of ^{14}C within the shoots was weak (images not presented) and changes in the distribution were minimal. We therefore did not further evaluate the ^{14}C distribution in shoots. Note that 6 h after labeling, the total ^{14}C activity in the shoots was more than 10 times higher than that in the roots (compare DLU scale for shoots and roots).

3.2 Evenness of the ^{14}C distribution within the roots and identification of ^{14}C hotspots

The root image-plate pictures of ryegrass harvested 6 h, 2 d, and 11 d after labeling (Fig. 1 b, c, d) showed a shift of the ^{14}C spots with increasing time after the ^{14}C pulse. Six hours after labeling (Fig. 1 b), substantial amounts of ^{14}C were allocated to the youngest parts, *i.e.*, to the root tips. In contrast, 11 d after labeling (Fig. 1 d), tracer accumulations were also found in the adventitious roots (black arrows in Fig. 1 d).

To verify the visual findings of Fig. 1, the evenness of the ^{14}C distribution in the roots was statistically evaluated (Fig. 2). If ^{14}C was evenly distributed, Fig. 2 would show a constant line parallel to the x-axis. However, the ^{14}C distribution obtained consisted of many points with low or very low relative ^{14}C activity and few points with high activity. Up to the threshold of 0.126, the frequency strongly decreased with increasing relative ^{14}C activity (Fig. 2). We defined hotspots as regions with low frequency and high relative ^{14}C activity (> 0.126). The threshold can clearly be identified through the change of the slope of the relative frequency line. Although the number of ^{14}C hotspots was generally low, their frequency after 11 d was higher than after 6 h or after 2 d. Nevertheless, the number of hotspots at the root tips 11 d after ^{14}C photoassimilation clearly decreased.

3.3 Longitudinal ^{14}C allocation in individual roots

In order to better understand the heterogeneity and dynamics of the ^{14}C activity along the roots, individual parts of ryegrass roots prepared 6 h, 2 d, and 11 d after labeling were analyzed for ^{14}C allocation. Due to root growth, the tracer accumulation zone changed continuously over the 11 d after the ^{14}C pulse. At the first harvest, ^{14}C activity was highest mainly in the first millimeters from the root tips, and it decreased with increasing distance from the tips (Fig. 3). Two days later, the maximum of ^{14}C-enriched tissue still occurred within the first 2 mm from the root tips. The maximum peak shifted 11 d after labeling. The highest activity was now located ≈ 10 mm from the tips. After ^{14}C application, the apical meristem grew mainly with unlabeled C. This resulted in unlabeled tissue at the root tip and explains the shift.

4 Discussion

4.1 Applicability of ^{14}C phosphor imaging for visualizing and tracing belowground C allocation

Significant amounts of the C allocated belowground are released as organic C *via* rhizodeposition into the rhizosphere (*Marschner*, 1995). Previously, *Meharg* and *Killham* (1990) found that the net photosynthetic C allocated to the roots of *Lolium perenne* ranges from 14% to 67%. Recent studies showed an allocation of ≈ 48% of the total assimilated ^{14}C (*Domanski* et al., 2001). An amount of 10%–15% of the total fixed C is respired by roots, and a further 15%–25% is released *via* exudation (*Domanski* et al., 2001). In comparison, *Farrar* et al. (2003) reported that the exudation rate of soil-grown plants, detected in ^{14}C-labeling investigations, ranges between 5% and 10% of the net assimilated C. Depending on the level of nutrient supply, *Werth* and *Kuzyakov* (2006) recovered 0.4%–0.8% ^{14}C in exudates and 15.1%–16.9% in respired CO_2. It was shown that plants growing in nutrient solution lost only 0.5%–1.5% of the fixed C. Under field conditions, plants import more C belowground than laboratory-grown plants (*Meharg* and *Killham*, 1990). Our investigations were carried out with ryegrass grown on a

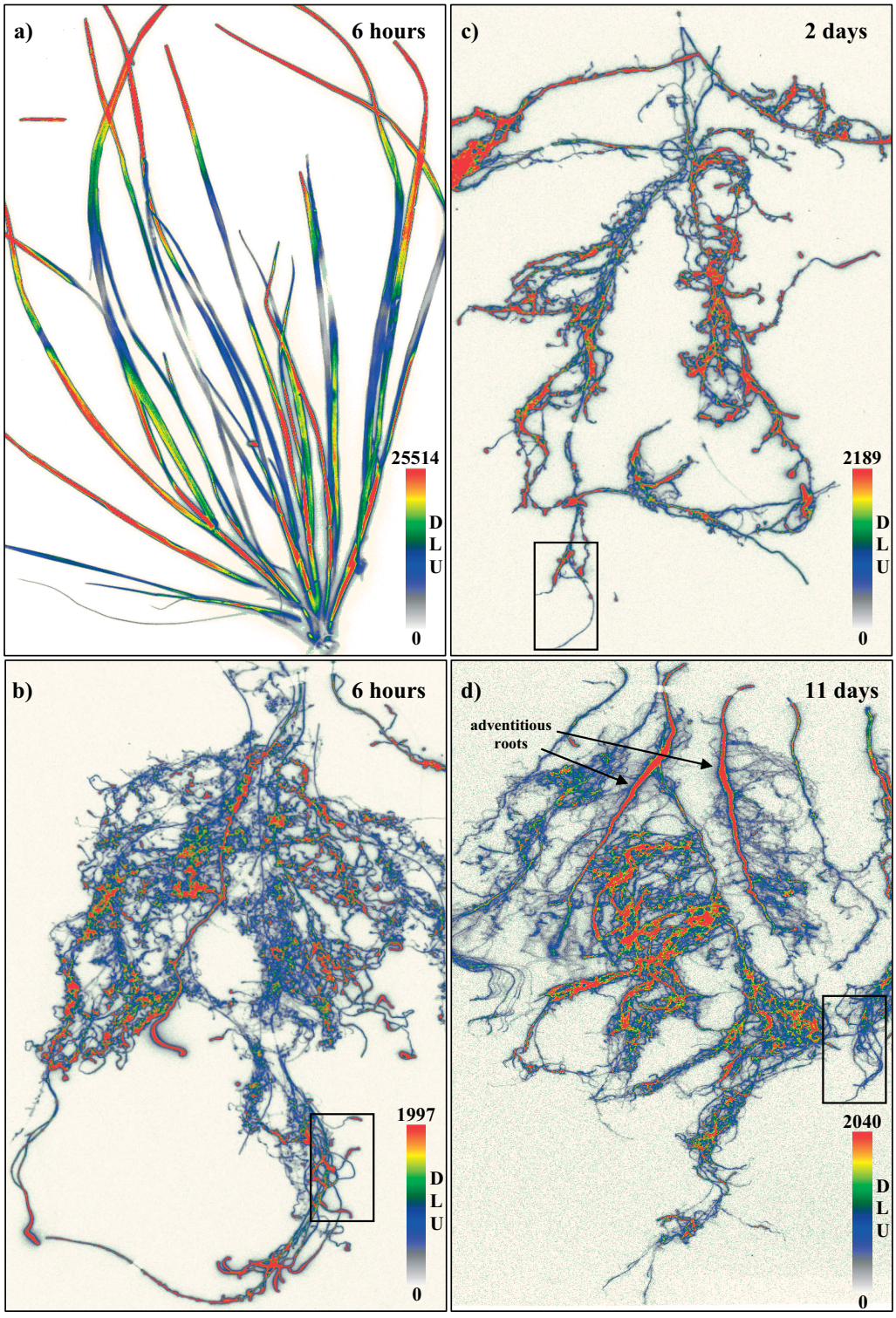

Figure 1: Phosphor images of a shoot 6 h (a) and of roots 6 h (b), 2 d (c), and 11 d (d) after labeling in $^{14}CO_2$ atmosphere. ^{14}C activity expressed as digital light units (DLU). The insets for Fig. 2 are marked by a black rectangle.

Cambisol, which is closer to field conditions than investigations with plants grown in nutrition solution.

^{14}C can either be released as exudates or CO_2 into the rhizosphere, or can be stored as a relatively stable fraction in growing tissues at the root tips and the lateral root emergence (*Henry* et al., 2005). The C translocated belowground is not released evenly by the roots, but in distinct areas, thus creating a gradient of rhizodeposits along each root axis. The ^{14}C-labeling technique allows distinguishing between root-borne C and C components that are already present in the soil. To date, one approach—the two-compartment rhizo-

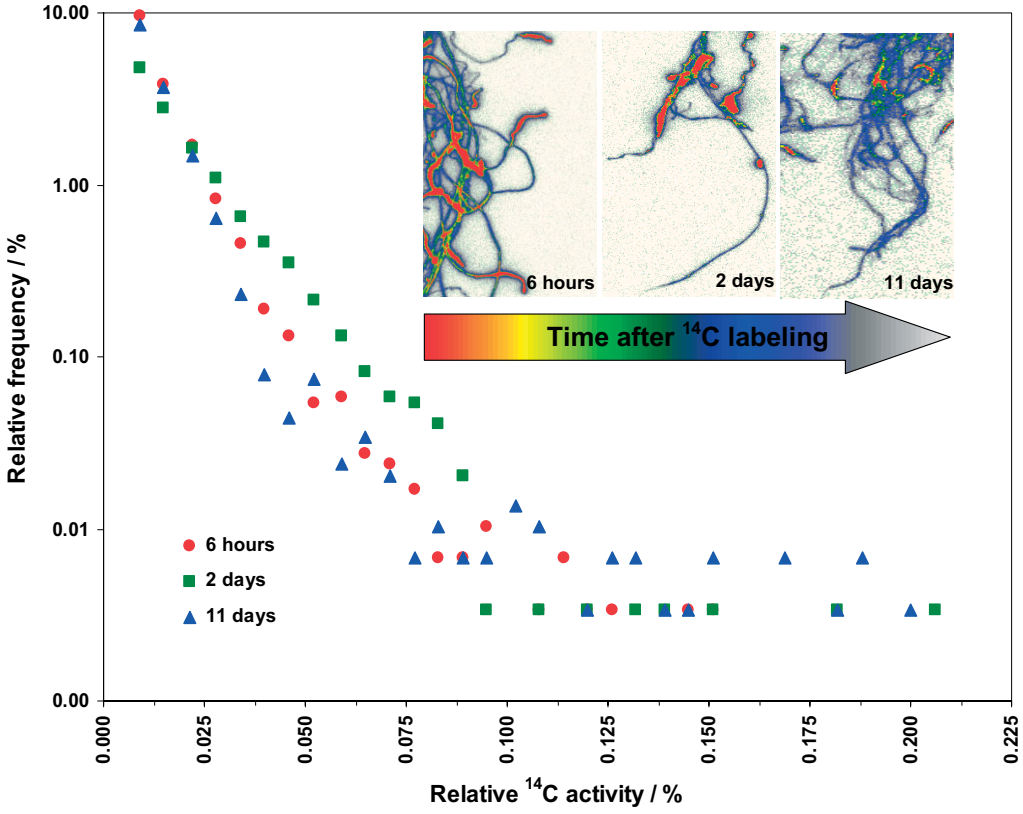

Figure 2: Relationship between the relative frequency of 1 mm × 1 mm squares and their ^{14}C activity at increasing time after tracer application: 6 h, 2 d, and 11 d. Note the logarithmic scale of relative frequency. The insets from Fig. 1 show enlarged hotspots at the root tips 6 hours after labeling and decreasing ^{14}C activity (DLU) at the tips with time.

box—was known to identify the gradient of rhizodeposition and thus the active microbial zones. In this rhizobox, the root-containing soil is separated from the root-free soil by gauzes with different hole sizes (0 ... 30 μm). After labeling the shoots in a $^{14}CO_2$ atmosphere, the soil compartment without roots is, after a certain period of time, cut into thin slices by a microtome, and the ^{14}C activity in the different slices is determined (*Sauer* et al., 2006). *Sauer* et al. (2006) found maximum ^{14}C accumulation within 2 mm of the root mat at the gauze. This was explained by the 0.08 to 2 mm long root hairs (*Esau*, 1965; *Zhu* et al., 2005) penetrating through the 30 μm gauze into the root-free soil. However, in the model of *Jones* et al. (1996), based mainly on diffusion of organic acids, the maximum distance for the recovery of organic acids was < 1 mm from the root surface.

The main shortcoming of this approach is the creation of a root mat at the gauze, followed by an overestimation of root effects due to specific conditions at the mat, undefined numbers of root layers, as well as variation in age and physiological development. Moreover, root effects may be underestimated due to solute mixing, sorption, and microbial metabolism (*Wenzel* et al., 2001). To avoid these problems, *Wenzel* et al. (2001) tested a novel rhizobox design: Plants were grown in a soil–plant compartment with roots penetrating through a slit into a soil-free compartment consisting of an acrylic window that moves horizontally when root growth pressure is exerted. This allows root growth, distribution, and morphology to be monitored. A membrane separates the soil-free compartment from the root-free soil compartment in which the rhizodeposition gradient can be determined afterwards. An important advantage of this design is the possibility to measure the exact root biomass. This approach enables the diffusion of exudates from the root compartment to be traced, but provides no information on the allocation of assimilated C along and within individual roots. To overcome this problem and to visualize the allocation of photoassimilates in individual roots, another approach based on ^{14}C phosphor imaging can be applied.

Phosphor imaging had already been used to visualize the allocation and the movement of labeled P (^{32}P and ^{33}P) in leaves of *Vicia faba* (*Hüve* et al., 2007). At the same time, ^{14}C-pulse labeling coupled with phosphor imaging was used to investigate the C flux of assimilated ^{14}C from *Pinus sylvestris* seedlings to the ectomycorrhizal mycelium (*Leake* et al., 2001) and between the seedlings of *Pinus densiflora via* ectomycorrhizal mycelia (*Wu* et al., 2001). This approach helps to visualize and quantify the spatial and temporal patterns of the allocation of ^{14}C assimilates.

In the present study, the ^{14}C phosphor-imaging approach enabled us to use the image-plate pictures to statistically evaluate the ^{14}C distribution. The methodology we used in preparing the herbariums did not include measuring rhizodeposition and CO_2 but focused on the ^{14}C allocation within the

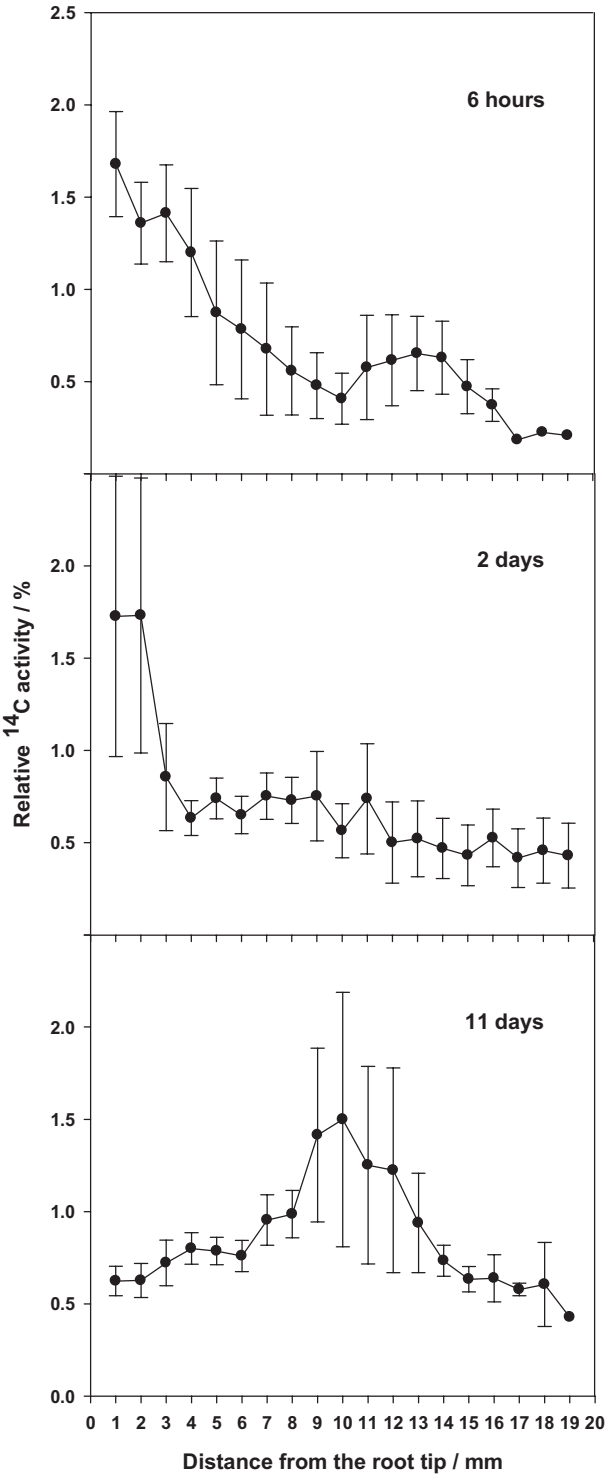

Figure 3: Relationship between the mean relative ^{14}C activities per mm^2 (%) and the distance from the root tip (mm).

roots. The limitations of this approach are mainly linked to the preparation of the root herbariums. Firstly, after carefully extracting the roots, their separation from mineral soil material and washing may lead to a significant loss of fine roots. Secondly, root exudates and rhizodeposits are completely lost during the washing procedure. Therefore, very fine roots, rhizodeposits, and exudates cannot be determined with this approach. Thirdly, the preparation of root (and shoot) herbariums may cause some parts of the roots to overlap (Fig. 1 b, c, d). Such overlapping of 2–3 roots could locally increase the ^{14}C specific activity of the area (DLU mm^{-2}), thus overestimating hotspots. The energy of β^- particles released by ^{14}C decay, however, is very low (E_{max} = 0.156 MeV), and the probability that they will penetrate the overlapped roots is very small. We therefore assume that the number of hotspots is not overestimated by the image of ^{14}C on the imaging plates.

The three shortcomings described above (loss of fine roots, loss of exudates, and overlapping) could be overcome using a specially constructed, thin rhizobox with a removable front wall. Placing the boxes in tilted position (20°–30° vertical) causes roots to grow along this wall. The front wall can be removed after labeling. Then, after drying or freezing the roots and the soil in the rhizobox, the phosphor-imaging plate can be placed instead of the wall to prepare the root image. After developing the image, the roots can be carefully removed, and the phosphor imaging plate can be placed again to the soil. This yields an image of rhizodeposition without roots.

4.2 Relative C allocation in shoots and roots

We compared the allocation of assimilated C in shoots with the allocation in roots based on the intensity of DLU mm^{-2}. The amount of assimilates in the shoots 6 h after labeling was ≈ 10 times higher than in the roots. Surprisingly, this ratio did not strongly decrease within 2 or 11 d after labeling. Other authors have also reported this interesting phenomenon (*Hill* et al., 2007). Similar results were frequently obtained for ^{13}C-pulse-labeling experiments based on higher ^{13}C enrichment in shoots *versus* roots (*Kastovska* and *Santruckova*, 2007). The difference cannot be adequately explained by less total assimilates allocated to roots compared to shoots: considering the ratio of ^{14}C shoot to ^{14}C roots of ≈ 10 (Fig. 1 a, b), the total shoot mass should also be ≈ 10 times higher. This phenomenon can be explained either by label storage in pools of the shoots with a long turnover time, or by a much higher turnover of roots compared with shoots and thus a much higher replacement of ^{14}C (or ^{13}C) from the pulse by subsequent unlabeled C.

Another surprising result clearly visualized by the ^{14}C distribution was the high inhomogeneity of the C allocation within the shoots. Even within a single leaf, the ^{14}C activity can differ by 5–7 times (compare parts of individual leaves in Fig. 1 a). This shows that it is impossible to produce homogeneously labeled plant biomass by pulse labeling (^{14}C or ^{13}C). Probably, repeated pulse labeling will also be insufficient to produce plant biomass with a homogeneous distribution of the label.

5 Conclusions

^{14}C labeling, coupled with phosphor imaging, can provide detailed insights into the C flows into and through plant roots. The spatial distribution pattern of allocated ^{14}C was already visible 6 h after assimilation. The spatial distribution of the C

allocation in the roots was uneven: up to 2 d after assimilation, it was associated with the root tips, whereas 11 d after assimilation, the ^{14}C allocation to the hotspots at the root tips disappeared. Moreover, it was impossible to produce homogeneously labeled shoot or root biomass by pulse labeling.

^{14}C phosphor imaging is a promising tool for visualizing C translocation in both plants and rhizosphere. This technique will yield further progress in describing and interpreting soil–root interactions.

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