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REGULAR ARTICLE

Bicarbonate as tracer for assimilated C and homogeneity of ^{14}C and ^{15}N distribution in plants by alternative labeling approaches

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Abstract
Aims Application of carbon (C) and nitrogen (N) isotopes is an essential tool to study C and N flows in plant-soil-microorganisms systems. When targeting single plants in a community the tracers need to be added via e.g., leaf-labeling or stem-feeding approaches. In this study we: (i) investigated if bicarbonate can be used to introduce ^{14}C (or ^{13}C) into white clover and ryegrass, and (ii) compared the patterns of ^{14}C and ^{15}N allocation in white clover and ryegrass to evaluate the homogeneity of tracer distribution after two alternative labeling approaches.
Methods Perennial ryegrass and white clover were pulse labeled with ^{15}N urea via leaf-labeling and ^{14}C either via a $^{14}CO_2$ atm or with ^{14}C bicarbonate through leaf-labeling. Plants were sampled 4 days after labeling and prepared for bulk isotope analysis and for ^{14}C imaging to identify plant parts with high and low ^{14}C activity. Subsequently, plant parts with high and low ^{14}C activity were separated and analyzed for ^{15}N enrichment.
Results Bicarbonate applied by leaf-labeling efficiently introduced ^{14}C into both white clover and ryegrass, although the ^{14}C activity in particular for white clover was found predominantly in the labeled leaf. Using ^{14}C imaging for identification of areas with high (hotspots) and low ^{14}C activity showed that ^{14}C was incorporated very heterogeneously both when using bicarbonate and CO_2 as expected when using pulse labeling. Subsequent analysis of ^{15}N enrichment in plant parts with high and low ^{14}C activity showed that ^{15}N also had a heterogeneous distribution (up to two orders of magnitude).
Conclusion Bicarbonate can efficiently be used to introduce ^{14}C or ^{13}C into plant via the leaf-labeling method. Both ^{14}C and ^{15}N showed heterogeneous distribution in the plant, although the distribution of ^{15}N was more even than that of ^{14}C.

Keywords Allocation of assimilates · Tracer allocation · ^{15}N distribution · N utilization · ^{14}C imaging · Isotopic applications · Pulse labeling

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Introduction

The application of C and N isotope tracers to living plants is a strong and essential tool to study C and N dynamics in many ecosystems (Bruggemann et al. 2011). In most studies the C and N isotopes are applied as $^{13}CO_2/^{14}CO_2$ and $^{15}NH_4^+/^{15}NO_3^-$ to the whole plant community to trace the fluxes in plant, soil and microorganisms as well as CO_2 and N_2O losses. However, in studies focused on single plant species in a mixed community or C or N derived from a specific plant, it is not possible to use CO_2 for C labeling and soil N application for N labeling. Instead targeted approaches have been used, introducing the tracers via leaf-labeling (McNeill et al. 1997) or stem-feeding (Russell and Fillery 1996) with urea as N-tracer carrier and a sugar as C-tracer carrier. When introduced via such targeted approaches neither the C- nor the N-tracer follow the ordinary transport and assimilation route into the plant; i.e., as photoassimilation of CO_2 and root uptake of N compounds. Therefore, the distribution of the tracers in the plant may differ compared to that by their natural routes of entry (Wichern et al. 2011), although detailed studies comparing tracer allocation are scarce.

Introducing the C-tracer has been done using a sugar, e.g., glucose, fructose or sucrose (Jones and Darrah 1994; Maffei et al. 2001), and by ^{14}C labeled urea (e.g., Clifford et al. 1973; Rasmussen et al. 2007). Urea splits up into NH_4^+ and CO_2 (partly converted to HCO_3^-) when entering the leaf (Hertenberger and Wanek 2004; Schmidt and Scrimgeour 2001). However, since urea has two N per C this carrier is not well suited for ^{13}C-labeling, as too much N is added to introduce enough ^{13}C. In studies of C flows in tree xylem bicarbonate/carbonate has been used as C-tracer carrier (Powers and Marshall 2011; Stringer and Kimmerer 1993). To our knowledge however, bicarbonate/carbonate is still to be tested as C-tracer carrier when using leaf-labeling and stem-feeding and its distribution has to be compared to other labeling methods.

In a recent study Pausch and Kuzyakov (2011) used ^{14}C imaging to study the distribution and flow of photoassimilates in perennial ryegrass shoots and roots. Very strong allocation of assimilates to root tips and consequently the production of hotspots were visualized and their life time were estimated to a few days. In the present study we used the same ^{14}C imaging approach after leaf-labeling (McNeill et al. 1997) (i) to determine if bicarbonate can be used as C-tracer carrier with the leaf-labeling method, (ii) to check the distribution of the ^{14}C label, and to detect the locations with preferable assimilate incorporations, and (iii) to clarify whether ^{15}N tracer follows the allocation of C-tracer within the plant. The allocations of ^{14}C and ^{15}N were evaluated based on two grassland species with and without N fixation: white clover and perennial ryegrass. The bicarbonate was determined as the bulk ^{14}C presence in plant parts and as distribution of C-tracer in ryegrass and white clover. We further used ^{14}C imaging to identify ^{14}C hotspots and areas with low ^{14}C activity; areas that were following used to study ^{15}N allocation in the plants.

Materials and methods

In a preliminary test we found that bicarbonate concentrations in the labeling solution higher than 0.01 M severely damaged the labeled-leaf of both grass and white clover while the leaves were in the labeling-tubes. For bicarbonate concentrations of 0.01 M or lower no leaf damage were seen at removal of the labeling-tube, but minor damages were observed at the labeled leaves after 3 days. Based on these observation further work was done with bicarbonate concentrations of 0.01 M. In a further test we looked at the disappearance of ^{14}C from a bicarbonate solution when labeled white clover leaves were either intact (McNeill et al. 1998) or the tip of the leaves were cut off (McNeill et al. 1997). We found that less ^{14}C remained in the bicarbonate solution when the leaf tip was cut off compared to intact leaves, and therefore we cut the leaf tip in this study. It should be noted, that the leaves where the tip was cut looked more damaged after removal of the labeling-tube than did the intact leaves. Thus, a shorter labeling-period is needed when cutting the leaf tips.

Main experiment

Plant growth conditions and experimental treatments

White clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) were grown in mixture on a loamy sand (Typic Hapludult, 7.7 % clay (<2 μm), 11 % silt (2–20 μm), 47 % fine sand (20–200 μm), 32 % coarse sand (200–2000 μm), and C_{org}=1.6 %) at

Foulumgaard Experimental Station, Jutland, Denmark. In late May, 10 days before the experiment start, we inserted 25 steel cylinders (7 cm in diameter and 10 cm depth) into the 2 year old sward to give ten perennial ryegrass dominated cores, ten white clover dominated cores, and five cores with approximately equal mixtures. The cores were excavated 1 week later and the perennial ryegrass and white clover dominated cores placed in four boxes (five replicates per box) with a sand layer in the bottom (5 cm) to allow irrigation of the cores. The cores were left outdoors to simulate in situ climatic conditions. Water was added to the sand and soil water content in the cores allowed to adjust for 3 days. During the week the experiment ran the average temperature was 13.6 °C (lowest 4.1 °C and highest 21.8 °C), and the precipitation was 27.5 mm. The five mixtures were separated before the start of the labeling to obtain unlabeled control samples of perennial ryegrass and white clover shoot and root material.

Pulse labeling procedure

The uptake and distribution of ^{14}C in perennial ryegrass and white clover were studied by two labeling methods. In CO_2 treatment ^{14}C was applied as $^{14}CO_2$, and ^{15}N as one 1 mL ^{15}N urea solution (0.008 M or 0.5 %w/v, 99 atom%) in 2 mL labeling-tubes via the leaf-labeling method (McNeill et al. 1997). In the bicarbonate treatment ^{14}C was applied as ^{14}C bicarbonate (0.01 M, 0.355 kBq/mL) mixed with ^{15}N urea and added through the leaf-labeling-tubes (1 mL labeling solution in a 2 mL tube). Thus, the method of ^{15}N labeling was the same for the CO_2 and bicarbonate treatments. Briefly, for both treatments the leaf-labeling was started on the morning of the first day and terminated in the afternoon of the third day, allowing the plants to assess to the labeling solution for two and a half days. After removal of the labeling-tubes the labeled leaves were dried with paper towel to remove labeling-solution on the outside of the leaves. Five plants in each core were leaf-labeled to give a total number of 25 leaf-labeled plants per species for each treatment. In the CO_2 treatment, the ^{14}C labeling was done on day two by covering each core with a plastic bag, and allowing the plants to assimilate $^{14}CO_2$ (1.775 kBq per core) for 2 h before a flush with unlabeled CO_2 for 1 h (Rasmussen et al. 2008).

Four days after termination of the labeling, the soil in the cores were taken out to recover as much intact roots as possible. The plant shoots and roots where then gently washed and while in water collected on a strip of blotting-paper to spread out the roots. The labeled plants were pressed during drying at 60°C. A Geiger-Muller counter was used to identify which plants had a clear ^{14}C signal and selected on this basis three to four plants for ^{14}C imaging. The rest of the labeled plants were divided in shoots and roots for perennial ryegrass, and shoots, stolons, and roots for white clover. The plant parts were bulked per replicate and ground on a ball mill before bulk ^{14}C and ^{15}N analysis. The ^{14}C activity was determined after combusting the samples in a sample oxidizer (Packard model 307, Packard Instrument Company, Meriden, CT, USA) and counting of ^{14}C activity on a liquid scintillation counter (Tri-Carb 2250LL, Packard Instrument Company, Meriden, CT, USA). The analysis of ^{15}N enrichment was done using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europe 20–20 isotope ratio mass spectrometer (Sercon Ltd. Chershire, UK). The atom% ^{15}N excess was calculated as the difference between atom% ^{15}N in labeled plant parts and unlabeled controls.

The plants selected for ^{14}C imaging were exposed to the imaging screens for 1 week. The imaging screens were recorded on a Cyclone™ scanner (Packard Bioscience) and the images were analyzed using the OptiQuant™ software (Packard Bioscience). The images were used to divide the plants in ^{14}C high (hotspots) and low ^{14}C activity areas using the drawing tool in the OptiQunat™ software. The division into hotspots and low ^{14}C activity areas was based on a comparison of digital light units (DLU) from each plant part having a ^{14}C signal above the background DLU-level defined as low ^{14}C activity areas. Plant material from hotspots and from low ^{14}C activity areas were ground separately before analysis for ^{15}N enrichment (see above).

Statistics on bulk analysis data

Data provided in Table 1 were analyzed on dry matter basis. Significant differences within species between plant parts were determined at α=0.05 using Tukey's test (HSD). The effect of plant species, labeling methods and plants parts as well as interactions among these factors on $^{15}N/^{14}C$ measurements were analyzed

on three-factorial Analysis of variance (ANOVA) basis using R-2.13.2 statistical software.

Results

Bicarbonate as C-tracer carrier

Bicarbonate led to uptake of ^{14}C in both ryegrass and white clover in comparable activities as when ^{14}C was introduced via photo-assimilation of $^{14}CO_2$ (Table 1). Note that the lower ^{14}C activity when introduced via $^{14}CO_2$ is due to ^{14}C being divided between all plants in the plant community. Therefore, the actual ^{14}C activities in plant are not directly comparable between the bicarbonate and CO_2 treatments, but the proportional distribution of ^{14}C activity among plant parts can be compared. There was a significant difference in the ^{14}C allocation depending on the C labeling approach. When ^{14}C was introduced in white clover via bicarbonate the proportion allocated to stolons and roots were lower than by ^{14}C application via CO_2. For ryegrass there were no significant differences in ^{14}C allocation comparing the two C labeling approaches. The bulk allocation of ^{15}N within the white clover did not differ between the CO_2 and bicarbonate treatments, whereas for ryegrass, bicarbonate led to significantly higher ^{15}N allocation to roots than in the CO_2 treatment.

Allocation of ^{15}N to ^{14}C areas with contrasting ^{14}C activities within the plants

The ^{14}C images showed a very heterogeneous distribution of recent assimilates within the plants (Figs. 1, 2, 3 and 4) both when using bicarbonate and CO_2 as ^{14}C carrier. Ryegrass and white clover both had heterogeneous ^{14}C distributions with clear ^{14}C hotspots in the roots. For white clover, ^{14}C hotspots were also found in

Table 1 The ^{15}N enrichment (atom% ^{15}N excess) and ^{14}C specific activity (dpm/mg) in bulk samples of white clover and grass parts, and the proportion of ^{15}N and ^{14}C in root or stolon as compared to leaves. Average and S.E. based on 5 replicates

Species	^{14}C applied		^{15}N measurements		^{14}C measurements	
			at% ^{15}N ex	% of leaf	^{14}C amount	% of leaf
Clover	Bicarbonate	Leaf	6.0±1.3a		63±21	
		Stolon	1.8±0.3b	30±4a	9.2±3.4	14±0.5b
		Root	1.0±0.3b	18±2b	11±4.7	16±0.9b
	CO_2	Leaf	6.0±1.3a		30±4.1	
		Stolon	2.1±0.5b	35±4a	11±3.5	35±8a
		Root	1.1±0.2b	20±2b	9.8±2.0	32±4a
Grass	Bicarbonate	Leaf	11.3±2.5a		55±20	
		Root	3.4±0.5b	33±4a	12±3.0	26±9a
	CO_2	Leaf	12.5±1.9a		17±2.6	
		Root	1.9±0.3b	16±2b	2.5±0.9	15±5a
Analysis of variance (F values)						
Plant species			20.61***	0.98		1.19
Labeling method			0.01	1.81		4.24
Plant part			38.97***	19.04***		0.001
Labeling method × Plant species			0.01	12.81**		9.72**
Plant species × Plant parts			6.83*	0.15		0.13
Labelling method × Plant parts			0.33			
Plant species × Labeling method × Plant parts			0.8			

Different letters show significant differences at $p<0.05$ (TukeyHSD test) within species between plant parts

Signif. codes: ***$p<0.001$, **$p<0.01$, *$p<0.05$, .$p<0.1$

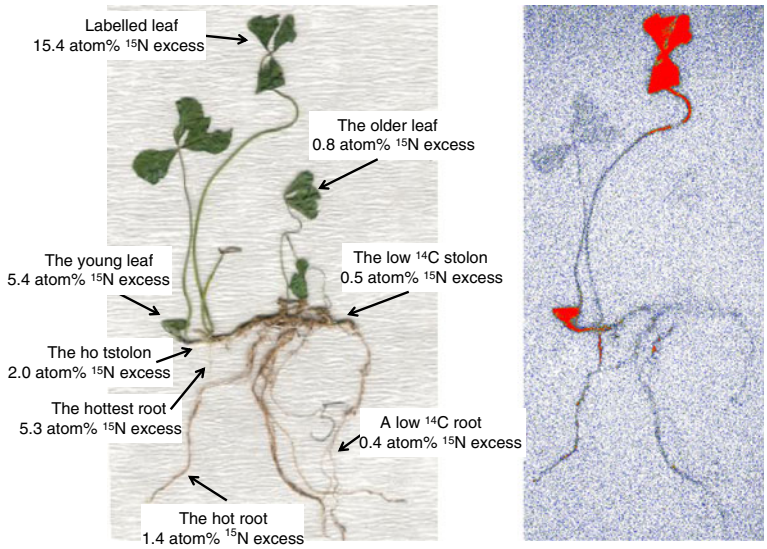

Fig. 1 ^{14}C distribution in white clover by labeling with bicarbonate; examples of the heterogeneous ^{15}N enrichment in plant parts are presented. The red color on the ^{14}C image to the right shows the most intense ^{14}C signal and the blue color shows the background

the shoots in younger leaves and flowers having stronger ^{14}C signals on the ^{14}C images than older leaves (Figs. 1 and 2). The main difference between ^{14}C allocations by the two C labeling approaches was that for ^{14}C bicarbonate the labeled leaf always had a strong ^{14}C signal, which for white clover accounted for 70–80 % of the total DLU recorded. In contrast, when CO_2 was the ^{14}C carrier then the leaf immersed in the ^{15}N urea solution did not have a ^{14}C signal (Fig. 5).

According to the distribution of ^{14}C on the ^{14}C images we divided the exposed plants into areas of high ^{14}C (^{14}C hotspots) and low ^{14}C activities and analyzed the ^{15}N enrichment of these. In general the ^{14}C hotspots had higher ^{15}N enrichments than the low ^{14}C activity areas (Fig. 5). There were a positive correlation between ^{14}C activity and ^{15}N enrichment in white clover labeled with bicarbonate (R^2=0.80 with the data of labeling-leaves included, and R^2= 0.63 when excluding the labeling-leaves). Thus, the recently assimilated C and N were allocated in the same areas. It could be tempting to compare the average ^{15}N enrichment in hotspots and low ^{14}C activity areas, but the variation in ^{15}N enrichment among plant parts is more interesting when comparing labeling methodologies. The variation in ^{15}N enrichment in white clover and grass labeled by bicarbonate and

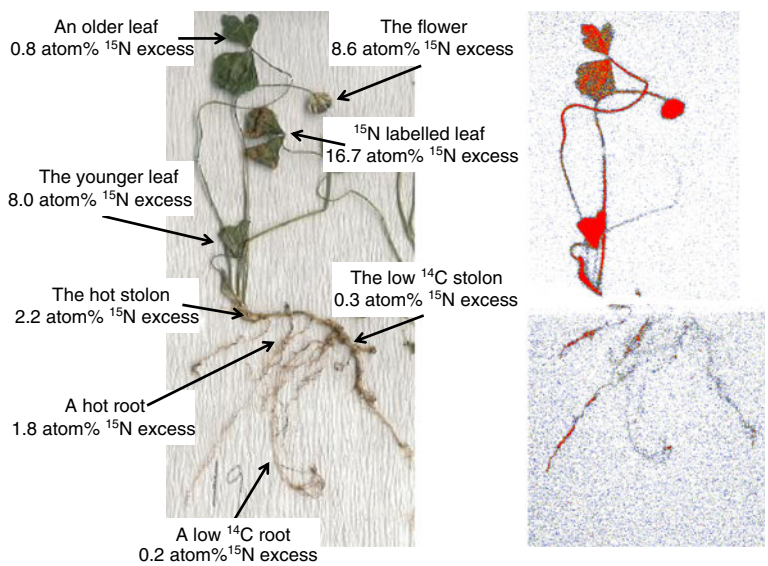

Fig. 2 ^{14}C distribution in white clover by labeling with CO_2; examples of the heterogeneous ^{15}N enrichment in plant parts are presented. The red color on the ^{14}C image to the right shows the most intense ^{14}C signal and the blue color shows the background

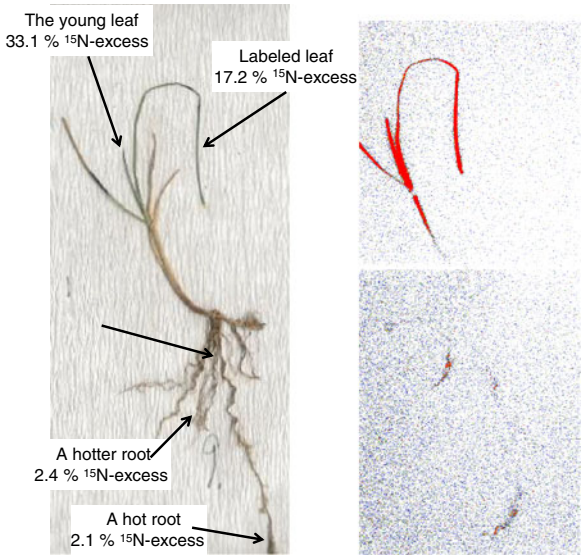

Fig. 3 ^{14}C distribution in ryegrass by labeling with bicarbonate; examples of the heterogeneous ^{15}N enrichment in plant parts are presented. The red color on the ^{14}C image to the right shows the most intense ^{14}C signal and the blue color shows the background

CO_2 is illustrated in Figs. 1, 2, 3 and 4. When excluding the leaf used for ^{15}N labeling, the ^{15}N enrichment ranged from 0.2 to 29 atom% ^{15}N excess for white clover and from 0.3 to 33 atom% ^{15}N excess for ryegrass (Fig. 5). Thus, differences in the ^{15}N allocation between and within the plant parts were about two orders of magnitude. For the ^{14}C hotspots there was a positive correlation between ^{14}C signal and ^{15}N enrichment, but also plant parts with weak or no ^{14}C signals had clear ^{15}N enrichments.

Discussion

Incorporation of C-tracers in living plants is an important and essential tool for understanding of the fate of assimilated C in plants and in the whole ecosystem. Ideally the C-tracer should enter the plant via its dominating physiological pathway as assimilation of CO_2 from the atmosphere. However, in many experimental setups e.g., by tracing of C assimilated by individual species in a plant community it is needed to add the C-tracer to specific plants excluding the possibility to use CO_2 assimilation for labeling. In these situations sugars have been used as the C-carrier (Wichern et al. 2007). The first aim of this study was to investigate whether bicarbonate can be used as an alternative C-carrier. The present data clearly support that bicarbonate can be used as an effective C-carrier, with an important advantage of e.g., leaf labeling being that in contrast to CO_2 labeling, high solar irradiation or light intensity is not necessary for the uptake of N and C tracers from the feeding solution. This means that leaf labeling can be applied even at cloudy days. However, it should be noted that the C-allocation within the plant using bicarbonate does not fully resemble that of CO_2. This is connected with the fact that by application of bicarbonate only one leaf (or a few leaves) of the plant will be soaked in labeling solution. In contrast, by the labeling in a $^{14}CO_2$ atm, all leaves have theoretically the same access to the label. Consequently, when using bicarbonate as C-carrier the labeled leaf will have a higher C-tracer enrichment. Using bicarbonate as C-carrier with the leaf-labeling method does bring a

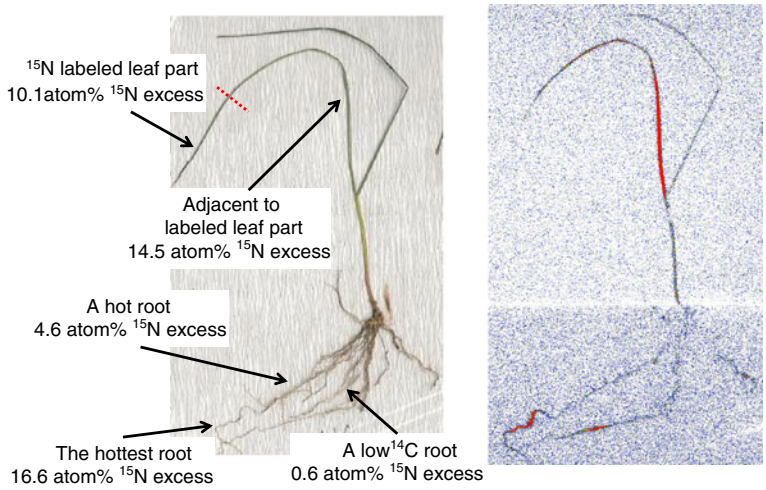

Fig. 4 ^{14}C distribution in ryegrass by labeling with CO_2; examples of the heterogeneous ^{15}N enrichment in plant parts are presented. The red color on the ^{14}C image to the right shows the most intense ^{14}C signal and the blue color shows the background

greater proportion of C-tracer into the roots of white clover than e.g., the "foliar brushing" method proposed by Putz et al. (2011). Further, additional studies are needed to elucidate to what extent the pathway of tracer application causes the observed higher allocation of ^{15}N to ryegrass roots when using bicarbonate as C-carrier compared to CO_2, as this will affect the interpretation of labeling studies.

The present study confirmed the findings of Pausch and Kuzyakov (2011) and Wichern et al. (2011) that newly assimilated ^{14}C is distributed heterogeneously in ^{14}C hotspots and low ^{14}C activity areas. The present study additionally showed a very heterogeneous distribution of ^{15}N in the plants. Inhomogeneous tracer distribution must be expected when using a pulse labeling approach, but finding that ^{15}N-enrichment differs up to two orders of magnitude is somewhat unexpected. Our findings underline that we have methodological challenges when estimating e.g., rhizodeposition (Rasmussen 2011) and N transfer through plant labeling studies. It might be needed to use more advanced calculation approaches (e.g., Remus et al. 2006) based on uneven tracer distributions. A "good practice"-suggestion from the present study is to remove the labeling-leaf after end of labeling since this leaf clearly is highly enriched. However, the feasibility of this recommendation is case dependent, as repeated removal of labeling-leaves e.g., in a multiple-pulse labeling experiment, could damage the growth of the plant.

The third aim of the present study was to investigate to what extent ^{15}N enrichment paralleled the C-tracer flow within the plant. Our data showed that ^{14}C hotspots were also ^{15}N hotspots, so that a highly heterogeneous distribution was apparent for both C and N tracers. However, some low ^{14}C activity areas were also well enriched with ^{15}N, meaning that the N-tracer did not completely parallel the allocation patterns of the C-tracers (Yasmin et al. 2010) and that the ^{15}N distribution was more homogeneous. This result has important consequences as it shows that independent on the labeling method, the N-tracer will

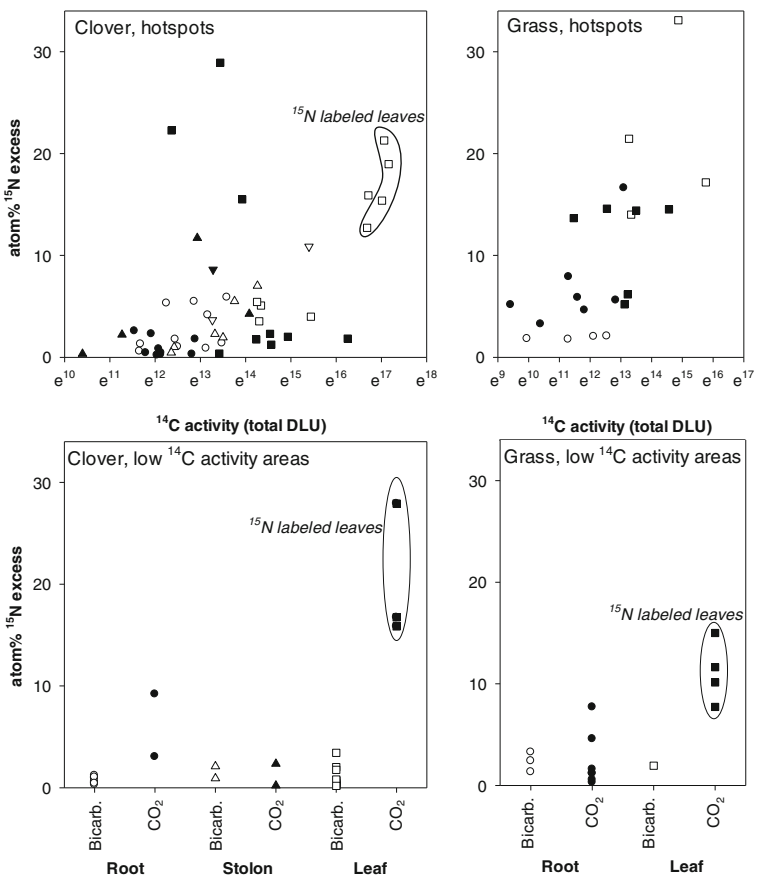

Fig. 5 Variation in atom% ^{15}N excess for clover and ryegrass ^{14}C labeled with bicarbonate (open) or CO_2 (closed) divided in plant parts: roots (*circle*), stolons (*triangle down*), flowers (*triangle up*), and leaves (*square*). On the two upper figures with ^{14}C hotspots, the correlation is shown between ^{15}N enrichment and the measured ^{14}C activity in Digital Light Units (DLU) on the ^{14}C images. On the two bottom figures with low ^{14}C activity areas (no signal on the ^{14}C images) the variation in atom% ^{15}N excess is shown

have a less heterogeneous distribution in the plant than the C-tracer. Accordingly, the correct selection of labeling approach is more important for C than for N tracing.

In conclusion, the present study shows the potential for using bicarbonate to add ^{14}C (or ^{13}C) to plants especially for targeted labeling. The study shows the expected heterogeneous tracer distribution of both C and N after pulse labeling both with $^{14}CO_2$, ^{14}C-bicarbonate, and ^{15}N-urea. However, the magnitude of the heterogeneity in ^{15}N distribution of up to two orders of magnitude is surprising. Nevertheless for targeted studies focused on individual plants within a plant community (e.g., a grass-clover sward), the leaf-labeling method still remains a very useful option without appropriate alternatives. Furthermore, the study shows that the distribution of N-tracer is more homogeneous than that of C-tracers.

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