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Microbial formation of stable soil carbon is more efficient from belowground than aboveground input

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The relative contributions of aboveground versus belowground plant carbon inputs to the stable soil organic carbon pool are the subject of much debate—with direct implications for how the carbon cycle is modelled and managed. The belowground rhizosphere pathway (that is, carbon exiting the living root) is theorized to form stable soil carbon more efficiently than the aboveground pathway. However, while several mechanisms have been invoked to explain this efficiency, few have been empirically tested or quantified. Here, we use soil microcosms with standardized carbon inputs to investigate three posited mechanisms that differentiate aboveground from belowground input pathways of dissolved organic carbon—through the microbial biomass—to the mineral-stabilized soil organic carbon pool: (1) the physical distance travelled, (2) the microbial abundance in the region in which a carbon compound enters (that is, rhizosphere versus bulk soil) and (3) the frequency and volume of carbon delivery (that is, infrequent ‘pulse’ versus frequent ‘drip’). We demonstrate that through the microbial formation pathway, belowground inputs form mineral-stabilized soil carbon more efficiently than aboveground inputs, partly due to the greater efficiency of formation by the rhizosphere microbial community relative to the bulk soil community. However, we show that because the bulk soil has greater capacity to form mineral-stabilized soil carbon due to its greater overall volume, the relative contributions of aboveground versus belowground carbon inputs depend strongly on the ratio of rhizosphere to bulk soil.

Efforts both to promote the retention and accrual of soil organic carbon (SOC), and to predict its fate under environmental change, are predicated on a mechanistic understanding of the specific carbon (C) sources that form slow-cycling SOC^{1,2}. Plant inputs, the dominant organic constituent of SOC, enter the soil as either aboveground or belowground C, and include both structural litter inputs (root and shoot detritus) and dissolved organic carbon (DOC; for example, rhizodeposition and leaf litter leachate). Historically, aboveground structural residues were assumed to be the dominant source of C to the soil and a primary control on the rate of SOC accumulation^{3,4}. However, there is growing recognition that the contributions from belowground C inputs have been vastly underestimated^{3,5}. Specifically, labile DOC inputs from rhizodeposition (for example, root exudates) are now appreciated as important sources of C to the soil⁶ and key precursors of slow-cycling SOC (hereafter, ‘stable SOC’)^{7–9}. At the same time, recalcitrant aboveground litter C is increasingly regarded as a comparatively inefficient C source to form stable SOC^{10–12}.

A suite of recent studies have found that the C contributions from belowground inputs can exceed those of aboveground inputs^{4,10–13}. In sharp contrast to the traditional paradigm, these findings have prompted many to start categorizing the belowground pathway as the dominant C source of stable SOC^{3,5,14,15}. This view has grown more widespread, even as research on leaf litter leachate DOC shows that this aboveground source can also deliver substantial amounts of C to the soil^{16–18}. Support for the belowground pathway has gained traction because its primacy is theorized to be more than simply a function of the quantity and quality of C flowing through it. Namely, unique features of the belowground pathway itself, which promote direct and intimate association between C inputs and the mineral soil, are posited to confer more efficient stabilization per unit of C relative to aboveground inputs^{4,19}.

Although the features proposed to lead to more efficient stabilization via the belowground pathway are compelling, they lack

definitive empirical support and have not been well-quantified²⁰—especially when controlling for the confounding roles of C quality and quantity. Here, we address three pathway effects that have been posited as key mechanisms in the specific context of the ‘DOC-microbial route’ to mineral stabilization²¹. That is, the route by which labile DOC compounds (for example, glucose) from aboveground and belowground plant sources are anabolized by the soil microbial community before their stabilization in the mineral-associated SOC (MASOC) pool^{7,8,22} (as opposed to other routes such as direct sorption onto soil metals/minerals²³ or aggregate formation¹⁹). Resolving the mechanisms underlying the stabilization of aboveground versus belowground inputs via the DOC-microbial route is critical because as much as 50–80% of stable SOC may be microbially derived^{24,25}.

First, aboveground DOC (for example, leaf litter leachate) must travel a longer and more circuitous route to the mineral soil than belowground root inputs, including passage through the organic horizon²⁶—where DOC may be absorbed or released back into the atmosphere via microbial respiration^{16,27} (Fig. 1a, mechanism 1). Second, while aboveground leachate DOC spreads diffusely across a region of relatively low microbial abundance and activity (that is, the bulk soil), belowground root DOC inputs enter directly into a spatially constrained, microbially dense area of the mineral soil (that is, the rhizosphere)¹⁰. Since microbial residues are the dominant source of C to the MASOC pool via the DOC-microbial route to stabilization (for example, microbial necromass and other byproducts)^{8,23,28}, the higher microbial abundance in the rhizosphere should increase the chance that C inputs are assimilated, anabolized and then transformed to microbial products before their incorporation in the stable MASOC pool (Fig. 1a, mechanism 2)¹⁴. Third, belowground rhizosphere DOC inputs generally enter the soil as a continuous, low-volume ‘drip’ throughout the growing season²⁹, which should promote steady microbial anabolism and turnover. In contrast, aboveground DOC inputs leach down the soil profile

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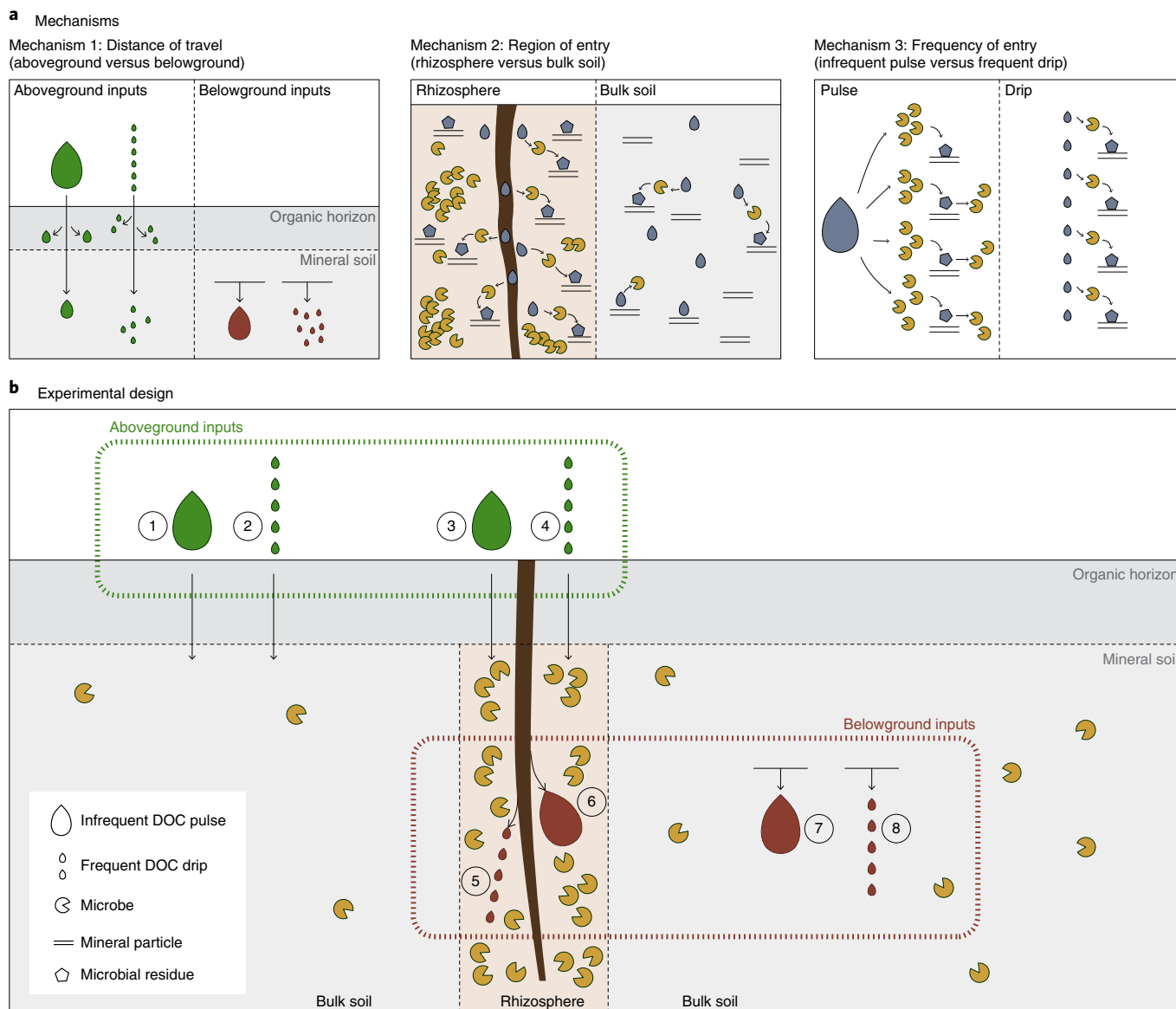


Fig. 1 | Proposed mechanisms favouring the efficiency of the belowground versus aboveground pathway in the DOC-microbial route to mineral stabilization. **a**, The tested mechanisms include (1) a shorter distance of travel to the mineral soil, (2) direct entry into the microbially dense rhizosphere and (3) entry as a frequent, low-volume drip versus an infrequent, high-volume pulse, promoting steady microbial anabolism and turnover. **b**, Fully crossed experimental design testing the interaction of all proposed mechanisms (a fixed quantity of ^{13}C -glucose was inserted into one of the eight location \times frequency combinations).

as infrequent ‘pulses’ during precipitation events²⁶. Although pulses may transiently spike microbial biomass and activity²⁸, a temporarily enlarged community may also co-metabolize a large proportion of the newly formed MASOC (Fig. 1a, mechanism 3).

These three mechanistic features of the belowground root pathway may substantively influence SOC dynamics, helping to explain why belowground inputs are retained in the mineral soil between two to five times more efficiently than aboveground inputs^{4,19}. If such pathway differences broadly apply across space and time, their inclusion is probably necessary for accurate portrayals and predictions of the terrestrial C cycle¹². Indeed, it has been argued that the stabilization of belowground C inputs cannot simply be modelled as a function of their quantity and quality, but must be additionally represented by a coefficient that accounts for their more efficient stabilization^{11,12,30}. However, although most conventional soil organic matter models include coefficients that distinguish the

quality of plant material, they do not model processes that differentiate the efficiency of the aboveground versus belowground C-input pathways for stable SOC formation^{12,20}. Further, most experiments that have explored aboveground versus belowground contributions to the stable SOC pool have typically used C compounds or shoot and root biomarkers (for example, cutin and suberin, respectively) that do not uncouple the role of C quality and quantity from specific pathway effects^{10,11,31–33}. Thus, on the basis of current empirical knowledge, it remains an open and pressing question as to whether the belowground DOC pathway should be characterized as a more efficient conduit to stable SOC formation—and if so, what specific mechanistic features underlie it.

DOC entry simulation

To specifically target potential belowground versus aboveground pathway effects, we used soil microcosms with a recreated organic

horizon, mineral horizon, bulk soil region and rhizosphere, which surrounded a microporous plastic ‘root’³⁴ (Fig. 1b; Supplementary Fig. 1). We tested the three different mechanisms as they specifically relate to the DOC-microbial route to mineral stabilization (Fig. 1a). Although belowground root DOC typically enters as a frequent drip into the rhizosphere via rhizodeposition, and aboveground DOC litter leachate inputs are typically pulsed into the bulk soil via precipitation events, we used a fully crossed experimental design to discern any interactions between the proposed three mechanisms. Thus, we employed eight different location × frequency combinations (four locations, two frequencies), in which the same quality and quantity of DOC was inserted: (1) aboveground and belowground, (2) proximally and distally to the rhizosphere and (3) as a drip and a pulse. Over the course of the experimental period, we inserted a solution containing a fixed amount of ¹³C-labelled glucose (99 atom% ¹³C) into one of these location × frequency combinations per treatment (see treatments 1–8 in Fig. 1b). Unlabelled glucose was simultaneously inserted into the other seven locations, ensuring all microcosms experienced exactly the same conditions. We selected glucose as a model DOC compound to test the posited pathway effects because monomeric carbohydrates are a common constituent of both belowground root exudates³⁵ and aboveground leaf litter leachate³⁶ and are typically rapidly and efficiently anabolized by soil microbes⁸.

An artificial rhizosphere environment (hereafter, ‘the rhizosphere’) was successfully generated around the plastic root. On average, the concentration of total microbial biomass (¹²C + ¹³C) in the rhizosphere was ~25% larger than the bulk soil—well within the range of values observed in the field³⁷ (Supplementary Table 3). Our design emulated a high-leaching temperate forest ecosystem context during the first months of the growing season (a ten-week experimental period), when both root exudation and litter leachate flux (via precipitation events) are high^{16,38}. SOC formation via the DOC-microbial route primarily occurs on these short timescales²¹.

We tracked the recovery of ¹³C-glucose in several different soil C pools, including MASOC, particulate organic C, microbial biomass C (MBC) and C remaining in soil solution as DOC. We focused primarily on the formation of MASOC because this pool contains the subset of stable SOC that is considered to have the longest turnover times (centuries to millennia). ‘Stable ¹³C-MASOC’ was identified by exposing the MASOC pool (total ¹³C-MASOC)—isolated via physical fractionation—to a chemical stabilization assay (refluxing the < 53 μm fraction with 6M hydrogen chloride)³⁹. There are several different methods of determining SOC stability; we selected this particular two-step approach because (1) the DOC-microbial route is predominantly associated with the organo-mineral fraction²¹ and (2) the chemical stability of organo-mineral sorption is an important determinant of its persistence in the soil and is generally correlated with other metrics of stability (for example, long-term incubation ‘biological stabilization’ assays)⁴⁰.

Pathway of entry

The belowground pathway, entering directly into the mineral soil, was the more efficient conduit of C to the stable ¹³C-MASOC pool. Across treatments, ~9% of belowground ¹³C-glucose inputs were transformed to stable ¹³C-MASOC, compared with ~5% of aboveground inputs (Supplementary Table 2). This nearly twofold difference was due, in part, to the fact that belowground inputs circumvented passage through the organic horizon (Supplementary Fig. 2). A far greater proportion of total aboveground glucose supply (~36 times) was retained in the organic horizon relative to belowground glucose inputs, reducing the amount of aboveground C ultimately delivered to the mineral horizon (Fig. 1a, mechanism 1).

Once glucose did enter the mineral soil—whether as an aboveground or a belowground input—a key determinant of its mineral stabilization was the proportion assimilated and transformed by

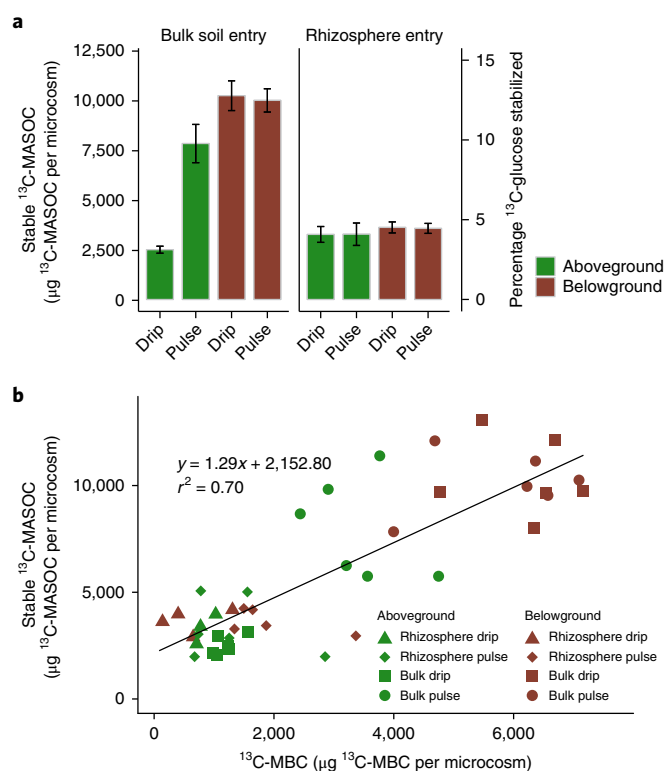


Fig. 2 | The formation of stable ¹³C-MASOC in a whole microcosm. **a**, Bars represent the mean mass (error bars are s.e.m.) of stable ¹³C-MASOC formed in a whole microcosm for each experimental treatment ($n = 6$). The y axis on the far right indicates the percentage of added ¹³C-glucose that was recovered in the stable ¹³C-MASOC pool. **b**, Relationship between the total amount of stable ¹³C-MASOC formed and the total amount of ¹³C-MBC in a whole microcosm. The positive linear relationship suggests that greater microbial anabolism of ¹³C-glucose led to greater formation of stable ¹³C-MASOC.

the soil microbial biomass. First, to test the strength of the direct interaction between glucose and mineral surfaces in our specific forest soil, we conducted an adsorption assay on sterilized samples of sieved mineral soil. Specifically, soil samples were shaken with a ¹³C-glucose solution for 24 h, which was added at the same concentration of ¹³C-glucose per dry gram of soil as was added to the rhizosphere of each microcosm. We then exposed the isolated MASOC fraction to a chemical stabilization assay (described above) and found that, in the absence of a microbial community, only ~0.3% of added ¹³C-glucose was recovered as stable ¹³C-MASOC (Supplementary Table 2)—consistent with previous studies that showed low reactivity of glucose with the mineral phase⁴¹. In contrast, ~3–13% of added ¹³C-glucose was recovered as stable ¹³C-MASOC when a microbial community was present (Fig. 2a; Supplementary Table 2). Moreover, the values within this range were positively associated with the total amount of ¹³C-glucose anabolized by the microbial community. That is, when considering the ¹³C budget of the whole microcosm, we found a strong, linear relationship between the total amount of ¹³C recovered in the MBC pool and the total amount of ¹³C in the stable MASOC pool (Fig. 2b; coefficient of determination $r^2 = 0.70$). Viewed together, these observations confirm that microbial uptake and transformation of ¹³C-glucose was an important precursor for its efficient mineral stabilization.

Overall, the bulk soil stabilized 170% more ¹³C-glucose than the rhizosphere (Fig. 2a), even though the rhizosphere soil formed a significantly higher concentration of stable ¹³C-MASOC per gram

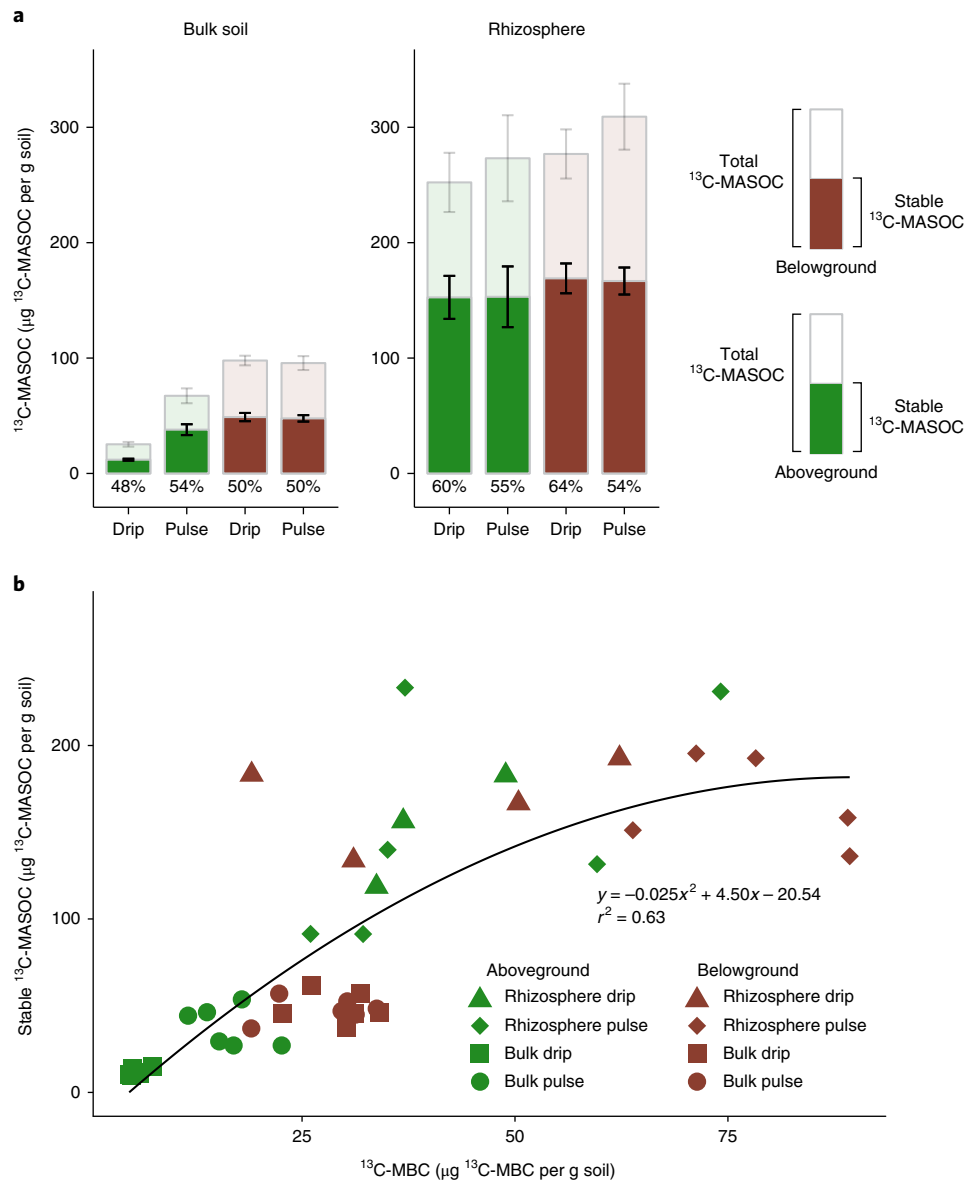


Fig. 3 | The concentration of stable $^{13}\text{C-MASOC}$ formed per gram of rhizosphere or bulk soil. a, Each bar represents the mean concentration (error bars are s.e.m.) of $^{13}\text{C-MASOC}$ formed per gram of soil in the specific region of entry that corresponds with each treatment ($n=6$). The height of the entire bar represents total $^{13}\text{C-MASOC}$ formed, and the opaque bar represents the subset of $^{13}\text{C-MASOC}$ that is stable. Percentages below the bars indicate the mean percentages of $^{13}\text{C-MASOC}$ that were stable (see Supplementary Table 4 for s.e.m. values of these percentages). **b**, Relationship between the concentration of stable $^{13}\text{C-MASOC}$ and the concentration of $^{13}\text{C-MBC}$ per gram of soil within the specific soil region of entry.

of soil (Fig. 3a). Because the same amount of ^{13}C -glucose was inserted into the bulk and rhizosphere regions—and their soil mass differed by one order of magnitude (ratio of rhizosphere to bulk soil = 1/10)—the lower total amount of ^{13}C -glucose stabilized in the rhizosphere suggests that the amount of ^{13}C -glucose added per gram of soil exceeded the rhizosphere's stabilization potential in the experimental period. Such an exceedance of the rhizosphere's stabilization capacity is consistent with the saturating relationship that we observed between the total amount of ^{13}C recovered in the MBC pool and the amount of ^{13}C per gram of soil in the stable $^{13}\text{C-MASOC}$ pool (Fig. 3b; $r^2 = 0.63$).

This saturating effect may be evidence for a maximal organic C loading rate onto available mineral surfaces in our soil (^{13}C saturation) and/or a finite capacity of the active microbial community to metabolize incoming C substrate. Regardless, this observation points to one potential benefit for DOC to enter as low-concentration

inputs into the bulk soil region (for example, litter leachate DOC via spring throughfall²⁶). Namely, the more widely distributed coverage of the DOC throughout the bulk mineral soil may help to avoid a potential maximum stabilization rate encountered in the more spatially constrained rhizosphere soil.

Region of entry

Although the rhizosphere formed less stable MASOC than the bulk soil overall, we found that ~59% of the $^{13}\text{C-MASOC}$ that formed per gram of rhizosphere soil was stable compared with only ~50% in each gram of bulk soil (Fig. 3a; Supplementary Table 4). This difference in the proportion of $^{13}\text{C-MASOC}$ that was stable suggests that the rhizosphere microbial community may form stable $^{13}\text{C-MASOC}$ more efficiently than the bulk soil community. Specifically, we found that the amount of stable $^{13}\text{C-MASOC}$ formed per unit of $^{13}\text{C-MBC}$ was 83% higher in the rhizosphere than the bulk soil. Thus,

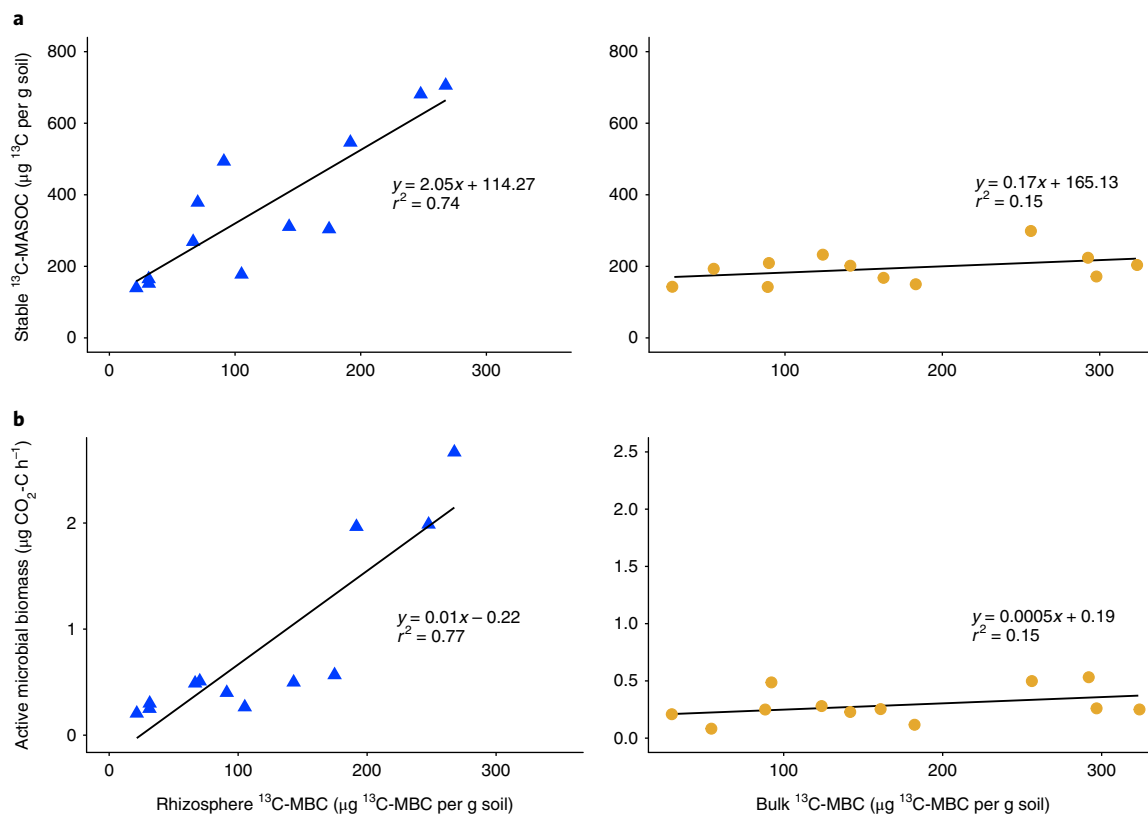


Fig. 4 | Follow-up assay with field-isolated rhizosphere and bulk soil microbial communities. **a**, Relationship between stable ¹³C-MASOC formation and ¹³C-MBC in field-isolated rhizosphere versus bulk soil communities ($n = 12$). **b**, Relationship between active microbial biomass (measured through substrate-induced respiration, which captures average microbial respiration rate in units of μg of CO₂-carbon respired per gram of soil per hour) and ¹³C-MBC in field-isolated rhizosphere versus bulk soil communities. Field-isolated rhizosphere communities are shown as blue triangles, while the bulk communities are shown as orange circles.

Table 1 | Summary of metrics differentiating the efficiency of stable ¹³C-MASOC formation from aboveground versus belowground ¹³C-glucose inputs

Mechanism	Metric				
	Percentage of ¹³ C-glucose stabilized in whole microcosm	Concentration of stable ¹³ C-MASOC per g soil	Stable fraction of total ¹³ C-MASOC	Stable ¹³ C-MASOC formed per unit ¹³ C-MBC ^a	Stable ¹³ C-MASOC formed per unit ¹³ C-MBC (with equal C concentration) ^b
(1) Pathway of entry	190% more stabilized via belowground entry	20% higher via belowground entry	^c	^c	Not applicable
(2) Soil region of entry	170% more stabilized in bulk soil	340% higher in rhizosphere	20% higher in rhizosphere	83% greater in rhizosphere community	121% greater in rhizosphere community
(3) Frequency of entry	120% more stabilized as aboveground pulse into bulk soil than belowground drip into rhizosphere	^c	In rhizosphere: 12% higher as drip; in bulk soil: 21% higher as pulse	In rhizosphere: 57% greater as belowground drip than all other modes of entry	Not applicable

^aThis metric is from the main experiment, where different concentrations of glucose were added to the rhizosphere and bulk soils of experimental microcosms. ^bThis metric is from the additional assay where equal concentrations of glucose were added to field-isolated rhizosphere and bulk microbial communities isolated from the forest site where mineral soil was collected (see Fig. 4). ^cNo significant difference between treatments.

assuming that the ¹³C pools we measured are representative of the flow of ¹³C-glucose through microbial biomass and into the stable ¹³C-MASOC pool³⁹, this suggests that once glucose has been anabolized, the rhizosphere community may be more efficient at forming stable MASOC from anabolized C (mechanism 2). Admittedly, our design cannot uncouple C concentration effects from community effects: each gram of rhizosphere soil received 10 times more glucose than each gram of bulk soil and contained 1.25 times more

microbial biomass. To separate these potential community versus concentration effects, we performed a follow-up assay (see ‘Substrate concentration versus microbial community effects’ below).

Frequency of entry

In our microcosm soils, the greatest percentage of total ¹³C-MASOC per gram of soil was stable when rhizosphere inputs entered as a frequent, low-volume drip (two-way interaction, $P = 0.02$,

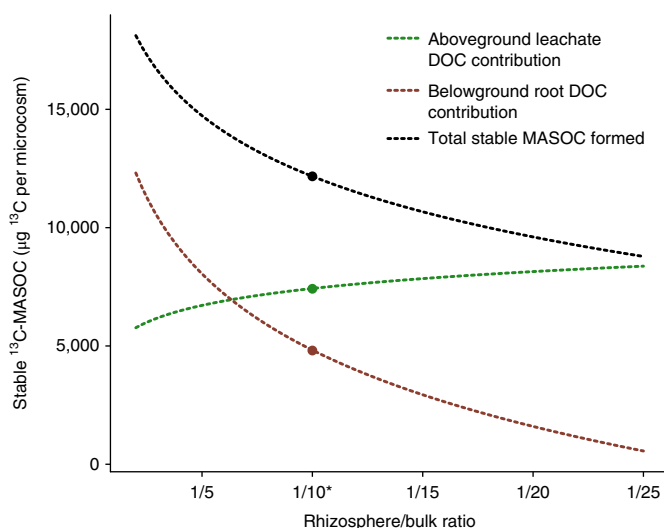


Fig. 5 | Projections of the relative importance of aboveground leachate DOC versus belowground root exudate DOC across a range of typical rhizosphere-to-bulk ratios. Data from the microcosm study (a 1/10 rhizosphere-to-bulk soil mass ratio, denoted with an asterisk on the x axis and as opaque circles on projection lines) were used from the belowground-rhizosphere-drip (root exudate DOC) and aboveground-bulk-pulse (leachate DOC) treatments, to project the relative importance of aboveground versus belowground DOC across a range of rhizosphere-to-bulk soil ratios in the upper mineral soil. As the rhizosphere-to-bulk ratio narrows (left side of graph), there should be greater formation of stable MASOC, because a greater proportion of C inputs are contributed from efficient belowground root exudate inputs.

Supplementary Table 8). Although pulsed rhizosphere inputs stimulated greater microbial anabolism of ^{13}C per gram of soil than rhizosphere drip inputs (Supplementary Fig. 3)—and were associated with the formation of more ^{13}C -MASOC per gram of soil—a smaller proportion of the ^{13}C -MASOC that formed was stable (Fig. 3a,b). Specifically, we found that the positive relationship between ^{13}C -MBC per gram of soil and stable ^{13}C -MASOC per gram of soil was nonlinear. At the higher end of ^{13}C -MBC values ($> 70 \mu\text{g } ^{13}\text{C}$ -MBC per g soil)—which represent those from pulsed rhizosphere inputs, greater microbial anabolism of ^{13}C -glucose per gram of soil did not generate the same concomitant gains in stable ^{13}C -MASOC per gram of soil as it did at lower ^{13}C -MBC values. That is, the formation of stable ^{13}C -MASOC per unit of ^{13}C -MBC was highest when belowground rhizosphere C entry occurred as a drip rather than as a pulse (Fig. 3b).

Previous research reveals that frequent, low-volume inputs of glucose promote more steady assimilation and turnover of the microbial biomass, as well as lower respiration, compared with infrequent, pulsed glucose inputs²⁸ (mechanism 3). Our results suggest a similar dynamic, whereby frequent belowground rhizosphere inputs may promote a more continual stream of assimilated ^{13}C -glucose into the stable MASOC pool.

Substrate concentration vs. microbial community effects

We attempted to emulate representative concentrations of rhizosphere versus bulk soil DOC inputs. As such, we could not disentangle potential effects of C concentration (per unit soil) from the possibility that our results might also, at least in part, be a product of differential efficiencies of rhizosphere versus bulk microbial communities. We therefore conducted a follow-up assay to investigate whether rhizosphere versus bulk communities might differentially stabilize glucose when all other conditions (for example, concentration of C inputs) were equal. For this assay, we isolated a

rhizosphere and bulk microbial community from the same location where we collected our mineral soil (hereafter, ‘field-isolated communities’ to distinguish them from the main microcosm experiment reported above). We separately added each type of microbial inoculum to sterile, artificial soil microcosms (clay + quartz sand), which contained minimal background C (removed via repeated washing with sodium hypochlorite, adjusted to 8 M)^{42,43}. This soil treatment was used to ensure the microbial community was primarily consuming ^{13}C -glucose, limiting potential substrate-identity effects arising from the use of other compounds contained in the soil organic matter.

To determine the relationship between ^{13}C -MBC and ^{13}C -MASOC, we generated a range in the density of the field-isolated rhizosphere and bulk soil microbial communities by using four different volumes of starting inoculum, each replicated in triplicate (giving $n = 12$ replicates per community). The two field-isolated microbial communities showed a similar range in the amount of ^{13}C -MBC across microcosms (Fig. 4). However, we found that—in line with our results from the main study—the rhizosphere community formed $\sim 121\%$ more stable ^{13}C -MASOC per unit of ^{13}C -MBC relative to the bulk community (Supplementary Table 9). Furthermore, the rhizosphere community demonstrated a strong, positive relationship between ^{13}C -MBC and stable ^{13}C -MASOC (Fig. 4a, left panel), whereas no relationship was apparent for the bulk community (Fig. 4a, right panel). We observed a similar relationship when we regressed ^{13}C -MBC against active microbial biomass (as measured through substrate-induced respiration⁴⁴), indicating that, per unit increase in ^{13}C -MBC, the rhizosphere community showed a significantly greater increase in microbial activity relative to the bulk community (Fig. 4b). These findings suggest that future research into the importance of the rhizosphere for stable SOC formation should include a consideration that differences among microbial communities may operate as an additional mechanism driving the efficiency of aboveground versus belowground pathways.

Implications for the carbon cycle

By focusing on proposed mechanisms specifically involved in the DOC-microbial route to mineral stabilization, we demonstrate several features that enhance the efficiency of the belowground rhizosphere DOC pathway to the stable MASOC pool (Table 1). Overall, belowground entry was 190% more efficient than aboveground entry, due to direct entry into the mineral soil (mechanism 1). Entry into the rhizosphere led to 340% more stabilized C per gram of soil than entry into the bulk soil, due to the greater efficiency and activity of the rhizosphere community (mechanism 2), although the bulk soil stabilized more C overall due to the saturating rate of stabilization observed in the rhizosphere. Finally, in the rhizosphere, the proportion of stable MASOC formed was highest when belowground inputs entered as a frequent drip versus an infrequent pulse (mechanism 3; regression coefficient = -10.8 , $P = 0.02$ for two-way interaction, Supplementary Table 8).

It is notable, however, that within the ^{13}C budget of the whole microcosm, more total ^{13}C was mineral-stabilized when ^{13}C -glucose entered as an aboveground pulse into the bulk soil (for example, leaf litter leachate) than as a belowground drip into the rhizosphere (for example, root exudates), due to spatial constraints of the rhizosphere (Table 1). The quantitative significance of aboveground versus belowground DOC was determined by the ratio of rhizosphere to bulk soil (1/10) in the microcosm, as the greater total soil volume in the bulk soil promoted greater total mineral stabilization. To broadly illustrate how the rhizosphere-to-bulk ratio affects the importance of each input pathway, as well as the total amount of ^{13}C stabilized, we used our measured bulk and rhizosphere ^{13}C concentrations from the main experiment to generate back-of-the-envelope projections across a range of representative rhizosphere-to-bulk ratios³⁷. We show that as the rhizosphere-to-bulk ratio

narrows (for example, to 1/4, such as the upper mineral soil of a grassland), the 'root exudate' simulated pathway contributes ~20% more C to the stable SOC pool than the 'leaf litter leachate' simulated pathway, and the total amount of C stabilized increases by ~25% relative to the measured 1/10 ratio. However, as the rhizosphere-to-bulk ratio widens (for example, to 1/25, such as in certain forest ecosystems), the relative importance of aboveground inputs increases, but the total amount of stable MASOC formed decreases by ~15% relative to the measured 1/10 ratio (Fig. 5).

There have been increasing calls to better incorporate into simulation models the differential stabilization of aboveground and belowground plant C inputs^{3,12,20,45}, especially from DOC inputs stabilized via the microbial formation pathway²¹. Our study sought to identify specific mechanisms purported to underlie the efficiency of the belowground rhizosphere DOC pathway to the stable MASOC pool, independent of C quality and quantity. Our work shows the potential for multiple specific features of aboveground versus belowground C input pathways to influence formation rates of stable MASOC, including potentially the composition of bulk versus rhizosphere soil microbial communities. Importantly, because our study was conducted in lab-based microcosms to precisely target the posited mechanisms, such a design simplifies key aspects of a natural soil system. A critical next step is therefore to discern the relative importance of the tested pathway features in studies that incorporate the multitude of factors that may influence the formation of stable MASOC in the field. For example, C compound identity, mycorrhizal status, microbial community structure, soil nutrient availability, soil depth and different temporal scales all probably shape the formation, size and persistence of the stable MASOC pool^{4,20,39,46–48}. Such work that considers the broad array of factors that ultimately govern SOC stocks seems essential if we are to accurately understand and project terrestrial C-cycle processes under a changing environment.

Online content

Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at <https://doi.org/10.1038/s41561-018-0258-6>.

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Author contributions

N.W.S. and M.A.B. conceived the project. N.W.S. designed and led the research and analysed the data. N.W.S. wrote the manuscript, with contributions from M.A.B.

Competing interests

The authors declare no competing interests.

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Methods

Soil sampling and microcosm design. Mineral soil and organic horizon material were collected from a temperate, mixed hardwood forest in southern Connecticut (41° 19' 57.6" N 72° 54' 36.9" W), where the dominant tree species include *Acer* spp., *Carya* spp., *Liriodendron tulipifera* and *Quercus* spp. Soils are Inceptisols, predominantly in the Holyoke series, classified as loamy, mesic Lithic Dystrudepts. Mineral soil was collected from the top 15 cm of the A horizon and sieved to 2 mm (clay = 13.0%, sand = 63.8%, silt = 23.2%, pH = 5.7, C = 4.1%, N = 0.2%). Organic horizon material was collected from directly below the litter layer, sieved to 4 mm, then repeatedly rinsed with de-ionized water in a 53 µm sieve to remove any mineral content, and then air dried at room temperature. All soil material was stored at 4 °C until further use.

Rectangular, acrylic soil microcosms were modelled after the design by Keiluweit et al.³⁴, built at a height of 20 cm, a width of 25 cm and a depth of 1 cm (see Supplementary Fig. 1). An artificial root (Micro Rhizon Sampler, length = 10 cm, diameter = 2.5 mm, Soilmoisture Equipment) was placed in the centre of each microcosm, and mineral soil was then packed into the microcosm frame, surrounding the artificial root, at field bulk density (0.92 g cm⁻³) and to a depth of 12 cm (slightly longer than the microporous section of the root, as shown in Supplementary Fig. 1). Organic horizon material was packed on top of the mineral horizon at a density of 0.25 g cm⁻³ and to a depth of 5 cm (total soil depth of mineral + organic soil = 17 cm). Following soil packing, microcosms were pre-incubated in the dark at 20 °C for one week, during which an inorganic nutrient solution (330 µM KCl, 70 µM KH₂PO₄ and 70 µM MgSO₄) was inserted daily into the artificial root (0.5 mL per day) via a syringe pump. Throughout the experiment, added glucose was dissolved in this same nutrient solution.

The rhizosphere soil was designated as the area with a radius of 8 mm from the root (total of 21 dry g equivalent). Even though the designated rhizosphere area was slightly larger than the typical area in which it is classically defined (~2 mm), it is well within the range of the observed distances in which root exudates can diffuse from the root³⁷, and was selected to allow for a sufficient soil sample to perform all required assays. The bulk soil was defined as the remaining mineral soil in the microcosm (total of 210 dry g equivalent). Overall, there was a mass ratio of rhizosphere to bulk soil of 1/10.

Importantly, although the artificial rhizosphere received labile sugar input and nutrient solution (similar to a true rhizosphere), it did lack several key components that are common to a rhizosphere in the field (that is, the full suite of carbon compounds that exit a root, root border cells, root mucilage, mycorrhizae and other components)⁴⁹. We selected this lab-based microcosm system to specifically target the DOC-microbial route to mineral stabilization, and not other forms of SOC stabilization such as aggregate formation or chemically recalcitrant particulate organic carbon, both of which are associated with shorter turnover times than mineral stabilization¹. While our experimental design may have excluded mycorrhizal fungi, saprotrophic fungi and high-molecular-weight root C inputs, evidence suggests that these elements are primarily involved in these other, co-occurring forms of SOC stabilization (for example, mycorrhizal fungi and other soil fungi contribute chemically recalcitrant residues⁵⁰ and play a key role in aggregate formation^{51,52}, as do root inputs such as root hairs and root mucilage³). In contrast, the DOC-microbial route to mineral stabilization is dominantly supplied by low-molecular-weight C inputs that are anabolized by surface-associated soil microbes, and some evidence suggests that these mineral surface-associated microbes (and thus their necromass contributions to the mineral-stabilized SOC pool) are primarily bacterial¹. Thus, while elements of the soil system were simplified in our lab-based design, our approach was intended to simulate dominant elements of the process of microbial formation of mineral-stabilized SOC as it would occur in a temperate forest ecosystem, permitting us to test the three proposed mechanisms of more efficient belowground formation in the absence of confounding factors.

Carbon additions. To test for the interaction between the three proposed mechanisms affecting the efficiency of stabilization (see Fig. 1b for all eight treatments), each treatment involved adding a fixed quantity (81.25 mg ¹³C over eight weeks) of 99 atom% ¹³C-labelled glucose (Cambridge Isotope Laboratories) into a single location × frequency combination of the eight possible treatment combinations (four locations, two frequencies; *n* = 6 per treatment; 48 total microcosms). We set up six additional microcosms that contained only unlabelled glucose to provide natural abundance atom% values to calculate the ¹³C label amounts in different C pools (see below).

For each of the four locations, C delivery was as follows: (1) aboveground entry into bulk soil: solution was delivered via syringe, distributed evenly along the surface of the organic horizon, with a 1 cm buffer zone on either side of the rhizosphere; (2) aboveground entry into rhizosphere: solution was injected on the surface of the organic horizon directly next to the artificial root; (3) belowground entry into bulk soil: solution was delivered via syringe with a 6 inch needle, and distributed evenly throughout all areas of the bulk soil, using a grid drawn on the glass of the microcosm (to avoid delivery into the same location twice); (4) belowground entry into rhizosphere soil: solution was delivered via syringe pump through the artificial root (allowing for sustained delivery of C into the same location). The two frequencies included a low-volume, frequent drip

(0.5 mL of solution per day, three times per week; 24 total insertions = 12 mL), and a high-volume, infrequent pulse (6 mL of solution once every four weeks; 2 total insertions = 12 mL). The volume of water for the pulse event was based on precipitation during relatively high-volume rainfall pulse events in northeastern US forests⁵³, whereas the rate for the root exudation was based on previous artificial root exudate experiments³⁴. We confirmed that there was minimal migration of rhizosphere C input into the bulk soil, and vice versa (see below and Supplementary Table 6).

To maintain similar conditions in all microcosms, unlabelled glucose was inserted into the seven location × frequency combinations that were not being specifically tested in an individual treatment, so that all microcosms were receiving the same amount of total glucose C in the organic horizon, rhizosphere and bulk soil. The only difference between treatments, therefore, was the location × frequency combination that contained the ¹³C-label. For treatments in which ¹³C-glucose was entering directly into the rhizosphere (treatments 3, 4, 5 and 6 in Fig. 1b), C addition rates averaged a daily rate of 0.5 mg ¹³C per week per dry g soil, representative of the intermediate range of daily C additions via root exudation^{38,39}. For treatments in which ¹³C-glucose was entering directly into the bulk soil (treatments 1, 2, 7 and 8 in Fig. 1b), C addition rates averaged a daily rate of ~0.05 mg ¹³C per week per dry g soil, representative of the higher range of DOC flux through the organic horizon in temperate forests³⁶. In total, we added 2.81 mg of C (¹³C-glucose + ¹²C-glucose) per dry g of mineral soil throughout the microcosm over the eight-week addition period. Following the eight-week C addition period, microcosms were left to incubate for one week to allow microbial assimilation and turnover of the final glucose additions.

In addition, an adsorption assay was conducted on sterilized mineral soil to determine the extent of glucose adsorption to the mineral soil in the absence of a microbial community. Soil was sterilized by autoclaving at 121 °C for 30 min; this procedure was performed twice, with a 24 h incubation period in between⁵⁴. Subsamples of sterilized soil (5 dry g equivalent) were then shaken for 24 h with 50 mL of solution⁵⁵, which contained the same concentration of ¹³C-glucose as was added to the rhizosphere throughout the eight-week period.

SOC fractionation and chemical stabilization assay. Microcosms were harvested by carefully excising the organic horizon, the bulk soil and the rhizosphere soil. A subsample of the air-dried rhizosphere and bulk soil (9 dry g equivalent) were shaken for 18 h with 30 mL of a sodium hexametaphosphate solution to disperse aggregates, and then thoroughly rinsed through a 53 µm sieve, to separate out the particulate organic carbon fraction (> 53 µm) and the MASOC fraction (< 53 µm) (ref. 44). We dried all soil fractions (including a subsample of organic horizon material) to constant mass, and ball-milled and weighed samples for elemental C and N percentages (Costech ESC 4010 Elemental Analyzer) and isotopic δ¹³C analysis (continuous-flow isotope-ratio mass spectrometer, precision ±0.2%, Thermo Delta Plus Advantage; δ¹³C = [(¹³C/¹²C)_{sample} / (¹³C/¹²C)_{standard}] - 1) × 1,000‰.

We then exposed a subset of the dried MASOC fraction to a chemical stabilization assay, whereby 0.5 g of MASOC was refluxed with 25 mL of 6 M HCl for 16 h at 100 °C (refs 39,40,56). After decanting the acid-hydrolysable fraction, the acid-unhydrolysable (mineral-stabilized) fraction was rinsed four times with de-ionized water, isolated by centrifugation, dried and subsequently run for elemental C percentage and isotopic δ¹³C. We selected this assay on the basis of the assumption that greater microbial processing of ¹³C-glucose would result in a lower proportion of ¹³C-MASOC that was acid hydrolysable. This was premised on the fact that the acid-hydrolysable fraction contains more chemically labile materials (for example, sugars, such as glucose), whereas the acid-unhydrolysable fraction contains more chemically resistant compounds⁴⁹. While the chemical recalcitrance of a carbon compound does not correlate with its long-term persistence as mineral-stabilized SOC⁵⁷, glucose specifically is known to show weak direct sorption with the solid mineral phase⁴¹, and is thus easily prone to leach down the soil profile via physicochemical stripping if it is not first transformed to microbial residues (many of which are more chemically resistant to acid hydrolysis than glucose alone). Thus, we assumed that a greater acid-unhydrolysable fraction of ¹³C-MASOC correlated with greater microbial processing of ¹³C-glucose and thus also a longer turnover time relative to intact ¹³C-glucose.

The values of the stable ¹³C-MASOC fraction were subsequently compared with the original ¹³C-MASOC fraction (total ¹³C-MASOC). Six samples were lost during this assay due to human error, with insufficient soil left to rerun the assay (two samples in the belowground-rhizosphere-drip, one in the belowground-rhizosphere-pulse and three in the aboveground-rhizosphere-drip treatments). A standard isotope mixing model was used to determine ¹³C values of all C pools: $C_{\text{label-derived}} = C_{\text{total}} \times (\text{atom}\%^{13}\text{C}_{\text{after}} - \text{atom}\%^{13}\text{C}_{\text{control}}) / (\text{atom}\%^{13}\text{C}_{\text{glucose}} - \text{atom}\%^{13}\text{C}_{\text{control}})$, where C_{total} is the total amount of C, atom% ¹³C_{after} is the atom% value of the C pool at the end of the experiment, atom% ¹³C_{control} is the atom% value of C in the unlabelled controls and atom% ¹³C_{glucose} is the atom% value of the labelled substrate.

We present our results on stable ¹³C-MASOC formation, total ¹³C-MASOC formation and all other ¹³C pools, both in terms of concentration (per dry g of rhizosphere or bulk soil) and in a whole-microcosm ¹³C budget. As our primary focus was on ¹³C formation, we primarily present our results graphically as µg of ¹³C. However, we also present the data for percentage of ¹³C-glucose recovered

in each C pool within the whole microcosm (see Supplementary Table 2) as well as the percentage recovered per g of soil (see Supplementary Tables 5 and 6). Graphically, the results are the same for μg of ^{13}C and 'percentage ^{13}C -glucose recovered' for the whole-microcosm ^{13}C budgets, but not for concentrations of ^{13}C per dry g of rhizosphere and bulk soil (because a greater concentration of ^{13}C -glucose entered the rhizosphere than the bulk soil).

Microbial biomass. MBC and DOC were determined through a modified chloroform-fumigation extraction procedure⁷. Fumigated and unfumigated (control) soil samples were both shaken for 4 h horizontally on a shaker table with 0.5 M K_2SO_4 . Total organic carbon of the extracts was measured on a total organic carbon analyser (Shimadzu), and the $\delta^{13}\text{C}$ of the extracts were measured on a Thermo GasBench coupled to a Thermo Delta Plus XP isotope-ratio mass spectrometer (Thermo Fischer Scientific). We calculated ^{13}C -MBC as the difference in ^{13}C between fumigated and unfumigated samples, which was then divided by a correction factor of 0.45 to convert extracted carbon to biomass carbon (ref. ⁵⁸). Total MBC was calculated as the difference in total C between the fumigated and unfumigated samples, and divided by the same correction factor. We used the same correction factor for both the main microcosm experiment as well as the additional assay involving field-isolated rhizosphere and bulk soil communities (see below).

Field-isolated rhizosphere versus bulk microbial community assay. Field-isolated rhizosphere and bulk soil microbial inoculum were collected from the same site as described above. Rhizosphere soil was isolated by gently shaking tree roots from the sampling area; the soil still clinging to the roots after shaking was then carefully removed and designated as the field-isolated rhizosphere microbial community⁵⁹. The bulk soil microbial community (non-rhizosphere soil) was isolated from soil that was in the same sampling area and that was not associated with living roots (and defined as the field-isolated bulk microbial community).

To prepare the microbial inocula, a soil slurry was created for both the rhizosphere and bulk microbial communities by vortexing a subsample of soil with Milli-Q water (1/50 mass (g)/volume (mL) concentration)³⁹. To generate the variable sizes of the microbial community, the amount of inoculum added to each treatment was increased by five times per level: 10 μL , 50 μL , 250 μL and 1,250 μL of starting inoculum (values based on Kallenbach et al.³⁹). Total replication of each microbial community was $n = 12$ ($n = 3$ for each level of starting inoculum). The soil + inoculum was thoroughly mixed with a dissecting needle to ensure that the microbial community was equally distributed throughout the mineral soil. To mirror the different levels of microbial inoculum, and to help constrain microbial growth over the experimental period, different amounts of full-strength Hoagland solution were added at the beginning of the experiment to provide the following amounts of N: 0.01 mg, 0.05 mg, 0.25 mg and 1.25 mg (values based on Kallenbach et al.³⁹). Gravimetric moisture was then standardized in all microcosms at 20%, so that all soils were at 65% water-holding capacity. Over the course of the three-week experiment, soils were maintained at 20 °C and 65% water-holding capacity.

Five times over the course of the three-week experiment (days 1, 5, 9, 13 and 17), 99.9 atom% ^{13}C -labelled glucose was syringe-injected into each microcosm at a rate of ~ 0.05 mg ^{13}C per week per dry g soil (the same as in the bulk soil of the main microcosm experiment). A sterile dissecting needle was used to mix ^{13}C -glucose additions thoroughly with the soil, to ensure uniform distribution of the ^{13}C -substrate. At the conclusion of the experiment, soil was harvested, and ^{13}C -MBC and stable ^{13}C -MASOC were measured through the same techniques as described above. In addition, active microbial biomass was measured on all samples by using a modified substrate-induced respiration technique, whereby a subsample of soil is mixed with excess, easily degradable C substrate (autolysed yeast)⁶⁰. Following the addition of C substrate, tubes were shaken on a shaker table for 1 h, incubated for 4 h at 20 °C and CO_2 efflux was measured using an infrared gas analyser (Li-Cor Biosciences, Model 8100).

Statistical analyses. All statistical analyses were conducted using the statistical package R, version 3.3.2 (<http://cran.r-project.org/>). Linear regression models were

fit using the factors 'pathway of entry' (aboveground versus belowground entry), 'region of entry' (rhizosphere versus bulk soil), 'frequency of entry' (frequent drip versus infrequent pulse) and their interaction. Non-significant interaction terms ($P > 0.05$) were dropped from the model. First, the models were fit for all response variables as they pertained to the C budget of the whole microcosm (for example, ^{13}C -MASOC per microcosm; see Supplementary Table 7). Second, the models were fit for the same response variables, but as they pertained to the concentration of a C pool in a single gram of rhizosphere or bulk soil (for example, ^{13}C -MASOC per g soil; see Supplementary Table 8). Residuals were checked for normality and homogeneity of variance. For retained factors, we considered $P < 0.05$ significant and $P < 0.10$ marginally significant⁶¹.

Code availability. The R code for the analytical models is available at <https://github.com/NoahSokol/root-pathway-efficiency>.

Data availability

Experimental data in support of these findings are available at <https://github.com/NoahSokol/root-pathway-efficiency>.

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