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Technical note: methodological choices influence root carbon exudation measurements

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Root exudation is an important pathway of belowground carbon (C) allocation in forest ecosystems, with profound implications for soil processes, nutrient cycling, and ecosystem functioning. Despite its importance, quantifying root exudation from mature trees *in situ* remains technically challenging, and methodological inconsistencies among studies hinder synthesis and upscaling. In this technical note, we systematically evaluated how variations in commonly used exudate collection protocols influence measured C fluxes. Specifically, we tested the effects of root resting, trap moisture, and trap solution composition on exudation rates in two contrasting biomes: a temperate forest in Germany and a Mediterranean forest in Israel. By incorporating both inter- and intraspecific root combinations, we also accounted for potential species interaction effects. Our results reveal several methodological sensitivities. Omitting a root-resting phase can streamline sampling without compromising measurements. Moisture conditions within cuvettes strongly influenced flux estimates, with saturated traps yielding up to six-times higher values than moist traps. Root exudation was further affected by trap solution composition, with initially increased exudation under P-deficient solutions in both biomes. Roots showed an adaptive response to the syringe environment, particularly to differences in trap-solution composition and moisture conditions; with effects partially diminishing after 48 *hrs* when a second flush was collected. However, the magnitude and direction of these responses differed between biomes. Within our experimental framework, first-flush sampling using a standard trap solution provided the most consistent basis for cross-biome comparison of root exudation fluxes. Species mixing had no detectable effect on exudation rates. Together, these findings emphasize that methodological variation can substantially alter root exudation C flux rates. Standardized approaches – or, at a minimum, transparent and detailed reporting – are essential to improve comparability across studies. Addressing methodological challenges will allow more accurate quantification of root exudation, strengthen its integration into terrestrial C models, and ultimately refine our understanding of belowground C allocation under global change.

KEYWORDS

phosphorus deficiency, plant–soil interaction, rhizodeposition, temperate and Mediterranean forest biomes, tree species mixing

1 Introduction

Root exudation is a critical yet understudied component of the forest carbon (C) cycle, playing a key role in soil processes and ecosystem functioning (Jones et al., 2009; Finzi et al., 2015; Obersteiner and Klein, 2022). Accurately quantifying root exudation is essential not only for advancing scientific understanding but also for improving estimates of net primary production (Clark et al., 2001). Similarly, for correctly informing climate policies, terrestrial C models are used where exudation is often inferred by budget closure (Luyssaert et al., 2007). Although a method for collecting exudates from field-grown, mature trees exists (Phillips et al., 2008), its varied implementation across studies complicates efforts to understand belowground C dynamics at the landscape scale. Exudation rates are highly sensitive to a tree's carbon status (Williams and De Vries, 2020; Brunn et al., 2025) and the surrounding soil environment (Meier et al., 2020), further emphasizing the need for methodological consistency and a systematic evaluation of how methodological variation influences exudation is still lacking.

Measuring root exudation in forest ecosystems remains technically demanding and time-intensive (Oburger and Jones, 2018; Brunn et al., 2025). The most widely used approach involves incubating excavated fine roots in cuvettes containing a trap solution, allowing quantification of exudates from first- and second-order roots (Phillips et al., 2008). Exudation rates are typically expressed as the amount of substance released per unit root mass or surface area over a 24 or 48-hour period, capturing diel variation (Brunn et al., 2025). As the number of studies reporting root exudation rates grows, so does the diversity of methodologies, particularly in root preparation (e.g., cleaning procedures, resting times) and incubation protocols (e.g., cuvette volume, flushing steps, nutrient concentrations, moisture in the cuvette). Yet, uncertainty persists regarding how methodological choices affect measurements. Without standardized sampling protocols, comparing results across studies remains difficult (Freschet et al., 2021) and can have considerable consequences for quantifying belowground C allocation. While any deviation in the protocol has the potential to alter outcomes, three methodological factors are often found to vary in the existing literature: (1) the performance of root resting prior to incubation, (2) moisture in cuvettes, and (3) the chemical composition of the trap solution itself.

First, Phillips et al. (2008) introduced a root resting period of 2–3 days in a sand-soil mixture before incubation in the cuvette system, aiming to minimize stress-induced artifacts. This approach was later supported by Williams et al. (2021), who confirmed that a 3-day recovery period is critical to avoid root damage bias. However, many studies report considerably shorter resting times – ranging from 2 to 48 *hrs* – often without justification (see supplementary material in Heinzle et al. (2023). For practical reasons, shorter recovery times are commonly employed, yet these may not allow sufficient time for physiological stabilization following excavation. This could lead to artificially elevated exudation rates due to stress responses. Conversely, longer resting periods may also introduce artifacts, as extended detachment from the soil matrix under hot and dry conditions can alter root physiology, including solute and nutrient fluxes, or even initiate necrotic processes. Taken together, while both insufficient and excessive recovery durations may bias results, there is currently no established scientific consensus on an optimal resting period for root exudation studies, likely because experimental approaches differ in terms of species investigated, vitality checks, and climatic conditions.

Second, after resting or not, the root is inserted into a syringe system filled with glass beads containing a trap solution that captures exudation. Already at the very start of tree exudate collection, Smith (1970) supported the idea that keeping roots in the glass-bead environment can uphold aerobic conditions. Thus, the idea to use the suggested materials is to simulate an environment for the root that is as similar as possible to the aerobic soil conditions in topsoil while preventing anaerobicity (Reid and Mexal, 1977). Despite differences among exudate collection techniques associated with the given condition and the question addressed (Oburger and Jones, 2018), it is unclear which moisture content is ideal in syringe systems, and huge variations are applied that are, unfortunately, not transparently reported in methodological descriptions in the research papers.

Third, Phillips et al. (2008) suggested to use a C-free trap solution that contains inorganic nitrogen (0.5 mM NH_4NO_3), inorganic phosphorus (0.2 mM KH_2PO_4), sulfate (each 0.2 mM K_2SO_4 and $MgSO_4$), chloride (0.3 mM $CaCl_2$), and the base cations potassium (0.6 mM), calcium (0.3 mM) and magnesium (0.2 mM). All these concentrations are relatively high in terms of comparative concentrations in forest soil solutions (Smethurst, 2000; Braun et al., 2020), with exceptions by season or dry/rewet events for N and K, and Ca in forest developed on limestone. Using the nutrient solution approach further hinders the measurement of N exudation rates and metabolomic profiles of plants *in situ*, which others circumvented by the use of $CaCl_2$ solutions (Gougherty et al., 2018; Heinzle et al., 2023), water (Preece et al., 2024), or by using a C- and N-free nutrient solution (Yin et al., 2014; Sun et al., 2017). In general, water can induce osmotic stress, eventually causing lysis and membrane leakage, and might therefore lead to different exudation rates. Also, a Hoagland solution is sometimes used (Maurer et al., 2021). Compared to the Hoagland solution – designed for optimal plant growth through promoting full metabolic activity – the trap solution is formulated to minimize background nutrient levels while keeping roots physiologically active enough to exude compounds – avoiding excessive uptake or metabolism. Yet, it is anticipated that plant roots likely respond to nutrient availability in the trap medium. Especially given that there are large variations in P availability in soil (McDowell et al., 2023), it can be assumed that roots grown in soils with different P availabilities respond differently to P in the trap solution. For example, grass roots grown in soils with low P availability tended to exude less C in nutrient-rich solutions compared to water, while roots grown in soils with P availability showed the opposite response (Maurer et al., 2021). Other studies found that roots incubated in a P-deficient trap solution increase C exudation, shown for maize (Carvalhais et al., 2011), rice (Schwalm et al., 2024), or beans (Juszczuk et al., 2004). Lambers et al. (2006) interpreted enhanced exudation in response to P deficiency as a

strategy to increase P acquisition, likely representing a general strategy to cope with limiting nutrient supply (Carvalhais et al., 2011).

To clarify which methodological steps are essential for measuring root exudation *in situ*, we systematically tested the effects of root resting time, trap moisture, and trap solution composition on exudate C release. The experiments were conducted in two contrasting ecosystems, a temperate forest in Germany and a Mediterranean forest in Israel, selected for their differences in P availability (McDowell et al., 2023). Given that trees in natural forests often grow in mixed-species assemblages, we also considered potential effects of species interactions by including both interspecific and intraspecific combinations in our design, which only affected exudate composition, but not the total C flux in previous studies (Weinhold et al., 2022; He et al., 2025).

We specifically addressed the following hypotheses:

1. Exudation rates do not differ between roots subjected to a resting period and those transferred immediately.
2. Saturating the trap solution increases C exudation and leads to overestimations of the root exudation C flux.
3. Phosphorus-deficient trap solutions increase exudation rates.

2 Methods

2.1 Sampling sites

We compared the response of root exudates to varying sampling techniques (resting time, saturation of the trap systems, composition of the trap solution) in two distinct forests belonging to two contrasting biomes: temperate and Mediterranean (Table 1).

TABLE 1 Characteristics of the two sampling sites.

Feature/ Sampling site	Temperate forest	Mediterranean forest
Coordinates	50°11`N, 7°49`E	31°43`N, 34°57`E
MAT (°C)	8.8	25.3
MAP (mm)	643	510
Biome	Cool temperate moist forest (HLZ #15)	Warm temperate dry forest (HLZ #21)
Altitude (m.a.s.l.)	379	320
Soil type (WRB)	Cambisol	Chromic Luvisol
Soil texture	Clay loam (5–45% clay; 30–50% silt; 5–45% sand)	Clay loam (30–35% clay; 40–50% silt; 15–30% sand)
P availability	> 40 mg kg^{-1} (Olsen P)[1]	8.4 ± 4.7 mg kg^{-1} (Olsen P)[2]
Tree species	*Fagus sylvatica* L. mixed with *Abies alba* Mill.	*Pinus halepensis* Mill. mixed with *Pistacia lentiscus* L.
Tree ages (years)	120	60
Weather during sampling	18.9 °C; 11 mm; 58.1% RH[3]	20.4 °C; 0 mm; 40.6% RH[4]

[1]Data from McDowell et al. (2023).
[2]Olsen P was extracted from six soil samples using 0.5M $NaHCO_3$ and quantified with Gallery™ Plus Discrete Analyzer (Thermo Fisher).
[3]Meteorological information (mean annual temperature (MAT), mean annual precipitation (MAP), and relative humidity (RH)) provided by the German Meteorological Service (DWD) at Berghausen, located at 18 km distance.
[4]Meteorological information (mean annual temperature (MAT), mean annual precipitation (MAP), and relative humidity (RH)) provided by the Israel Meteorological Service (IMS) at Beit Jimal weather station, located at 1.4 km distance.

2.1.1 Temperate forest

The study was conducted in the low mountain range of Rhineland-Palatinate, Germany. Sampling took place between June 14 and June 22, 2023, during a hot and dry early summer period, with rainfall occurring only on the final day of sampling. The forest plot was dominated by mature *Fagus sylvatica* L. trees (European beech), approximately 120 years old, with 15-year-old *Abies alba* Mill. (silver fir) individuals growing within 1–2 meters of some *F. sylvatica* trees (Figure 1). The understory was densely populated by juvenile *F. sylvatica*.

Six *F. sylvatica* trees with a diameter at breast height (DBH) of approximately 2 meters were selected for root exudate collection. Of these, three *F. sylvatica* trees had *A. alba* as neighboring tree (interspecific combination), and three grow in monospecific (intraspecific combination) *F. sylvatica* stands (Figure 1). For each tree, two fine roots were excavated and used for exudate collection: one incubated in a standard trap solution and one in a phosphorus-free (P-free) trap solution (see Section 3.2), yielding a total of 12 exudate samples (2 roots × 6 trees).

To assess whether recovery time after root excavation influenced exudation rates, an initial test was performed using four roots per tree. These were analyzed for potential differences based on resting time before transfer into the syringe system. As no significant effect of resting time was observed ($F_{1,17}$ = 0.53, p = 0.476), data were subsequently averaged at the tree level to test for the impact on trap composition.

To investigate the influence of trap composition, the same root systems incubated in either standard or P-free trap solution were used in a first (flush 1) and a second sampling after an additional 48-hour incubation period (flush 2). This yielded 24 samples in total; however, one sample from the second flush in the P-free treatment under mixed-species conditions was lost due to animal interference (final n = 23).

2.1.2 Mediterranean forest

The second study site was located in the Yishi Forest Research Plot in the Judean Foothills, Israel. Sampling was conducted during a hot and dry spring period between February 28 and March 12, 2023. The forest is dominated by the planted gymnosperm species *Pinus halepensis* Mill. (Aleppo pine), accompanied by the native Mediterranean angiosperm *Pistacia lentiscus* L. Other, not investigated species include *Cupressus sempervirens* L., *Quercus calliprinos* Webb, and *Ceratonia siliqua* L. Conifers were planted approximately 45 years ago, with broadleaf species colonizing the afforestation area shortly afterward. The canopy is primarily formed by tall *P. halepensis* trees, while *P. lentiscus* along with other lower-

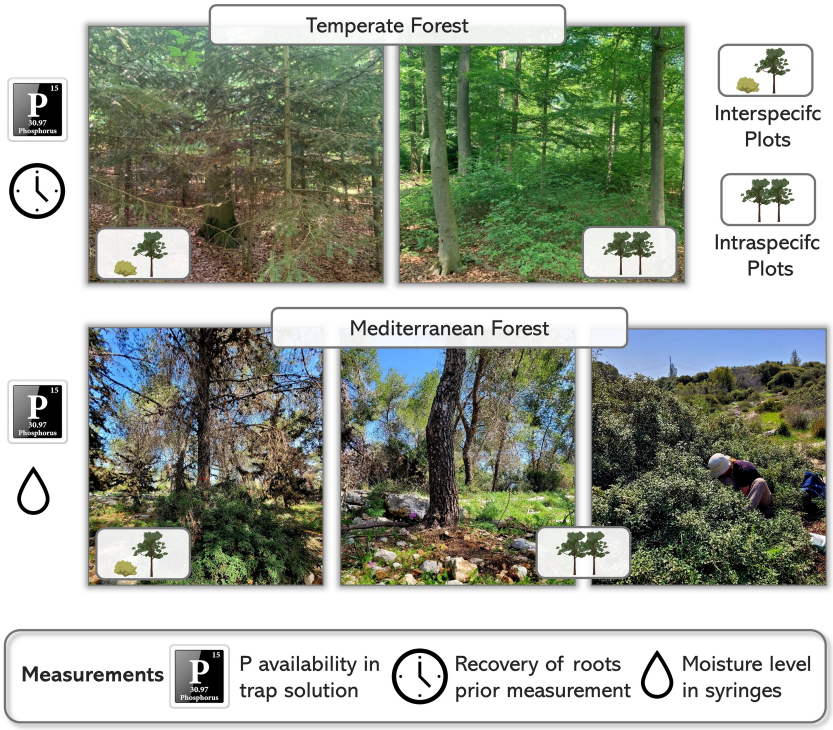

FIGURE 1
Research plots in a temperate forest with interspecific plots including *Fagus sylvatica* and *Abies alba* (upper left) and intraspecific plots with *F. sylvatica* (upper right), and in a Mediterranean forest with interspecific plots including *Pinus halepensis* and *Pistacia lentiscus* (lower left), and intraspecific plots with *P. halepensis* (lower middle) and *P. lentiscus* (lower right). Symbols indicate the measurements conducted at each site; the same symbol legend is used in all subsequent figures.

stature species such as oak and carob, comprises the understory (Jakoby et al., 2020; Rog et al., 2024).

We established three plots where *P. halepensis* and *P. lentiscus* were growing in proximity (hereafter referred to as "interspecific" combination; Figure 1). In each of these plots, three individuals per species were selected. On two additional plots where the species occurred in isolation, we selected four individual trees per species (hereafter "intraspecific"). For each tree, two fine roots were excavated and incubated separately: one in a standard trap solution and the other in a phosphorus-free (P-free) trap solution (see Section 3.2). The same roots were used for a second exudate collection after an additional 48-hour incubation period (flush 2).

For analysis, the three sampled trees per species on the interspecific sites were considered technical replicates, and plot-level means were calculated, resulting in 24 samples (3 trees × 2 species × 2 trap solutions × 2 flushes). For intraspecific sites, the four trees per species were treated as biological replicates from which two roots were investigated for incubation in the two trap solutions. However, due to interference by animals and species correction (see Section 3.2), the number of valid samples was reduced. For most parameters, a minimum of $n = 3$ was retained, except for *P. halepensis* in flush 2, where $n = 2$. This yielded a total of 24 usable exudate samples for trees at intraspecific sites.

To quantify the effect of trap saturation, we included an additional set of root samples randomly distributed across all study sites ($n = 7$). These samples were analyzed separately and excluded from all other analyses.

2.2 Root exudate collection and analysis

We sampled intact root branches from topsoil (0–5 cm soil depth) according to the cuvette-based approach from Phillips et al. (2008). Root branches, comprising 1st–3rd order roots attached to a single transport root, were randomly selected for sampling. Sampled root branches had an average dry weight of 75.4 ± SD 15.4 mg in the temperate forest and 97.3 ± 23.7 mg in the Mediterranean forest, an average fine-root (≤ 2 mm diameter) surface area of 30.0 ± 7.1 cm^2 in temperate and 3.2 ± 0.5 cm^2 in Mediterranean forest (Supplementary Table S1) with positive relations between root dry weight and surface area (Supplementary Figure 1). To test the impact of the composition of the trap solution, we used two different solutions (Table 2): a standard solution (0.5 mM NH_4NO_3, 0.1 mM KH_2PO_4, 0.2 mM K_2SO_4, 0.15 mM $MgSO_4$, 0.3 mM $CaCl_2$) and a phosphorus-free solution (0.5 mM NH_4NO_3, 0.2 mM K_2SO_4, 0.15 mM $MgSO_4$, 0.3 mM $CaCl_2$).

Root branches were carefully excavated, and the soil was gently removed with tweezers and by rinsing with either the standard or the P-free solution to limit osmotic stress. We excluded dead roots and roots that did not pass a vitality check (i.e., no lateral roots present or black tissue color) from sampling and evaluation. All roots were handled carefully and visually inspected to minimize mechanical damage. Afterwards, root branches were left to recover (48 *hrs* in temperate forest, 24 *hrs* in Mediterranean forest) in a 1:1 mixture of sand and native soil from the site, cleaned again, and

TABLE 2 Nutrient composition (mg L^{-1}) of the two trap solutions used in the study.

Trap solution/ Nutrient	N (mg L^{-1})	P (mg L^{-1})	K (mg L^{-1})	Mg (mg L^{-1})	Ca (mg L^{-1})
Standard trap solution	14.0	3.1	19.5	3.6	12.0
P-free trap solution	14.0	0.0	15.6	3.6	12.0

In the P-free trap solution, KH_2PO_4 was omitted; consequently, K concentrations were lower than in the standard trap solution.
Nitrogen (N) was added as NH_4NO_3, phosphorus (P) as KH_2PO_4, potassium (K) as K_2SO_4, magnesium (Mg) as $MgSO_4$, and calcium (Ca) as $CaCl_2$.

placed into syringes containing sterile and nitric acid-washed glass beads simulating a physical soil environment. To test whether this resting time affects exudation, we immediately inserted a set of roots into the syringe without giving the roots time to recover in the sand-soil mix. This study was done at the temperate forest site only. After the transfers of the root into the syringe system (temperate forest: 30 ml; Mediterranean forest: 20 ml), syringes were flushed three times with the trap solution (30 ml/20 ml) in which excess trap solution was removed by applying positive pressure after flushing and then incubated for 48 *hrs*, wrapped in aluminum foil, and covered with leaf litter. We are aware that different incubation volumes can affect exudation rates. However, the root-biomass-to-sampling-solution-volume ratios (RSVR, g L^{-1}) of 2.51 g L^{-1} in the temperate forest and 4.81 g L^{-1} in the Mediterranean forest fall within the range where no volume effects were detected (Otxandorena-Ieregi et al., 2024). After 48 *hrs*, we extracted root exudates trapped in the syringes after adding trap solution the first time (flush 1) and after 48 *hrs* the second time (flush 2). In order to prevent root damage bias in exudate measurements, we wanted to test if longer incubation times further reduce any bias. To test for saturation effects, we established additional saturated syringe systems with maintained high trap solution levels (saturated treatment: 20.6 ± 0.3% water content; non-saturated treatment: 11.8 ± 0.9% water content). Blank syringes (n = 6 in temperate forest and n = 8 in Mediterranean forest) with glass beads, flushed with both trap solutions but without root branches, and distributed over the plots served as a reference and were subtracted from exudate samples. In addition, three blank saturated traps were installed in the Mediterranean forest. After collection, root exudates were filtered through sterile syringe filters (0.22 µm, ROTILABO® MCE, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and stored at 4 °C until analysis. All consumables were acid-washed in 1% HNO_3 before use.

Exudate samples were quantitatively analyzed for total non-purgeable organic carbon concentration (TOC) with TOC analyzers – a Dimatec analyzer (Dimatoc 2000 N, DIMATEC Analysentechnik GmbH, Essen, Germany) for the samples in Germany and a Shimadzu VCPH-Carbon and Nitrogen analyzer (Kyoto, Japan) for the samples in Israel. The method included the removal of total inorganic carbon by adding 50 µl 2 M HCl and flushing with synthetic air (180 s). This measurement captures net exudation (i.e., gross exudation minus root reabsorption and microbial consumption) (Brunn et al., 2025).

All root branches were harvested after exudate collection and scanned at 1200 dpi (Epson Tokyo, Japan). Root-surface area and the number of root tips were determined using Rhizovision Explorer (Seethepalli et al., 2021). Root branches were dried, and the total dry biomass was recorded. Measured exudate TOC was expressed per root dry mass and refers to specific exudation rates (SER) reported in other studies (Chari et al., 2024; Brunn et al., 2025).

While root identification in the temperate forest was easy using visible classification, we performed DNA analysis in order to verify the species identity of the roots in the Mediterranean forest, using the tree ITS-2 region. DNA of roots used for exudation analysis was extracted according to Jakoby et al. (2020). Briefly, 3–4 mg of frozen, ground root tissue was mixed with 500 µl of 0.5 M NaOH containing 1.5% polyvinylpyrrolidone (PVP). Samples were vortexed, incubated for 10 min at 60 °C, and centrifuged for 5 min at 20,000 × g. Two microliters of the supernatant were diluted into 150 µl of 100 mM Tris-HCl (pH 8.0), mixed, and used for PCR amplification.

PCRs were set up in 25 µl reactions with 1.25 units Dream Taq DNA polymerase (Thermo Scientific), 2 µl DNA, and 1 µl each of primers ITS2-S21 (5′-ATGCGATACTTGGTGTGAAT) and ITS2-S3rR (5′-GACGCTTCTCCAGACTACAAT) (Livne-Luzon et al., 2024). Thermocycling conditions were: 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 1 min; followed by a final extension at 72 °C for 8 min. Ten µl of each PCR product was run on a 1% agarose gel; amplicons showing a single clear band were purified with Exo-1 and rSAP and Sanger-sequenced (Biological Services Department, Weizmann Institute of Science, Rehovot, Israel) unidirectionally with primer ITS2-S3rR. ITS2 sequences were manually BLASTed against the NCBI dataset, and samples with clear tree species identity were retained for further analysis. Three samples from interspecific plots mismatched their expected host but matched the other target species and were retained, while only one sample did not match any target species and was excluded.

2.3 Statistics

Data analyses were conducted using the statistical software R (version R 4.1.1, R Development Team (2021)) in the RStudio environment. To analyze the relationship between exudation rates and the explanatory variables, we used linear mixed-effects models fitted with lme in the nlme package. In all models fixed effects included species combination (intraspecific vs. interspecific), trap solution (standard vs. P-free) and flush (flush 1 vs. flush 2). Forest-specific subset models tested in addition prior root recovery in the temperate forest (root exudation ~ inter_intra * solution * flush* Recovery; Figure 2) and saturation in the Mediterranean forest (root exudation ~ inter_intra * solution * flush * saturation; Figure 3), while the overall model included the two forests (temperate vs. Mediterranean forest; root exudation ~ inter_intra * solution * flush * forest; Figure 4). In all models, repeated measurements across the two flushes per tree were accounted for by including tree

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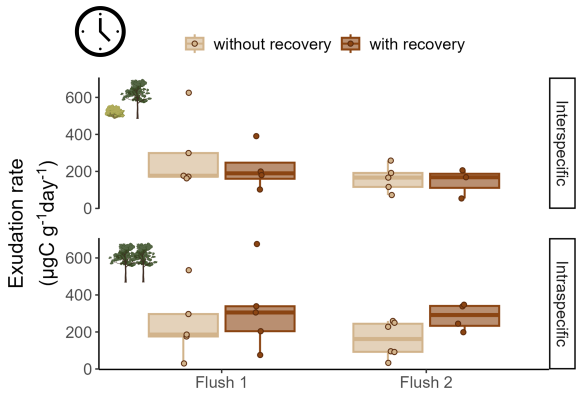

FIGURE 2
Root exudation rate (µg C day^{-1} cm^{-2}) in the temperate forest for *F. sylvatica* in interspecific (upper panel) or intraspecific (lower panel) combinations. Exudation was collected either 48 *hrs* (flush 1) or 96 *hrs* (flush 2) after roots were transferred to the syringe, and roots either rested after excavation (i.e., with recovery) or were immediately transferred to the syringes (i.e., without recovery). Boxes denote the 25th and 75th percentiles with median lines. Resting did not have a significant impact on root exudation (n.s.).

identity (Treenumber) as a random intercept (random = ~1 | Treenumber) and modelling within-tree residual correlation using compound symmetry (corCompSymm(form = ~1 | Treenumber)); models were fitted using REML. Results were extracted with the *anova* function in the nlme package with *post hoc* testing using the emmeans package (Searle et al., 1980; Lenth, 2026). Model assumptions were verified by inspecting residual plots for homoscedasticity and normality. Residual diagnostics indicated no violations of model assumptions. For statistical results of models, see Supplementary Table 3. All data are presented as means ± standard deviation (SD).

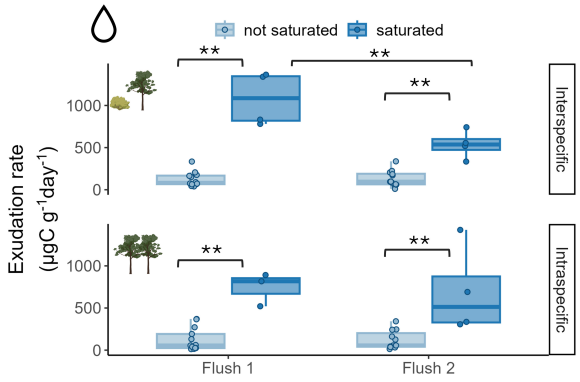

FIGURE 3
Root exudation rate (µg C day^{-1} cm^{-2}) in the Mediterranean forest for *P. halepensis* and *P. lenticus* in interspecific (upper panel) or intraspecific (lower panel) combinations. Exudation was collected either 48 *hrs* (flush 1) or 96 *hrs* (flush 2) after roots were transferred to the syringe, and syringes were either saturated or not saturated with incubation solution. Boxes denote the 25th and 75th percentiles with median lines. Significant differences (pairwise comparison) are displayed using asterisks, p ≤ 0.01** and p ≤ 0.05*.

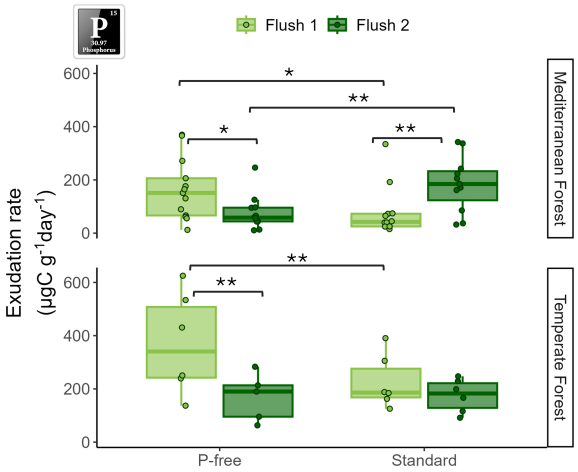

FIGURE 4
Exudation rates (µg C g^{-1} day^{-1}) in the Mediterranean forest (*P. halepensis* and *P. lentiscus*; upper panel) and temperate forest (*F. sylvatica;* lower panel). Roots were collected with either P-free (left panels) or standard trap solution (right panels), either 48 *hrs* (flush 1) or 96 *hrs* (flush 2) after roots were transferred to the syringe. Root exudation was collected in non-saturated syringes. Boxes denote the 25th and 75th percentiles with median lines. Significant differences (pairwise comparison) are displayed using asterisks, p ≤ 0.01** and p ≤ 0.05*.

3 Results

3.1 Sensitivity of root exudation to prior resting

Resting did not have an impact on exudation rates (Figure 2; Supplementary Table S3). However, in intraspecific combinations, there were trends towards lower exudation when roots were immediately transferred to the syringe system (198 ± SD 144 µg C g^{-1} day^{-1}) compared to roots that rested in the sand-soil mixture before the transfers (303 ± 165 µg C g^{-1} day^{-1}). These trends were not found in interspecific species combinations (Figure 2).

3.2 Sensitivity of root exudation to saturation of the trap solution

Overall, root exudation was significantly influenced by saturation ($F_{1,37}$ = 142.62, p < 0.001) (Figure 3; Supplementary Table S3). This effect also depended on flush (flush × saturation: $F_{1,37}$ = 10.76, p = 0.002) and, more weakly, on whether combinations were intra- or interspecific (inter_intra × flush × saturation: $F_{1,37}$ = 4.09, p = 0.050; Supplementary Table S2). Roots that were incubated in saturated traps released on average 6.1-fold higher exudate C fluxes than those in moist traps (763 ± 368 vs 125 ± 104 µg C g^{-1} day^{-1}; t_{37} = -11.68, p < 0.001), which was true in intra- and interspecific combinations (Figure 3). However, we observed an adaptation over time: in saturated traps, average exudation rates in intra- and interspecific combinations were highest in flush 1 (935 ± 309 µg C g^{-1} day^{-1}) and declined 614 ± 368 µg C g^{-1} day^{-1} in flush 2 (t_{37} = 3.31, p = 0.002).

3.3 Sensitivity of root exudation to the composition of the trap solution

The composition of the trap solution significantly affected exudation rates in flush 1 in both the Mediterranean and temperate forests (t_{41} = 2.08, p = 0.044 and t_{41} = 2.70, p = 0.010, respectively; Figure 4; Supplementary Table S3). Mean exudation rates in P-free solution were 2.1-fold higher compared to the standard solution in the Mediterranean forest (163 ± 114 vs 79 ± 93 µg C g^{-1} day^{-1}) and 1.6-fold higher in the temperate forest (369 ± 190 vs 226 ± 101 µg C g^{-1} day^{-1}) in flush 1. Additionally, in the P-free solution, exudation declined from flush 1 to flush 2 in both forests (Mediterranean: 163 ± 114 to 76 ± 63 µg C g^{-1} day^{-1}, t_{41} = 2.07, p = 0.045; temperate: 369 ± 190 to 169 ± 90 µg C g^{-1} day^{-1}, t_{41} = 3.81, p < 0.001). In flush 2, the pattern reversed in the Mediterranean forest, with the standard solution showing 2.4-fold higher exudation than P-free (184 ± 104 vs 76 ± 63 µg C g^{-1} day^{-1}; t_{41} = -2.72, p = 0.010). In contrast, in the standard solution, root exudation increased from flush 1 to flush 2 by 132% (79 ± 93 to 184 ± 104 µg C g^{-1} day^{-1}; t_{41} = -2.73, p = 0.009).

Thus, using a P-free solution reduced exudation rates with time, with roots responding to P-free incubation solution with elevated exudation in both ecozones, suggesting an immediate response of roots to a lack of P.

3.4 Abiotic, biotic, and technical controls: sensitivity to biome, species mixing, and equilibration time

Overall, we found that root exudation rates differed between the two biomes ($F_{1,41}$ = 14.40, p < 0.001), with overall higher exudation in the temperate forest compared to the Mediterranean forest (Supplementary Table S3). In addition, the effect of the solution depended on the flush (solution × flush: $F_{1,41}$ = 15.12, p < 0.001), and flush effects differed between forests (flush × forest: $F_{1,41}$ = 10.00, p = 0.003).

To test whether the second flush produces similar results than the first flush allows to answer the question whether the collection process can be sped up. Between the flushes, the results were the most stable for the standard solution in the temperate forest. However, using the P-free solution, average exudation rate in both forest sites was more than double in the first flush compared to the second flush (228 ± 169 vs 103 ± 82 µg C g^{-1} day^{-1}; t_{41} = 4.32, p < 0.001). In contrast, in the Mediterranean forest, exudation rates increased in the second flush in the standard solution (see Section 4.3).

Inter- and intraspecific combinations did not have an effect on exudation rate, no significant interaction was found between species combination and trap solution or flush in both combinations (Supplementary Figure 3; Supplementary Table S4). Tree species significantly influenced root exudation ($F_{2,35}$ = 7.81, p = 0.002), and this effect depended on flush (flush × species: $F_{2,35}$ = 4.77, p = 0.015). We found species-dependent differences in exudation rates only in the P-free solution, and only during flush 1: *F. sylvatica* showed higher exudation than *P. halepensis* in the interspecific combination (t_{35} = 2.53, p = 0.042) and higher exudation than *P. lentiscus* in the intraspecific combination (t_{12} = 3.68, p = 0.008; Supplementary Figure 2).

4 Discussion

This study evaluated how methodological variations in a hybrid root exudate collection approach – where trees are soil-grown but individual root branches are temporarily transferred to a hydroponic system – influence root C exudation. Specifically, we tested the robustness of this method with a focus on identifying steps that could be streamlined to improve exudate collection efficiency. Consistent with our first hypothesis, re-burying root branches before hydroponic incubation had no significant effect on exudation rates, suggesting that this step may be omitted without compromising data quality. Testing our second hypothesis revealed that using saturated trap solutions increases C exudation and leads to overestimations of the root exudation C flux. In line with our third hypothesis, the composition of the trap solution matters in regard to the initial response, while using the suggested standard solution produces stable results in the temperate forest but not in the Mediterranean forest. Species mixing itself had no impact on the exudation rates; however, methodological effects were more pronounced in interspecific species combinations.

4.1 Resting time before sampling does not affect exudation rates

The re-burial step was intended to allow excavated roots time to recover from mechanical stress or damage incurred during sampling and rinsing (Phillips et al., 2008). There is a huge variation in the literature regarding the resting duration, which varies between direct transfer (Phillips et al., 2011; Yin et al., 2013; Tückmantel et al., 2017; Sun et al., 2021), 2 *hrs* (Heinzle et al., 2023) to 24 *hrs* (Preece et al., 2021), to 48 *hrs* (Phillips et al., 2008; Brunn et al., 2022) to up to 72 *hrs* (Michalet et al., 2013; Gougherty et al., 2018). To test the relevance of this recovery period, we compared exudation rates from roots that were immediately transferred into the syringe system with those that were re-buried in a soil–sand mixture for 48 *hrs*. We hypothesized that exudation rates do not differ between roots subjected to a resting period and those transferred immediately and our results did support this hypothesis (Figure 2). Thus, under our field conditions, a 48-hour resting period did not systematically reduce exudation rates compared to immediate transfer. Although we observed no difference between treatments, we found a non-significant trend toward slightly higher exudation in re-buried roots of the intraspecific sites. This unexpected pattern may be partially explained by environmental conditions during sampling. The experiment was conducted during a hot and dry period, potentially inducing drought stress in the re-buried roots, which was more pronounced in intraspecific sites with less dense canopy closure. As drought has previously been shown to stimulate root exudation (Williams and De Vries, 2020; Brunn et al., 2022), this may have counteracted any recovery-related decline in exudation.

Overall, our findings suggest that the resting period prior to hydroponic incubation in glass bead-filled syringe systems is not essential for capturing reliable exudation rates in mature trees under field conditions. Omitting this step could help streamline sampling protocols and reduce the overall time required for exudate collection in forest settings.

4.2 Root exudation rates are higher in saturated trap solutions

Our data show that saturated traps strongly affect measured root-exudate C fluxes, yielding exudation rates up to six-times higher than those obtained with non-saturated traps (Figure 3). Although interspecific roots appeared to adapt to the syringe conditions – evidenced by substantially lower exudation rates during the second flush – our results emphasize the importance of maintaining the aerated conditions originally intended when placing roots in a glass-bead environment. If this is true for roots in subsoil, under seasonally shifting soil moisture conditions, or in fine-textured soil where the formation of anoxic conditions is more likely (Lacroix et al., 2025) requires further testing. Similarly, the moisture status within glass-bead-filled syringes deserves closer consideration. Given the very coarse texture of the 0.75–1 mm beads, water contents decline rapidly following drainage, and water content is expected to be low across the large range of the matric potential. Although we quantified soil water content in saturated and non-saturated traps, the corresponding matric potentials remain unknown, and pressure-assisted removal of solution may have reduced water contents below field capacity. As moisture conditions are rarely characterized in detail in comparable studies, improved reporting and clearer definitions would enhance methodological comparability among root-exudate collection approaches. In addition, our findings also call into question the comparability of existing root-exudate collection techniques. Because several studies employ fully hydroponic systems (Oburger and Jones, 2018; Döll et al., 2024; McLaughlin et al., 2025), it remains unclear whether such systems may substantially overestimate C-exudation fluxes. In contrast to many hydroponic approaches, roots in our cuvette system remain in the trap for extended periods – often for at least an entire diurnal cycle – to capture the strong coupling between exudation and photosynthesis (McLaughlin et al., 2023). A shorter, one-hour immersion in a non-glass-bead trap, as used in hybrid approaches, might yield more comparable results between the syringe system and the hydroponic immersion. However, implementing such short incubations under field conditions is technically infeasible because trees are spatially dispersed and measurements would need to occur under closely matched photosynthetic conditions. Overall, our results highlight that trap moisture content is critical. Non-saturated traps better align with the original intent of the glass-bead method to mimic aerobic topsoil conditions, suggesting that incubation traps should be maintained moist rather than fully saturated, at least when simulating topsoil environments. Studies aiming to inform forest C budgets should therefore prioritize or re-evaluate data obtained under non-saturated conditions to avoid overestimating belowground C allocation via root exudation. As root exudation in the subsoil may respond differently to trap moisture conditions compared to topsoil (Brunn et al., 2022), further methodological testing across soil depth profiles is needed.

4.3 Root exudates respond sensitively to the composition of the trap solution

We investigated root-exudation fluxes in two contrasting forest biomes and found that trees from both sites responded similarly to the two trap solutions – a standard solution and a P-deficient one. Thus, despite the contrasting soil conditions in which the roots had developed (Table 1), their exudation responses to nutrient availability in the incubation medium were comparable. This finding aligns with hypothesis 3 that roots exhibit only an immediate, short-term response to P deficiency. Consistent with studies on maize, rice, and beans (Juszczuk et al., 2004; Carvalhais et al., 2011; Schwalm et al., 2024), tree roots also released more C when incubated in a P-free solution than when P was present (Figure 4). As omission of KH_2PO_4 also resulted in lower K availability, the observed exudation responses cannot be attributed exclusively to P deficiency, particularly as K availability may indirectly influence root carbon release (Kraffczyk et al., 1984). Interestingly, although overall exudation rates differed between biomes, the magnitude of the initial response to the P-deficient trap solution did not differ between the two forest sites, which contrasts with previous results from grasses grown in soils with contrasting P availability, where roots from high-P soils tended to exude less C in nutrient-rich solutions than in water (Maurer et al., 2021). Whether increased C exudation under P-deficient conditions reflects an adaptive strategy to cope with nutrient limitation (Carvalhais et al., 2011) cannot be fully resolved here, as transient stress responses, minor root damage during cleaning, or contributions from rhizoplane-associated microorganisms may also have influenced the initial exudation rates. However, we found that the initial response to P-deficiency was lower at interspecific sites, suggesting that the lower P status at interspecific sites (unpublished data) might decrease the initial response (Supplementary Figure 3), further supported by the weak positive relation between C exudation and P uptake (Supplementary Figure 4).

Similar to the adaptation to saturated syringes (Section 5.2), we observed a rapid adjustment of C-exudation fluxes over time. In the second flush, exudation rates in P-deficient trap solutions converged, as roots previously exposed to P-free conditions showed a marked decline compared to the first flush (Figure 4). For experiments aiming to quantify root-derived C fluxes in temperate forests, we therefore recommend using second-flush data to avoid overestimating C allocation driven by transient responses to unfamiliar nutrient conditions. However, this recommendation cannot be directly extended to Mediterranean forest sites. Although exudation rates in the P-deficient trap solution declined in the second flush to levels comparable to those measured in the first flush with the standard solution, prolonged incubation in the standard solution resulted in higher C-exudation rates during the second flush than during the first (Figure 4). We interpret the second flush as reflecting root

acclimation to the incubation environment; in this context, the elevated exudation observed in the standard solution suggests increased C allocation following adaptation to comparatively high P availability. Such an increase was not observed in the temperate forest, where trees are likely adapted to higher soil P availability. We recommend using the standard trap solution when the objective is to generate comparable exudation estimates across sites, such as for inclusion in global datasets (Chari et al., 2024). In temperate forests, the standard solution can be applied in either the first or second flush, as no significant differences were observed between them. In Mediterranean forests, however, we recommend restricting its use to the first flush, given the increase in exudation observed during the second flush. By contrast, for longer-term measurements – such as labeling experiments (Hikino et al., 2022) – soil-specific trap solutions may be more appropriate.

We found higher exudation in the temperate forest compared to the Mediterranean forest, which seemingly contrasts with a recent global synthesis showing no significant differences in branch-level root-exudation rates among biomes (Chari et al., 2024). The relatively low exudation fluxes observed in the Mediterranean forest likely reflect natural seasonal variability. Root exudation is known to vary strongly over the year (Abramoff and Finzi, 2016; Tückmantel et al., 2017; Jakoby et al., 2020; Gao et al., 2023; Rog et al., 2024), and samples from the Mediterranean site were collected during the cool, wet winter season. During this period, *P. halepensis* and *P. lentiscus* exuded 206% and 406% less C, respectively, compared with the hot, dry summer, as reported in long-term seasonal investigations (S. Obersteiner et al., *under review*). In contrast, exudation rates measured in the temperate forest were likely close to their seasonal maximum. For example, Tückmantel et al. (2017) showed that exudation rates of *F. sylvatica* decline later in the growing season and thus, lower rates are expected earlier or later in the season.

5 Conclusion

This study demonstrates that the hybrid root-exudate collection approach is generally robust, but also sensitive to specific methodological decisions that can substantially influence estimates of C allocation to root exudation. We showed that a commonly adopted procedural step – re-burying root branches before hydroponic incubation – does not affect measured exudation rates and may therefore be omitted to improve sampling efficiency. In contrast, the moisture status of the trap solution represents a critical parameter: saturated traps markedly increased C-exudation fluxes and are likely to overestimate belowground C allocation up to 6-fold. Our results also confirmed that the composition of the trap solution shapes the initial exudation response, reinforcing the importance of using standardized solutions for comparability within and among studies. However, more tests with site-adapted solutions need to be performed, especially if longer-term measurements are conducted. Species mixing did not alter exudation rates, underscoring that methodological context, rather than biotic interactions, drives variability in the investigated systems. Overall, our findings highlight the need for greater transparency and standardization in root-exudate collection protocols and provide clear guidance for improving the reliability of field-based measurements of tree C allocation.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

SO: Writing – original draft. TK: Writing – review & editing. MB: Writing – original draft.

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Conflict of interest

The author(s) declared that this work was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fagro.2026.1778756/full#supplementary-material

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