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Rhizodeposit Carbon Gradients: Potentials and Limitations of Destructive Rhizosphere Sampling on a Millimeter-Scale

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ABSTRACT

Background: Despite the availability of modern techniques for high-resolution non-destructive rhizosphere analysis, destructive examinations yielding a certain minimum soil amount are often required to provide detailed insights into organic matter composition.

Methods: We compared an established approach for destructive rhizosphere sampling via root brushing to a new millimeter-scale gradient sampling approach, expecting that the latter allows to characterize spatial patterns of rhizodeposit-carbon (C) distribution and relate them to root traits and soil texture. A tool to sample soil in 2 mm steps around a root was developed. Maize with and without root hairs was grown under field conditions until the end of tassel emergence, either in loam or in sand, and labeled with $^{13}CO_2$ one day before harvest.

Results: Both approaches showed an enrichment of C and ^{13}C in sandy and partially in loamy rhizosphere, but no δ^{13}C gradient could be statistically demonstrated due to high variability. The major uncertainty of both approaches was the potential masking of bulk soil organic C concentration and isotopic composition by non-target roots. The new gradient sampling approach offers uniform, pre-defined, and thus neutral conditions with respect to sampling distance independent of root and soil properties; yields at least 100–200 mg of soil on a millimeter-scale from one individual root segment; and can be applied in natural settings without root growth artifacts. The presented techniques integrated signals from fine roots and root hairs.

Conclusions: For root systems with longer unbranched segments, the new approach has potential for tracing ^{13}C released by roots and for analyzing plant and microbial remains at the millimeter-scale.

1 | Introduction

The rhizosphere, defined as the volume of soil around roots that is influenced by root activity in chemical, physical, and biological regards (e.g., Uren 2007), is a highly dynamic and diverse zone where numerous processes interact with each other. It is a hotspot of carbon (C) input from roots, as well as C turnover by microorganisms (Kuzyakov and Blagodatskaya 2015). Numerous studies have investigated C release and distribution in the rhizosphere, as reviewed, for example, by Jones et al. (2009), often using assimilated ^{13}C as a tracer (e.g., Pausch et al. 2016; Schmitt et al. 2013).

Depending on the objective, the rhizosphere extent can be determined by the steepness of the gradient of the respective compound (Darrah 1993) and accounts for a range between a few micrometers and up to several millimeters around the root, depending on the compound in question and its mobility (Hinsinger et al. 2009). Models commonly give a gradient of decreasing C concentrations from root toward soil (e.g., Jones et al. 2009; Landl et al. 2021; Schnepf et al. 2022) due to C release by the root and microbial consumption. It remains unclear, however, to what degree external drivers (see Vetterlein et al. 2020), such as root hairs and soil texture–related pore space or soil moisture, control the extent and the shape of rhizosphere C gradients.

Traditional methods of rhizosphere analysis distinguish between the two compartments "rhizosphere soil" adhering to roots after gently shaking and/or washing and/or brushing them on the one hand and "bulk soil" on the other hand (e.g., Gocke et al. 2011; Schreiter et al. 2014). *Sensu stricto*, bulk soil refers to the soil not influenced by roots to an extent that can be detected, whereas the soil adhering to roots is defined as the rhizosheath, which itself "can be considered the most biologically active fraction of the rhizosphere" (Ndour et al. 2020). Unfortunately, the terms "rhizosphere soil," "rhizosheath," and "bulk soil" are not used in a uniform way by different disciplines. The sampling approach described above, where rhizosphere soil is obtained by shaking/washing/brushing off roots, allows for a rough distinction between soil affected and soil not affected by roots. The approach does, however, not yield precise information on spatial context, as the amount of adhering soil depends on root age, plant species, soil texture, and moisture during sampling (Vetterlein et al. 2020). However, concentration and flux profiles around roots decrease with the square root of distance from the root surface, resulting in specific gradients (de Parseval et al. 2017). New opportunities for the investigation of these specific gradients were developed in the last decade, reviewed by Oburger and Schmidt (2016) and Vetterlein et al. (2020). They include mainly in situ non-destructive visualization and imaging techniques such as microtomography, giving high-resolution (nm-to-µm scale) three-dimensional insight into rhizosphere processes and spatial arrangement (Lippold et al. 2023). Simultaneously, the destructive sampling of rhizosphere (reviewed by Luster et al. 2009) on the millimeter-scale was further developed on the basis of compartment box studies. In these particular plant pots, which were invented in the 1960s, the root surface is separated from the adjacent soil via a fine-meshed gauze (e.g., Hafner et al. 2014; Sauer et al. 2006; Vetterlein and Jahn 2004). Unfortunately, the growth of thick root mats on the gauze can lead to artificial conditions with altered aeration, water availability, and other factors in such settings. Using rhizoboxes entails further artifacts due to the sensitivity of some components of root exudation to the sampling setup (Oburger et al. 2013) and due to the required freezing prior to slicing (Fitz et al. 2003).

An innovative approach of three-dimensional rhizosphere sampling under field conditions has been introduced by Gocke et al. (2014) for fossil, calcified roots as well as thick tree and shrub roots, and was successfully applied in soils and various sedimentary deposits for analysis of organic and inorganic C concentrations, as well as root- and microorganism-related biomarkers. The authors collected concentric slices of loess with radii between 5 and 25 mm around fossil roots to show the rhizosphere extent in soils and sedimentary deposits. Petzoldt et al. (2020) adapted this sampling approach for the investigation of earthworm- and taproot-derived biopores in steps of 2–4 mm radius. To the best of our knowledge, there are currently no studies available that determined rhizosphere gradients under natural field conditions (i.e., without pre-installed compartments affecting root growth) on a millimeter-scale.

We hypothesized that (i) root exudates form gradients that are detectable using recently assimilated ^{13}C as an isotopic tracer and that (ii) root traits (presence or absence of root hairs) and soil texture (loam or sand) shape these rhizosphere gradients in terms of extent and steepness. The second hypothesis is based on the fact that root hairs may increase the soil volume explored by an individual root segment, like previously shown for barley (Holz et al. 2018), and soil texture controls porosity and moisture of soil and hence water and nutrient fluxes (Jarvis 2007). We further hypothesized that (iii) a millimeter-scale gradient sampling approach yields sufficient soil material for investigation of rhizosphere C distribution. We here compare an established, conventional sampling approach for rhizosphere sampling via root brushing and washing to a new millimeter-scale gradient sampling approach via metal cylinders. Both approaches were applied in a field experiment (Bad Lauchstädt, Germany) with maize with root hairs and maize without root hairs, both grown either in loam or in sand and pulse-labeled with $^{13}CO_2$ one day before sampling (Vetterlein et al. 2021).

2 | Material and Methods

2.1 | Experimental Design and ^{13}C Isotope Labeling

Soil samples were collected from the central field experiment of the DFG priority program 2089, "Rhizosphere Spatiotemporal Organisation—a Key to Rhizosphere Functions," which had been established in 2018 in the research station Bad Lauchstädt, Germany (N51°22′0″, E11°49′60″). The field experiment had a 2-factorial, randomized block design with six field replicates, resulting in 24 individual plots. Factor one was the maize (*Zea mays* L.) genotype with different root hair characteristics, and factor two was soil texture. Besides the *Z. mays* wild-type (WT) with normal root hair growth, the *Z. mays* mutant *rth3* was included in the field experiment because it showed normal root

hair initiation but disturbed elongation. For simplification, the mutant genotype is called root hair-less in the following. The two soil textures included a loam (L) and a sand (S), where the former was obtained from 0 to 50 cm soil depth of a haplic Phaeozem and the latter represented a mixture of L with quartz sand (16.7% loam, 83.3% quartz sand; Vetterlein et al. 2021). These substrates differed in their texture, bulk density, initial C and N concentrations, and further chemical properties (Vetterlein et al. 2021).

Original soil was removed from the plots, and the respective substrate, that is, loam or sand, filled in to a depth of 75 cm and packed with a defined procedure to obtain homogenous conditions without layering or grain sorting. Maize plants were then grown under fertilization conditions that are slightly nutrient deficient, as well as no tillage (Vetterlein et al. 2021).

To trace the fate of assimilated C, maize plants received two short-term ^{13}C pulses over a 4 h period in a gas-tight chamber (Pausch et al. 2016) at the end of tassel emergence (corresponding to the phenological development stage BBCH 59; Bleiholder et al. 2001). For this purpose, ^{13}C-enriched CO_2 was released from 32 g of $Na_2{}^{13}CO_2$ dissolved by sulfuric acid, whereas the soil was sealed with a plastic foil to avoid direct gas exchange (Vetterlein et al. 2021).

2.2 | Sampling and Sample Preparation

One day after ^{13}C labeling, in each of the 24 plots, six soil gradients around roots, each consisting of four concentric soil slices of increasing distance from the root were collected.

For the concentric soil slices, a metal drawer (size 100 × 150 × 35 mm^3, length × width × height; Figure 1A) and matching lid, both having sharpened fronts, were inserted with the open side into the soil at an angle of approximately 45° and a depth interval from 25 to 32 cm at several places around one maize plant at a distance of approximately 15 cm from the stem (Figure 1B). To recover the drawer and lid from the soil, surrounding soil was first removed by hand as far as possible, then a stainless-steel knife was inserted along the open front of the drawer and lid. Thereby, smaller roots, which were partially inside the drawer and partially outside, were carefully cut. This ensured that the soil inside the drawer stayed in spatial context while taking the drawer out in the next step. After removing the lid, one to four roots at least 5 cm distant from each other and from other roots were chosen for gradient sampling (Figure 1D2). Main criteria for root selection were a light, healthy color and the root diameter (~0.5 mm), as very small roots bear the risk that they end above the bottom of the drawer. For the next step, a set of four cylinders with inner radii 2, 4, 6, and 8 mm was used, which also had a sharpened front (Figure 1C). The four cylinders had in their lower part a wall thickness of 0.4 mm each and were designed in such a way that all of them stayed in concentric position to each other due to the thickened upper part of each cylinder, as each cylinder had in its upper part an outer diameter almost as large as the inner diameter of the next larger cylinder (Figure 1C). The smallest cylinder was placed over a selected root, with the root in the central position of the cylinder. The cylinder was carefully pushed into the soil down to the bottom of the drawer, which was then repeated with the three larger cylinders (Figure 1D1), respectively. With an estimated average diameter of roots of 317 µm (Vetterlein et al. 2022), this means that the distances from root surface in the four soil slices were 0–1.84 mm, 1.84–3.84 mm, 3.84–5.84 mm, and 5.84–7.84 mm, respectively. Afterward, the soil from each of the cylinders was removed using a piston (Figure 1D3). These steps were repeated until a total of six gradients of four soil slices each were available from every plot (n = 24 plots × 6 gradients × 4 slices = 576 gradient samples). Each soil slice sample was checked for macroscopic root remains and, after removing these with tweezers, transferred to a glass vial with a plastic lid. Shaking off the root obtained from the innermost soil slice was not necessary, as there were no major portions of soil adhering to the root due to the short length of 35 mm and subsequently minor or absent branching.

All samples were transported on dry ice, then immediately frozen and dried under controlled conditions at 40°C.

For each of the 24 plots, soil slices with identical distances to the root from the six gradients were pooled for analyses, resulting in a total of 96 gradient soil samples. These pooled samples were carefully crushed and homogenized using a quartz mortar and pestle.

Additionally, bulk soil samples were collected prior to planting in 0–20 cm depth, which represent the initial soil conditions before plant growth and before isotopic labeling. These samples were therefore called reference soil. Reference $\delta^{13}C$ values were −26.26‰ and −25.94‰ for loam and sand, respectively.

Data obtained with the new gradient sampling approach were compared with those generated with the conventional sampling approach used within the DFG priority program 2089 (Ganther et al. 2022). For the conventional approach, soil monoliths with dimensions 20 × 20 × 20 cm^3 were excavated and carefully broken up. Then roots were separated from soil with tweezers to obtain bulk soil. Roots as well as rhizosphere soil were obtained by brushing off adhering soil from roots with soft toothbrushes. Soil monoliths were excavated within the same ^{13}C labeled subplots, which were used for the new gradient sampling technique. For comparison with the gradient sampling approach, where the sampling depth was 25–32 cm, here we used the monolith data from 20 to 40 cm depth extracted at BBCH 59 (1 monolith per plot, 6 plots per treatment, 4 treatments = 24 samples of bulk soil, rhizosphere, and roots each, resulting in a total of 72 samples). To remove the remaining soil adhering to the roots, these were vortexed in 0.3% (w/v) NaCl, then snap-frozen in liquid nitrogen and stored at −80°C. Root samples were crushed and homogenized under liquid nitrogen using a mortar and pestle. Subsequently, the homogenized root powder was dried at 40°C for 3 days. Soil samples were stored at −20°C and dried at 40°C for three days prior to homogenization with mortar and pestle.

2.3 | Analysis of Organic Carbon Concentrations and Carbon Stable Isotope Composition

The sample set from the gradient sampling approach (n = 96 soil slices pooled from six roots per plot) was analyzed for C

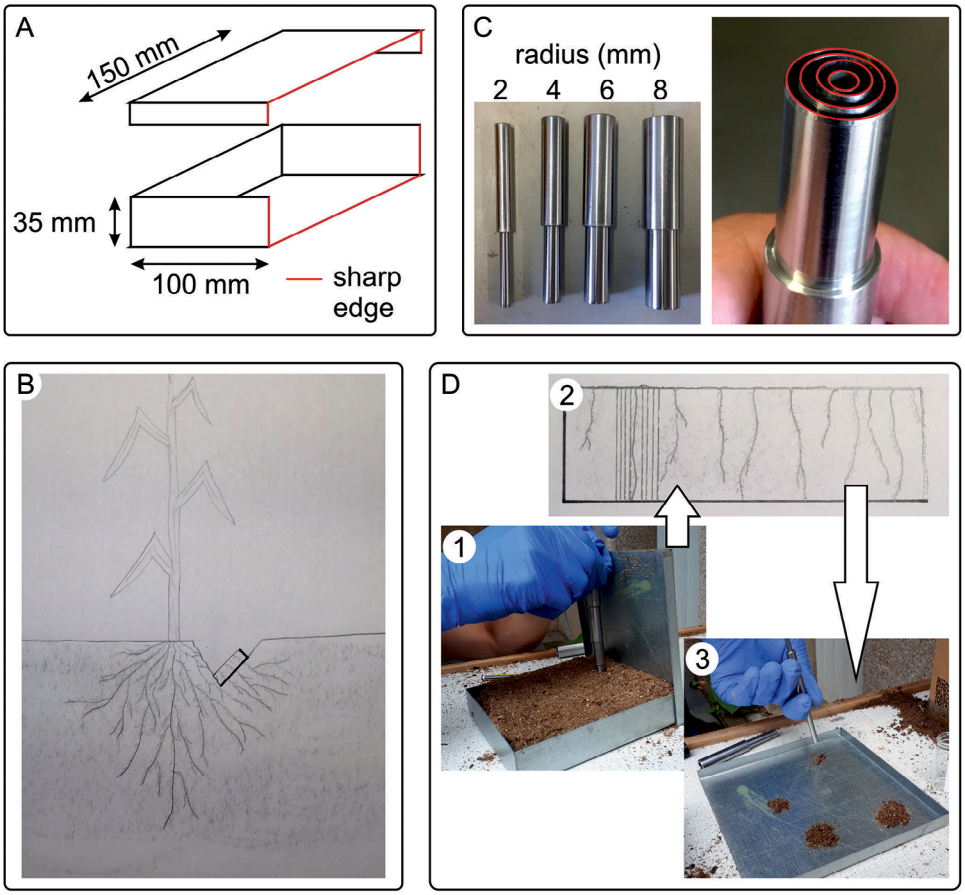

FIGURE 1 | Equipment (A and C) and strategy (B and D) of the gradient sampling approach. (A) Drawer used to obtain a soil block. (B) Scheme of sampling approach, where the drawer was inserted several times around one plant. (C) Cylinders used to obtain concentric slices around a root with increasing distances. (D) Application of cylinders within the drawer, as well as the procedure of obtaining individual soil slice samples.

concentrations and $\delta^{13}C$ isotope ratios at Bayreuth University, using an elemental analyzer (NA 1108, CE Instruments, Milano, Italy) coupled via a ConFlo III interface (Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta S, Finnigan MAT, Bremen, Germany).

The sample set from the conventional sampling approach ($n = 72$) was analyzed for C concentrations and $\delta^{13}C$ isotope ratios at Kompetenzzentrum Stabile Isotope, Göttingen, on an isotope ratio mass spectrometer (DELTAplusCP for soil samples, DELTA V Advantage for root samples; both Thermo Fisher Scientific, Waltham, Massachusetts).

Acetanilide was used as reference material. Raw data were expressed as $\delta^{13}C$ relative to V-PDB ($R = 0.011237$).

2.4 | Presentation of Data and Statistics

Diagrams show averages from six field replicates ± standard error of the mean. Along the gradients extending from the root surface, differences between distances were tested for significance by one-way ANOVA with significance levels of 0.05 (significant) and 0.01 (highly significant), respectively, followed by post hoc Scheffé test using STATISTICA 7.0 (StatSoft).

3 | Results

3.1 | Sample Yields

For the conventional sampling approach, 7–12 g of dry soil per g of root fresh weight were obtained by brushing off the adhering soil. This corresponds to 0.04 g of soil if we assume an individual root segment of 35 mm length (as sampled with the new gradient sampling method) with an average root diameter of 317 µm and a conversion factor from root fresh weight to volume of 1.25 (Vetterlein et al. 2022). For this given geometry and soil bulk density, a weight of 0.04 g represents a distance from the root surface of 360 µm. Rhizosphere samples obtained with the conventional method thus were sampled closer to the root surface than the innermost sample obtained with the new gradient sampling method (360 vs. 1840 µm).

Concerning the new gradient sampling approach, yields of dry soil from individual roots and transects (i.e., before pooling of samples from equal root distance) were in the range of 0.2–0.3 g for the innermost soil slice, independent of soil texture and maize genotype ($n = 576$ samples, Table 1). With increasing distance, sample yields increased to a range between 0.5 and 1.3 g for the second slice and then further to 0.8–2.3 g in the third slice and

TABLE 1 | Sample amounts of dry soil in grams (range and median [in parantheses]) per soil slice obtained with the gradient sampling approach from the four distances from the root surface (in mm) for the two substrates, loam and sand, as well as the calculated sample amount of rhizosphere soil obtained with the conventional approach (for calculation, see Section 3.1 and Vetterlein et al. 2022).

Sampling technique		Loam	Sand
Gradient sampling approach	0–1.84 mm	0.20–0.35 (0.28)	0.17–0.34 (0.27)
	1.84–3.84 mm	0.48–1.29 (0.71)	0.76–1.08 (0.91)
	3.84–5.84 mm	0.84–2.29 (0.85)	0.84–2.06 (1.22)
	5.84–7.84 mm	0.65–1.32 (0.99)	0.58–2.59 (0.84)
Conventional sampling	Rhizosphere soil	0.04	0.04

Note: For the gradient sampling approach, each range comes from six individual sampling gradients within 12 field plots of the respective substrate. Height of soil slices was 35 mm each. No significant difference was found between maize genotypes (not shown).

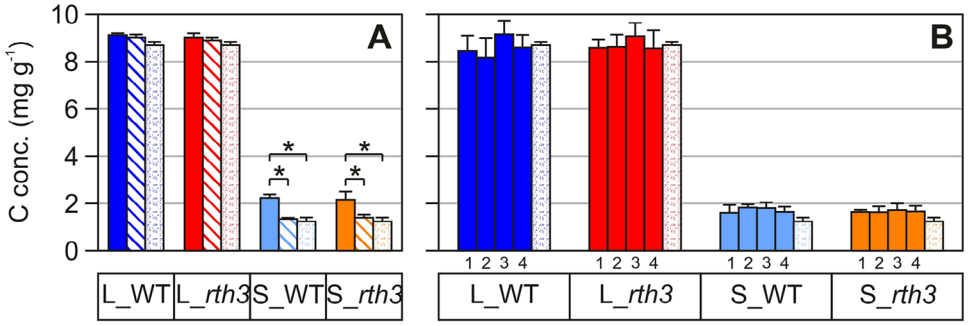

FIGURE 2 | Carbon (C) concentrations in rhizosphere and root-free soil obtained via (A) the conventional sampling approach and (B) the gradient sampling approach for the four treatments: loam × wildtype (L_WT), loam × mutant (L_*rth3*), sand × wildtype (S_WT), and sand × mutant (S_*rth3*). Filled bars represent rhizosphere soil (gradient sampling approach: 1 = 0–1.84, 2 = 1.84–3.84, 3 = 3.84–5.84, 4 = 5.84–7.84 mm). In the conventional sampling approach, hatched bars represent bulk soil from identical sampling dates (depth 20–40 cm). Dotted bars represent reference soil of initial conditions prior to planting (depth 0–20 cm). Please note that for both approaches, identical reference soil samples were included in the sample set. An asterisk indicates a significant difference at $p < 0.05$ between the soil samples within a given treatment collected by conventional sampling. No significant differences were found between the soil samples within each treatment collected by gradient sampling.

0.6–2.6 g in the fourth slice, again without significant differences between soil texture and maize genotypes.

3.2 | Carbon Concentrations in Soil

From conventional sampling, a range of 8.3–9.4 mg C g^{-1} (Figure 2A) was detected for rhizosphere soil, bulk soil, and reference soil in loam treatments. In sand, C concentration in bulk soil and reference soil was ~1.3 mg g^{-1}, in contrast to significantly elevated C concentrations in rhizosphere soil of 2.2 mg g^{-1}. There was no difference between maize genotypes.

Over the whole gradient sample set, C concentrations varied between 5.8 and 11.2 mg g^{-1} in loam and between 0.3 and 2.5 mg g^{-1} in sand, with no significant differences between the maize genotypes. No clear trend from innermost rhizosphere toward reference soil was visible (Figure 2B).

3.3 | $\delta^{13}C$ Isotope Composition of Soil and Roots

From root isotopic composition it was obvious that assimilated ^{13}C reached belowground within 24 h after labeling, resulting in root $\delta^{13}C$ values of 287‰ ± 97‰, 123‰ ± 16‰, 188‰ ± 13‰, and 355‰ ± 26‰ for the treatments L_WT, L_*rth3*, S_WT, and S_*rth3*, respectively, at 20–40 cm depth (data not shown here).

For conventional rhizosphere sampling, $\delta^{13}C$ values in the loamy treatments were only slightly elevated in the rhizosphere (−22.6‰) compared with reference soil, with no difference between genotypes (Figure 3A). In sand, $\delta^{13}C$ values were considerably higher in the rhizosphere (on average −14.68‰ and 79.57‰ for WT and *rth3*, respectively) compared with reference soil, but due to the large variability within treatments (S_*rth3*), no significant difference between genotypes was found.

With the gradient sampling approach, $\delta^{13}C$ gradually and consistently decreased from innermost rhizosphere to reference soil. However, the variation in the data set was too large to show significance (Figure 3B). The only exception to this trend was the treatment loam with root hair-less maize, in which labeling clearly did not lead to elevated $\delta^{13}C$ in soil close to roots. In all other treatments, $\delta^{13}C$ was highest in the first two soil slices, that is, until 3.84 mm distance from the root. Then, it decreased and nearly reached reference soil $\delta^{13}C$ levels in the fourth slice, that is, after 5.84 mm distance from the root, in loam. In contrast, in sand there was still a difference in ^{13}C levels between the outermost soil slice and the reference soil of 6‰ and 16‰ with the wildtype and mutant, respectively (Figure 3B).

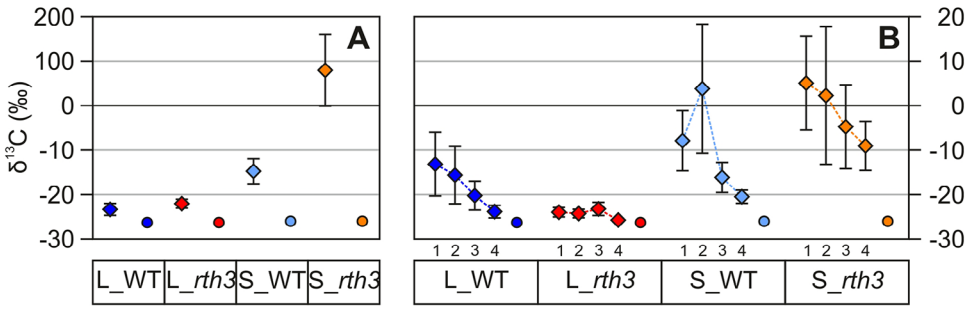

FIGURE 3 | Stable isotopic composition of C (δ^{13}C) in rhizosphere and root-free soil obtained via (A) the conventional sampling approach and (B) the gradient sampling approach for the four treatments: loam × wildtype (L_WT), loam × mutant (L_*rth3*), sand × wildtype (S_WT) and sand × mutant (S_*rth3*). Diamonds represent rhizosphere soil (for distances of slices in gradient sampling approach 1–4, see Figure 2). Circles represent reference soil of initial conditions prior to planting (depth 0–20 cm). Please note that for both approaches, identical reference soil samples were included in the sample set. Note the different scales of positive *y*-axes. No significant differences were found between the soil samples within each treatment, neither collected by conventional nor by gradient sampling.

4 | Discussion

Both of the here presented destructive rhizosphere sampling approaches have been designed for the application on plants grown under field conditions without pre-settings such as root windows or ingrowth tubes, that is, on root systems with natural architecture. This allows for the prevention of artificial growth conditions occurring in the mentioned field settings as well as in greenhouse experiments, such as thick root mats or alterations in aeration and water availability, for example, in compartment boxes or rhizoboxes. This is an important aspect, for example, for studies on root exudates, root respiration, and oxygen diffusion (Sauer et al. 2006; Oburger et al. 2013; Uteau et al. 2015; TIziani et al. 2021).

The main difference between the two rhizosphere sampling approaches here is the fact that the established approach via brushing represents findings from the root system of a whole maize plant—or from the respective monolith, potentially containing root systems of more than one plant. In contrast, the new gradient sampling approach yields data from one specific, 35 mm long root segment within the overall root system. For the new approach, target roots were chosen on the basis of their diameter, with the aim of selecting gradients around young active roots, but the age and function of these selected roots were not necessarily the same in each collected gradient. Further, average root diameter was larger in sand than in loam and larger in the maize mutant than in the wildtype (each by a few tens of micrometers; Vetterlein et al. 2022), potentially impairing a direct comparison of results from both approaches.

4.1 | Spatial Arrangement of Carbon Dispersal

Numerous studies have investigated C distribution in the rhizosphere (reviewed by Jones et al. 2009). The fate of C starts as roots push through the soil, releasing mucilage and sloughed root cap cells as well as exudates, and only a bit later most of it will be incorporated into microbial biomass and finally immobilized in microbial necromass (e.g., Liang et al. 2017), absorbed to minerals, or released as CO_2.

We expected an enrichment in C for the rhizosphere soil (slices) compared with bulk soil (conventional sampling) and reference soil (conventional and gradient sampling) due to organic matter input via root and microbial remains (Hinsinger et al. 2009; Philippot et al. 2013). An increase in C concentrations was observed solely in C-poor sand, with significant differences by the conventional approach and as a tendency without statistical significance also by the gradient sampling approach (Figure 2A,B).

Similarly, the ^{13}C isotopic composition of bulk C was not suitable to distinguish rhizosphere soil (slices) from root-free soil, either due to the close proximity of values, especially in loam, or due to high variations between replicates, especially in sand. Yet, similar to root samples, rhizosphere soil from both sampling methods was systematically enriched in ^{13}C (Figure 3A,B), demonstrating the incorporation of recently assimilated ^{13}C into soil.

The estimation of the rhizosphere extent is possible by gradients and their steepness, as concentration and flux profiles around roots decrease with the square root of distance from the root surface (de Parseval et al. 2017). With analysis of bulk C concentration, the new sampling approach presented here failed to detect such gradients from the root surface toward root-free soil, which might be the result of masking by non-target roots (see Section 4.3.3).

Our first hypothesis was that root exudates form gradients that are detectable using recently assimilated ^{13}C as a tracer. This was partially confirmed, as both approaches were suitable to detect incorporation of recently assimilated ^{13}C into the soil, and trends for δ^{13}C gradients were revealed for the wildtype in loam and for both maize genotypes in sand, respectively.

4.2 | Effect of External Drivers on Root Gradients

For the gradient sampling approach, we found some heterogeneity in C concentrations and δ^{13}C values between the six field replications within each treatment, as reflected by error bars mostly larger than those from conventional sampling (Figures 2 and 3). Such variations tended to be largest in the innermost

two soil slices (e.g., $\delta^{13}C$ for maize wildtype, Figure 3B), which was likely a result of variations in root diameter, age, and function of the roots chosen for transect sampling (see above). The variations could be further caused by soil properties like, for example, the pore system at the very place of sampling. X-ray CT images showed that local variability can be greater in sand (Vetterlein et al. 2021), which is in line with larger error bars in sand compared with loam treatments for the gradient sampling approach. These factors cannot be distinguished by our new sampling approach—even more so if samples are pooled before analysis. Similarly, the conventional rhizosphere sampling approach is not capable of this, as it averages over the whole root system both in terms of root orders and soil inhomogeneities, thus masking respective differences in root and soil properties in different parts of the root system.

Nevertheless, we expected the following soil and root properties to affect C and $\delta^{13}C$ data: Distribution of root exudates is controlled only partially by the root itself, that is, permeability of the plasma membrane and spatial location of the solutes in roots (Jones et al. 2009). It is, in addition, driven by other factors, including, for example, root–soil concentration gradients of the compound in question, root–soil contact, soil sorption and transport properties; the latter are controlled by soil texture, among others. Further, it is known for barley that root hairs alter pore structure and thus diffusion and permeability in the first millimeter around the root (Koebernick et al. 2017) and extend the volume affected by root exudates on the millimeter-scale (Holz et al. 2018).

Altogether, the strongest effect on rhizodeposit dispersal was exerted by the soil texture, as loam had systematically higher C concentrations than sand, which was already expected. As a result, in sandy rhizosphere higher $\delta^{13}C$ values were reached than in loamy rhizosphere because of a lower signal-to-noise ratio in the former (see Sections 4.1 and 4.3.3). Our second hypothesis was thus partially confirmed concerning the effect of soil texture.

For the two maize genotypes with contrasting root traits, we would have expected, using the gradient sampling approach, to detect differences in terms of gradient steepness between wildtype and mutant, and thus a different rhizosphere extent (WT > *rth3*), as previously shown for barley (Holz et al. 2018). Unfortunately, the above-mentioned methodologic issues did not allow for statistically solid results. Yet, in loam, an enrichment of recently assimilated ^{13}C was visible solely for WT (Figure 3B), which could mean that in the case of a fine pore system, the distribution of C from the root zone was more efficient when root hairs were present.

The abundance of mycorrhiza-infected roots at 20–40 cm depth was between 20% and 40% at BBCH 59 but did not differ either between soil texture or between maize genotypes (Vetterlein et al. 2022). Therefore, we assume that this was not an additional source of error here. Nevertheless, mycorrhiza can introduce additional variability for our single root sampling approach.

Due to methodological issues and large variations between field replicates, we could thus neither confirm nor reject our second hypothesis with regard to the influence of root traits on rhizosphere C gradients.

4.3 | Suitability of Destructive Rhizosphere Sampling for Different Research Questions

4.3.1 | Size of Soil Samples

Although a study by Burak et al. (2021) showed significantly higher amounts of soil adhering to maize wildtype roots than to maize root hair-less mutant, we did not find differences between the genotypes in the current study using the conventional rhizosphere sampling approach. The latter yielded lower amounts of rhizosphere soil for a respective root segment than the innermost soil slice obtained with the gradient sampling approach (Table 1), thus necessitating the collection of longer or more root segments to obtain similar sample amounts. Generally, this would be especially relevant for sandy soils with low or lacking aggregation, in which soil particles may not stick well to each other or to the root, which can result in lower sample amounts (Burak et al. 2021) and thus potentially lead to an underestimation of rhizosphere extent with the conventional approach. The established conventional approach thus strongly depends on the cohesiveness of the soil, which in turn is controlled by further factors such as soil moisture (see above; Vetterlein et al. 2020). It therefore requires strict control of soil physical parameters and especially thorough instruction of field staff for uniform/consistent sampling as well as representative and reproducible results.

With the gradient sampling approach, we aimed to collect sufficient amounts of soil material for elemental and isotopic analyses and other analyses to follow, such as nutrient analyses (e.g., plant-available phosphorus via calcium–acetate–lactate extraction, Schüller 1969) and markers for microbial bio- and necromass (e.g., amino sugars, Joergensen 2018). This was most critical for the innermost soil slice 0–1.84 mm distant from the root surface, and our sampling approach enabled us to collect on average 270 mg at this distance within one single transect (Table 1), that is, using the set of four cylinders (Figure 1C) once for a single root segment. The minimum amount of soil sample obtained with the gradient sampling approach was at this time point insufficient for non-traditional isotope analyses such as $\delta^{18}O$ in soil phosphate for determination of nutrient dynamics (Bauke 2021) and rather at the lower range of sample amount required, for example, for analysis of lipids for allocation of plant and microbial sources of soil organic matter (Wiesenberg and Gocke 2017). It was, however, plentiful for elemental and isotopic analyses, thus supporting our third hypothesis.

4.3.2 | Spatial Resolution and Spatial Context

In the case of the established conventional rhizosphere sampling approach, the amount of collected soil corresponded to an average distance from the root of 360 µm (see Section 3.1). This means that the conventional approach does not resolve spatial context in distances >360 µm. Rhizosphere sampling with the new gradient sampling approach, in contrast, gives a pre-defined setting with respect to sampling distances and does not depend on plant species, root age, soil texture, or moisture. It further resolves also soil properties at distances >360 µm up to 8 mm on a millimeter-scale, whereas this soil volume is part of the bulk soil when applying the conventional sampling. The disadvantage of

these pre-defined sampling distances with the gradient sampling approach is that it cannot capture gradients of rhizosphere processes or compounds that are restricted to <2 mm around the root. The conventional approach, otherwise, cannot depict the exact spatial context of obtained data because it is applied to the root system of a whole plant. At locations with stronger adhesion, for example, due to mucilage (root tips), however, greater soil amounts will stick to the root compared with other places in the root system. Therefore, the estimated rhizosphere extent of 360 µm is an approximation, which itself depends on further estimated parameters (see Sections 3.1 and 4.3.1).

The spatial resolution of the gradient sampling approach is at a similar level to that of other destructive methods such as compartment boxes combined with cutting of the root-free compartments (e.g., Sauer et al. 2006), at the same time avoiding artificial root growth conditions and giving more realistic information on spatial context.

Compared with recently developed and permanently improved non-destructive imaging techniques (Lippold et al. 2023), both of the here presented approaches lack a high spatial resolution. The conventional approach only distinguishes two soil fractions, and a sampling distance can only be assigned on the basis of average root diameter within the sample (see Section 3.1). The gradient sampling approach can provide a resolution at millimeter-scale at best. In situ laser ablation techniques on undisturbed soil cores have shown that rhizosphere extent may only reach about 100 µm in terms of ^{13}C dispersal within 24 h (Rodionov et al. 2019; Lippold et al. 2023). The millimeter-scale extent of ^{13}C label detected in the current study via the gradient sampling approach seems to be in conflict with this. The difference is a result of scales: Laser ablation techniques observe micrometer extent of C distribution around a root segment and can resolve the signal of fine roots, whereas methods resolving millimeters reflect the composite signal of a root segment together with its fine roots (thus leading to masking of the actual gradient; see Section 4.3.3).

In summary, if regarding compounds or processes that are limited to <2 mm distance from the root, the conventional approach represents more realistically the soil volume directly influenced by roots (rhizosheath or "hotspots") but lacks information on the exact spatial context and extent of the rhizosphere. The latter can be revealed by the mentioned non-destructive imaging techniques.

4.3.3 | Limitations of Conventional and Gradient Rhizosphere Sampling and Outlook

Limitations of our new gradient sampling approach were identified by unexpected deviations in C concentrations. Typically, C accumulates in the rhizosphere relative to bulk soil (Hinsinger et al. 2009; Philippot et al. 2013). Here we could not find such enrichment in loam (both sampling methods), most likely due to high background C concentrations. In sand with low C background, C concentrations were elevated in the rhizosphere compared with bulk soil and reference soil when using the conventional approach (Figure 2A), as expected from the literature. For the gradient sampling approach, in sandy treatments, rhizosphere soil C concentrations were higher than those in reference soil but lower than those in the rhizosphere soil from the conventional sampling approach. The latter could result from one or both of the following factors: (i) Rhizosphere soil obtained by the conventional sampling can still contain root debris like hairs or fragments of fine roots after brushing, thus entailing high C concentrations, and (ii) soil slices obtained by the gradient sampling might be diluted by soil less influenced by roots due to pre-defined distances. Further, with the gradient sampling, the same values were observed across the entire gradient within each of the four treatments (Figure 2B). This may be the result of root material contributing to all soil slices and masking a potential rhizosphere gradient, as the removal of macroscopic root remains by tweezers (see Section 2.2) cannot guarantee that no very small root fragments or root hairs remained in the soil sample, for example, included in aggregates. Although this issue applies also to bulk soil obtained by the conventional sampling—treated with tweezers as well—it has less impact on C data there due to "dilution" of the root effect by larger soil amounts.

The new gradient sampling technique implicitly assumes a vertical root segment without laterals in the center of the sampling device. Obviously, this assumption did not hold true for the sampled maize roots. Segments of laterals, or roots derived from other parts of the root system crossing the chosen sampling area, cannot be ruled out, and the visual check for any non-target roots was obviously not sufficiently rigorous. As roots contain much greater amounts of C than soil (ca. 40% C in root dry matter) and greater amounts of ^{13}C from labeling ($\delta^{13}C > 100‰$), small fragments constituted a substantial contamination of the soil sample. For this reason, we recommend improving the isolation of root remains from gradient samples, either by more efficient exclusion using a microscope or by analytical inspection of the soil samples using, for example, root-specific methods such as suberin biomarkers (Mendez-Milan et al. 2011). Moreover, the plants in the current study had reached the phenological development stage of the end of tassel emergence. Younger and/or less branched root systems are likely to yield more unambiguous results with the gradient sampling approach.

The problem of masking of C and ^{13}C dispersal by non-target microscopic roots could be further overcome by investigation of certain fractions of soil C or of specific organic compounds instead of bulk C, as the latter gives a signal integrated over several compound classes of various origins (e.g., neutral sugars and fatty acids). This requires either collection of sufficient soil material or adaption of the analytical methods to lower sample amounts, as already demonstrated for certain low molecular weight compounds of root exudates by Lohse et al. (2021). Thus, more detailed insights into root input and microbial turnover under contrasting root traits and soil texture could be achieved, eventually allowing for the detection of gradients and consequently the determination of rhizosphere extent.

Using the formula for the half mean distance (i.e., half of the medium distance between roots, if all roots were aligned parallel; described by Schlüter et al. 2018), the maximum possible rhizosphere extent in the sampled field experiment in 20–40 cm depth at the end of tassel emergence would be 4.6–5.5 mm. This means that statistically, a rhizosphere extent beyond 5.5 mm could

not be expected, as zones of influence of neighboring roots would overlap with one another. However, for the gradient sampling approach, it is possible to choose a root segment far away from other roots. Therefore, after exclusion of the above-mentioned insecurities of root contamination, and if using specific fractions instead of bulk C, this new approach might detect rhizosphere extents larger than 5.5 mm, depending on the compound in question.

5 | Conclusions

A gradient sampling approach originally developed for fossil tree roots was adapted for annual crop plants to collect distinct soil slices with increasing distance (0–8 mm) around roots on the millimeter-scale. The method was compared with conventional rhizosphere sampling via brushing rhizosphere soil off the roots. The soil sample amounts obtained by the gradient sampling approach from maize grown in loam or sand were in the range of some hundreds of milligrams; thus, they were large enough for carbon elemental and stable isotopic analysis. Both approaches were capable of revealing the incorporation of recently assimilated ^{13}C in soil surrounding the root, and the new approach additionally enabled the detection of decreasing $\delta^{13}C$ values with increasing root distance as a trend. However, when compared with micrometer-scale in situ detection methods, it became clear that the millimeter sampling approach rather reflects a composite signal of one root segment, including its fine roots and potentially associated mycorrhizal hyphae.

For a more detailed insight into rhizodeposit distribution and microbial community composition, the here introduced gradient rhizosphere sampling approach can be used in root systems with low number of lateral roots for biomarker analysis, including, for example, phospholipid fatty acids or neutral sugars.

Author contributions

All authors contributed to the study conception and design. Manuel Vergara Sosa performed the gradient sampling and sample preparation. Eva Lehndorff, Doris Vetterlein, and Eva Lippold performed or induced analysis of samples. Johanna Pausch contributed major parts to the discussion on $\delta^{13}C$ values. Martina I. Gocke compiled data and prepared the first draft of the manuscript. Manuel Vergara Sosa, Andrea Scheibe, Doris Vetterlein, Johanna Pausch, Eva Lippold, and Eva Lehndorff commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

Data Availability Statement

The dataset analyzed during the current study is available from the corresponding author on reasonable request.

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