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Subsoil rhizosphere carbon enrichment and depletion: processes and scaling in tree-based systems

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ABSTRACT

Tree roots have the potential to release carbon into deep soil layers, where this carbon is generally considered to exhibit greater stability. However, field studies that investigate the drivers of the soil organic carbon (SOC) balance in the rhizosphere of trees across soil depths and that upscale this balance to the whole soil profile are lacking. This study presents an innovative approach integrating normalized rhizosphere sampling and root density mapping to a depth of 1.5 m under trees from Mediterranean agroforestry and a tree plantation. The estimated SOC balance in the rhizosphere of the *Robinia pseudoacacia* trees varied from −38 kg C ha^{-1} to +53 kg C ha^{-1} at the different soil horizons, with a neutral balance at 0–0.3 m, a negative balance at 0.3–0.5 m and a positive balance at 0.5–1.0 m and 1.0–1.5 m of soil depth. When scaled up to the whole profile, the value was +50.6 kg C ha^{-1} for the tree plantation and +72.4 kg C ha^{-1} for the tree row for the agroforestry system, with no significant difference between these two estimates. The balance between hydrolytic and oxidative enzyme activities and between fungal guilds indicated increasing nutritional constraints for microbial saprotrophs at depth. In the subsoil, these nutritional constraints were locally attenuated in the rhizosphere, inducing a substantial increase in microbial abundance and triggering a pronounced shift from oligotrophic to copiotrophic communities, which in turn supported SOC enrichment. In the topsoil, the lower chemical complexity of substrates available to microorganisms increases susceptibility to saprotrophic activity, which likely underlies the observed neutral or negative SOC balances in the rhizosphere. This field study presents a scalable approach for quantifying the rhizosphere SOC balance in deep soil horizons and disentangling its biogeochemical drivers.

1. Introduction

Root-derived carbon (C) has considerable potential for soil organic carbon (SOC) sequestration, as studies have indicated that this C may be 2 to 5 times more stable in soil compared to aboveground litter C (Rasse et al., 2005; Jackson et al., 2017). Roots release C into the soil through rhizodeposition and root turnover (Gill and Jackson, 2000; Pausch and Kuzyakov, 2018). The sequestration of this C in soil organic matter may be particularly favoured in deep soil horizons (Kell, 2011; Bertrand et al., 2019; Thorup-Kristensen et al., 2020; Button et al., 2022). In fact, root-derived C inputs are expected to remain sequestered for a longer time in subsoil (i.e., below the ploughed horizon) because of the reduction in the microbial decomposition rate with depth (Hicks Pries et al., 2018) and because more mineral surfaces can be available for stabilizing organic molecules (Button et al., 2022). However, soil incubation also suggested that deep soil layers may exhibit greater sensitivity to the priming effect (Fontaine et al., 2007), a stimulation of native SOC mineralization by saprotrophic microorganisms in response to fresh C inputs (Kuzyakov, 2010). A potential limitation of these incubation experiments is the use of sieved bulk soil, which typically receives C input in a labile form (Button et al., 2022). Processes could differ under field conditions where subsoils have a very high density and lower oxygen concentration (Button et al., 2022). Under field conditions, root growth is concentrated in a limited number of soil pores, which may be

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sequentially used by multiple roots, leading to new rhizospheres overlapping with older ones (Kuzyakov and Blagodatskaya, 2015). This spatial organization indicates that most of the old C in the subsoil can remain protected from rhizosphere priming (Chabbi et al., 2009; Heitkötter and Marschner, 2018). Overall, the conditions under which root-derived C stabilization or the rhizosphere priming effect dominate the SOC balance remain unclear, particularly given the complexity of soil *in situ*, where measurements are both challenging and scarce.

Microorganisms exert a strong control over the SOC balance in the rhizosphere (Allison et al., 2010a; Schimel and Schaeffer, 2012). Their influence depends on nutritional constraints that regulate their growth and decomposition activity (Allison et al., 2010b; Mooshammer et al., 2014; Manzoni et al., 2017), and these constraints change with depth (Bertrand et al., 2019; Siegwart et al., 2023). The stoichiometry of soil resources is a central component of microbial nutritional constraints that control the balance between microbial immobilization and mineralization (Zechmeister-Boltenstern et al., 2015; Bertrand et al., 2019). In addition, the high chemical complexity of the substrates decomposed by microbes can also increase microbial C mineralization over immobilization (Sinsabaugh and Shah, 2011; Takriti et al., 2018). Indeed, microorganisms must synthesize extracellular enzymes to acquire C and nutrients from organic substrates. Given the metabolic cost of enzyme production, its optimization is expected to depend on the available resources (Allison et al., 2010b). Depending on the chemical complexity of the available resource, microorganisms produce more or less of these different types of extracellular enzymes. Hydrolytic enzymes are secreted to degrade macromolecules such as polysaccharides and proteins and oxidative enzymes to degrade more complex compounds, such as lignin (Sinsabaugh and Shah, 2011). Therefore, the ratio between hydrolytic and oxidative enzymes can be used to evaluate changes in the chemical complexity of substrates utilized by microorganisms (Takriti et al., 2018; Malik et al., 2019). Variation in nutritional constraints drives changes in the community composition. Oligotrophic microorganisms are adapted to foraging for chemically complex C sources, as opposed to copiotrophs that rely on simple C (Fontaine et al., 2003; Fierer et al., 2007). Changes in the microbial enzymatic traits in the subsoil and rhizosphere support that root presence favours copiotrophs, which utilize easily degradable carbon sources (Loeppmann et al., 2016; Takriti et al., 2018). Some microorganisms, such as mycorrhizal fungi, can instead rely on symbiotic associations with roots to obtain C, thereby circumventing the challenge of obtaining C from complex soil organic matter. The reduced dependence of symbiotic fungi on soil C has been proposed to explain their lower sensitivity to depth compared to saprotrophic fungi (Heitkötter and Marschner, 2018; Carteron et al., 2021). However, we are lacking field studies that investigate how these influences of roots on the stoichiometry and chemical complexity of available resources vary with depth and the consequences for the microorganisms that control the SOC balance in the rhizosphere.

The deep rhizosphere has substantial ecological significance, especially in tree-based systems (Jackson et al., 1996; Thorup-Kristensen et al., 2020). Agroforestry systems, which integrate trees into cropping systems, are increasingly advocated to increase root-derived C inputs in the subsoils of arable land, while data remain limited for temperate regions (Cardinael et al., 2018, 2020). In an alley-cropping system located in southern France, it has been reported that competition between crop and tree roots can stimulate deeper root growth in trees compared to those in pure tree plantations (Mulia and Dupraz, 2006; Cardinael et al., 2015), which would supply more root-derived C in deep horizons. These studies described the distribution of tree roots and estimated the associated C inputs and changes in SOC in the subsoil. However, they did not account for the limited volume of soil colonized and influenced by rhizosphere processes in deep soil horizons (Chabbi et al., 2009). Any potential change in SOC stocks in such restricted soil volumes is thus blurred by its dilution in the much larger volume of bulk soil. Considering the heterogeneity associated with the specificity of rhizosphere *vs*. bulk soil in deep soil horizons might be the key to better understanding the drivers of deep SOC dynamics in tree-based systems.

While the study of the rhizosphere *in situ* across soil horizons is crucial for assessing how roots shape the carbon cycle, the study of the rhizosphere in the field across whole soil profiles is hampered by several challenges. To date, most of our knowledge of the rhizosphere has been derived in artificial settings. While these studies have provided considerable insights into rhizosphere processes (Hinsinger et al., 2009; Kuzyakov and Razavi, 2019), extrapolating such findings to field scales is challenging. In contrast, field studies have been mostly descriptive, mapping the root-influenced zone with imagery (Heitkötter and Marschner, 2018) or comparing rhizosphere and bulk soil properties. A substantial body of research has operationalized the definition of the rhizosphere as “the soil adhering to roots after gentle shaking” (Finzi et al., 2015). However most of these investigations have focused on the topsoil and only a limited number explored rhizosphere properties in deeper horizons (Pradier et al., 2016). Characterizing soil adhering to roots has been useful to confirm *in situ* that soil around roots is different from bulk soil (Finzi et al., 2015; Lv et al., 2023). However, a major limitation of the sampling of soil adhering to roots as a proxy for the rhizosphere is the considerable variation in sampled soil volume due to difference in soil texture or moisture, which strongly influence soil adhesion to roots and often changes with depth (Phillips and Fahey, 2006). In addition, architectural and morphological variations in roots can affect soil–root adhesion (Rabbi et al., 2018), potentially influencing the observed rhizosphere effect along e.g. the texture gradient (Rüger et al., 2023). Poor root–soil adhesion may also cause parts of the rhizosphere to remain in the bulk soil during sampling and can limit the amount of soil collected for laboratory analysis. Consequently, critical interpretation is required when comparing rhizosphere samples from different soils or different soil horizons within a soil profile. Furthermore, strong assumptions about the roots and rhizosphere volume are required to upscale the empirical results obtained with this sampling strategy (Finzi et al., 2015). The development of methodologies that facilitate the quantification, comparison and upscaling of rhizosphere properties across whole soil profiles is essential.

In this study, we combined normalized rhizosphere sampling and field root mapping in a Mediterranean agroforestry system and a tree plantation. We aimed to characterize the changes in SOC around tree roots and to upscale the effect of the rhizosphere on SOC stock to the entire rooted soil profile. We hypothesized that (1) roots induce greater differences in SOC between the rhizosphere and the bulk soil in deep soil horizons than in shallow soil horizons, thereby offsetting the decline in root density with depth and compensating for it in the rhizosphere effect's contribution to SOC stocks at the soil profile scale; (2) trees in agroforestry system develop deeper roots than those in tree plantations, with consequences for rhizosphere SOC; (3) a positive rhizosphere SOC balance at depth is favoured by a substantial shift from oligotrophic microbial communities foraging on chemically complex substrates to copiotrophic microbial communities relying on root C inputs.

2. Materials and methods

2.1. Study site

The DIAMS experimental site is a long-term experimental area of 5 ha located 10 km east of Montpellier in southern France at the INRAE experimental station UE DiaScope (43.612°N; 3.976°E). The climate is Mediterranean, with a mean annual air temperature of 15.5 °C and a mean annual rainfall of 552 mm (2012–2022). The soil is a Skeletic Rhodic Luvisol according to the IUSS classification (Fig. 1C). The topsoil (0–0.2 m) has 19.2 % clay, 32.8 % silt and 48 % sand, and a pH of 6.8. The volumetric portion of stones and clay content increased substantially in the subsoil, with 47 % stones and 47 % clay below 0.5 m (Siegwart et al., 2022).The experiment is structured as a split-plot design with land uses repeated in 3 blocks and separated by a distance ranging from 100 to 500 m (Martin-Blangy et al., 2025). Two land uses are

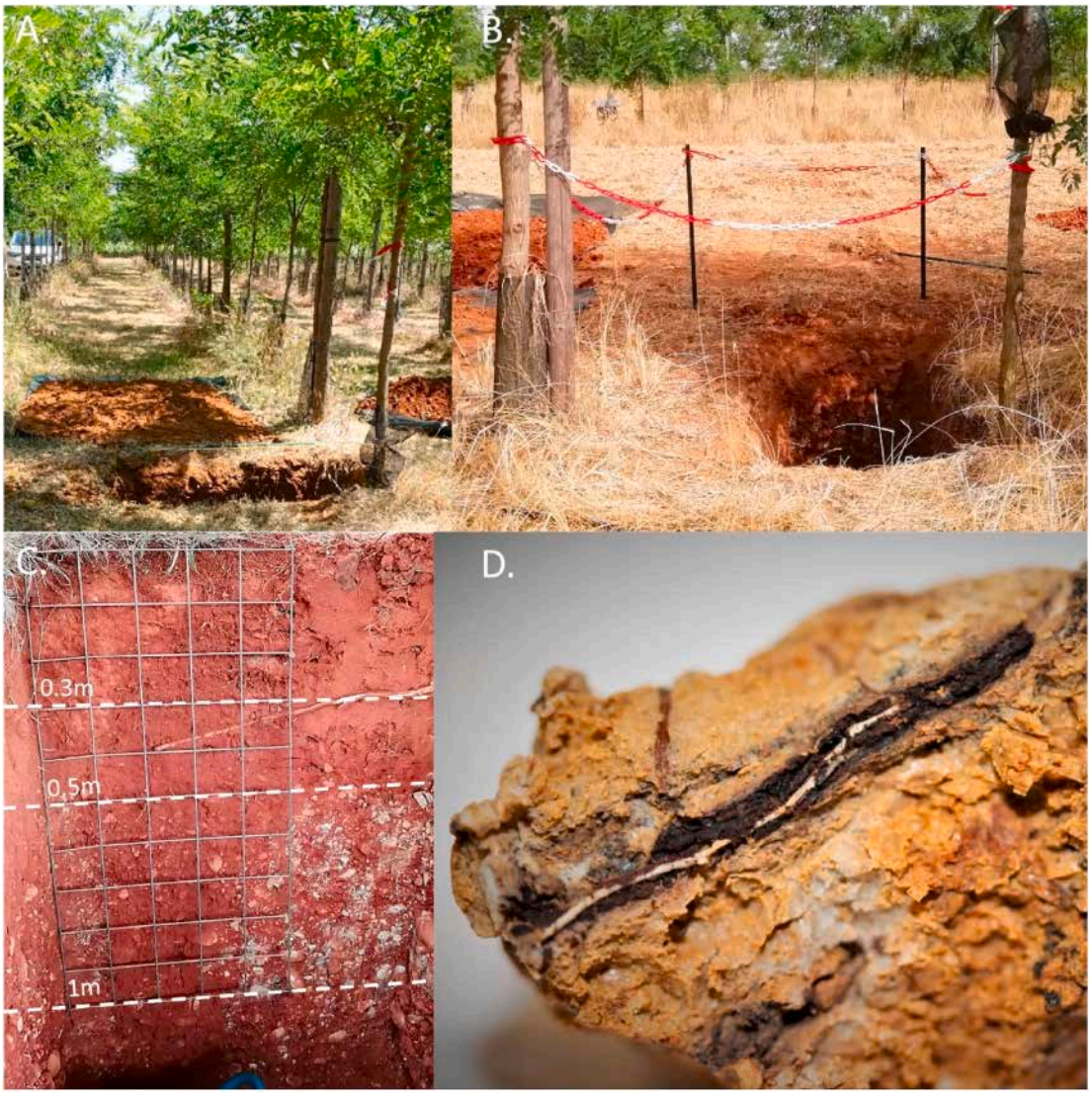

Fig. 1. Land use and soil at the study site (pictures were taken by GP and SMB). A. Tree plantation with its sampling pits. B. Agroforestry plot with its sampling pits. C. Soil profile with the 4 sampled horizons (0.0–0.3 m: 0.3–0.5 m, 0.5–1 m, 1–1.5 m) and the 0–1 m root mapping grid. D. Observation of the reuse of a biopore below 1 m in a sampled pit, with the presence of a black colour around the roots that could be attributed to metal oxide and/or organic matter accumulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compared in this study: an alley-cropping agroforestry system (AF) and a tree plantation monoculture (TP). In 2017 and 2018, black locust (*Robinia pseudoacacia* L.) trees were planted at 2 m intervals within rows, with rows separated by 3 m on the TP (1667 trees ha^{-1}) and 17 m in the AF plots (294 trees ha^{-1}) (Fig. 1A and B). Under the trees, a mix of sown (dominated by *Festuca arundinacea* S.) and spontaneous herbaceous flora is presentacross the entire TP plot and restricted to a 2-m wide strip below treesfor the AF plots. The AF crop alley followed a wheat (*Triticum turgidum* L. *subsp. durum*), barley (*Hordeum vulgare* L.) and pea (*Pisum sativum* L.)) rotation, with 20 cm depth tillage and biannual fertilization limited to cereal crops. During the year of sampling, the crop was pea, and no fertilization was applied.

2.2. Black locust rhizosphere sampling

From May–June 2022, 4- to 5-year-old black locust trees were excavated from the AF and TP plots of each block (6 trees in total). In each block, a tree was selected based on a diameter at breast height reflecting the mean value for the respective land use (see Martin-Blangy et al., 2025). Fine roots and soil (bulk and rhizosphere) were sampled from one-quarter of each tree root system (Fig. S1). This volume was defined based on the Voronoi tessellation sampling procedure (Levillain et al., 2011). More precisely, the sampling pit was dug from the observed tree stem base to a half distance from the closest neighbouring trees, extended to 0.5 m into the crop alley (1.5 m from the tree) in the AF plots, as no tree roots were previously observed in the crop alley (Siegwart et al., 2022). The final surface area of each pit was 1 m × 1.5 m. Within this sampling area, tree density was standardized at 1667 trees ha^{-1} for both TP and AF plots. It should be noted that this sampling was limited to the tree row (+0.5 m into the crop alley), representing 11.8 % of the total AF plot surface, the rest being covered by the crop (Martin-Blangy et al., 2025). Thus, the quantities reported in this study are expressed per hectare of tree row ($har^{-1}_{tree\ row}$)for the AF plots. Four soil layers were carefully excavated in each pit, 0–0.3 m, 0.3–0.5 m, 0.5–1.0 m and 1.0–1.5 m, corresponding to the pedological soil horizons previously described at the site (Siegwart et al., 2022). Subsequently, 10 to 30 dm^3 of soil containing fine roots were randomly subsampled in each horizon until at least 20 g of rhizosphere soil was collected. The rhizosphere was operationally defined as the soil adhering to the living roots and located at a distance of less than 7 mm from a root. This

distance, which exceeds the 2 mm zone around roots where the steepest parts of the chemical gradients occur (Kuzyakov and Razavi, 2019), was chosen to ensure that our samples encompassed the entire zone where soil biological activity and associated SOC dynamics were affected by roots. Additionally, such approach provides sufficient material to conduct multiple analyses. It should be acknowledged that this method can dilute some of the rhizosphere effects, thus reducing our ability to capture low or very localized rhizosphere effects. This rhizosphere sample was normalized. First, the soil was sieved through a 14-mm mesh to remove large soil–root aggregates. Second, soil aggregates containing living roots of black locust trees were collected. The tree roots were distinguished from those of the herbaceous plants or crops based on morphological traits (flaky aspect, with short and densely branched fine roots, a ramification angle less than 90°, and the presence of nodules), colour (yellow−brown) and a distinct odour. The soil remaining once all soil–root aggregates were sorted was considered the bulk soil sample, leading to a total of 48 samples (rhizosphere and bulk soil from 4 horizons in 2 land uses repeated in 3 blocks). Third, the rhizosphere samples were carefully transported to the laboratory, and roots extending beyond the volume of the root–soil aggregates were removed. Fourth, the soil within the soil–root aggregates was separated from the roots. Finally, for each soil horizon, the total amount of the rhizosphere sample and its associated root biomass were weighed. These portions of the sampled roots (from within the root–soil aggregates) were scanned to determine their total length (300 dpi scans in deionized water using an Epson Expression© 10,000 XL instrument and image analysis with the software WinRHIZO v. 2005b Regent, Canada) and dried at 50 °C for 72 h to determine their dry mass. This process allowed for the calculation of the root dry mass density (RMD_{rhizo}) and the root length density (RLD_{rhizo}) per g of rhizosphere for each rhizosphere sample and thus to verify if the rhizosphere sampling in the different soil horizons was comparable in terms of rooting density and the rhizosphere radius, as expected.

2.3. Soil properties

Immediately after sampling, a portion of the bulk and rhizosphere soils was frozen at −20 °C for microbial analyses, another portion was used for the soil solution measurement of fresh soil, and the remaining soil was air-dried to measure the soil pH and soil organic C and N contents. Dissolved organic C (DOC), total dissolved nitrogen (TDN) and mineral nitrogen were extracted from 10 g of fresh soil using a 0.5 M K_2SO_4 solution with a 1:4 soil/solution ratio, except for the rhizospheres collected in the deepest horizon that were available in limited quantities (Blocks 2 and 3), for which 2.5 or 5 g was used instead of 10 g of fresh soil. The TOC and TDN contents were measured with a TOC/TN analyser (TOC-Vsch-TNM SHIMADZU), and nitrate and ammonium contents were measured by flow colorimetry (Continuous Flow Analyser, Skalar). The available P content was measured in 2 g of fresh soil using an anion exchange resin (Hedley et al., 1982). The soil water content was calculated based on fresh soil mass loss after an incubation at 105 °C for 48 h. Soil pH was determined in deionized water with a soil/solution ratio of 1:5. Total soil organic C and total N contents were analysed by dry combustion (CHN Thermo Flash, 2000) with phosphoric acid pretreatment to remove carbonates (Bisutti et al., 2004). The absence of an effect on soil organic carbon resulting from pretreatment with this acid was verified in 8 samples without carbonates ranging from pH 7.4 to 7.9 ($R^2 = 0.98$ and slope = 1.04 for the correlation between the total soil carbon contents with and without acid pretreatment).

2.4. Potential enzyme activities and microbial community composition

The activities of four hydrolytic enzymes, namely, β-1,4-glucosidase (a cellulose-acquiring enzyme), alkaline phosphatase (an ester phosphatase, a P-acquiring enzyme), N-acetyl-glucosaminidase (chitin and peptidoglycan, N-acquiring enzymes), and leucine aminopeptidase (leucine and other hydrophobic amino acids, N-acquiring enzyme), were measured (Sinsabaugh et al., 2008). Potential phenol oxidase and peroxidase activities were additionally assessed, since these enzymes degrade more complex compounds, such as lignin. The potential activities of hydrolytic enzymes were quantified with fluorometric assays as described by Bell et al. (2013), and oxidative enzymes were quantified using the colorimetric methods described by Saiya-Cork et al. (2002). For both types of enzymes, a buffer corresponding to the soil pH (± 0.3) was used. The activity of each enzyme in each sample was measured in 4 technical replicates, with a specific standard curve and 2 negative controls (substrate + enzyme inhibitor).

The composition of the bacterial and fungal communities was characterized in soil DNA using Illumina metabarcoding and qPCR targeting the 16S and 18S rRNA genes (Supplementary information 1). The sequences were processed using the FROGS pipeline (Escudié et al., 2018) using the SILVA database for bacteria (Quast et al., 2012) and the UNITE database for fungi (Abarenkov et al., 2023). FunGuild (Nguyen et al., 2016) was ultimately used to calculate the relative abundance of fungal guilds (Supplementary information 1).

2.5. Root mapping

The root impact density (RID) was mapped on the two sides of the pits perpendicular to the tree rows and combined with the sampling of soil cubes to estimate the root length density (RLD) across the soil profiles (Chopart and Siband, 1999; Maurice et al., 2010; Siegwart et al., 2022). More precisely, root impacts were counted on 0.1x0.1-m grid tiles. Two grids with a height of 1.5 m (depth, Z coordinate) were positioned perpendicular to the tree rows across the 1.5-m length (distance from the tree, Y coordinate) of the pit, with one grid on the pit side below the tree trunk and one on the side at half distance between two trees (X coordinate). Roots from black locust trees and herbaceous species were distinguished based on their characteristics described above. Subsequently, in each grid, a 5x5x5-cm cube of soil was extracted at three distances from the tree (0.25 m, 0.75 m, and 1.25 m) and at three depths (0–0.3 m, 0.3–0.5 m and 0.5–1.5 m). The impact of black locust roots on each face of these soil cubes as well as the dry mass and length of the roots they contained were measured. These data were utilized to fit a linear regression (with the intercept fixed at 0) between RID and RLD for 0–0.3 m and 0.3–1.0 m depth ranges in each land use (AF and TP) for each type of plant (black locust and herbaceous species). These models (R^2 values between 0.56 and 0.68) for black locust and between 0.33 and 0.73 for herbaceous species were subsequently employed to estimate the RLD from the RID in all the grid tiles.

2.6. Data analysis

We tested the effects of soil horizons, the rhizosphere (as compared with bulk soil), land use (agroforestry or tree plantation) and their interactions on soil variables using mixed-effect models with the blocks as a random factor in the *nlme* R package (Pinheiro et al., 2017). All the variables were log or square root transformed to meet the assumptions of normal distribution and homoscedasticity of the model residuals according to the Shapiro and Bartlett tests respectively, using the *stats* R package. Mixed effect models were also applied on the relative abundances of bacterial and fungal phyla, but their distributions did not meet the assumptions of a normal distribution and homoscedasticity in the model for many phyla and were complemented with an analysis more adapted to abundance data. The habitat preference of bacterial and fungal taxa was thus assessed with point-biserial correlation coefficients with the option "r.g" (single and multiple habitat preferences tested) of the "multipatt" function from the *indicspecies* package. Their significance was calculated with 999 permutations computed within each pit to consider the sampling structure (Cáceres and Legendre, 2009). The p values were adjusted for multiple testing with the false discovery rate correction of Benjamini and Hochberg (1995) compiled in the *p.adjust*

package. We simplified the analysis of the preference of different taxa for different habitats and all their combinations by aggregating 0–0.3 m and 0.3–0.5 m into a topsoil habitat and 0.5–1.0 m and 1.0–1.5 m into a subsoil habitat based on the results from the soil properties of the rhizosphere and bulk soils. This analysis was run at the phylum and genus levels for bacteria and fungi and for the fungal guilds. Lower phylogenetic levels (OTU or species level) were not considered due to their excessively high number, which, combined with the false discovery rate correction, precluded the detection of indicator taxa. The difference between the rhizosphere and the bulk soil (Δ_{rb}), which was calculated for each pit at each soil horizon, was used to illustrate the variations in the rhizosphere effect on soil properties and microbial phyla with depth. In addition, we synthesized the multivariate coupling in the rhizosphere response by performing PCA on the $\Delta_{r\text{-}b}$(difference between the rhizosphere and bulk soil) of all the variables (soil, enzyme activities, microbial phyla and guilds) that responded to the rhizosphere in the analysis of the mixed effect model. Finally, we tested the linear mixed models between the $\Delta_{r\text{-}b}$ of the SOC and the soil properties in the associated bulk soil, with the block as a random factor. For this analysis, we selected soil variables expected to capture the microbial nutritional constraints induced by resource stoichiometry (soil C:N, DOC:TDN, root C:N, and C-acquiring enzyme/N-acquiring enzyme ratios), the chemical complexity of substrates preferentially decomposed by microorganisms (hydrolase/oxydase ratio, specific hydrolase activity and specific oxidase activity) and the microbial community composition (fungal guild abundance, phylum abundance, and OTU richness).

2.7. Rhizosphere effect and SOC upscaling

First, we calculated the difference between the soil properties of the rhizosphere and bulk soil ($\Delta_{r\text{-}b}$) observed in each horizon *h* and each pit *p* as follows:

$$(Equation\ 1)\ \Delta_{r-bph} = Rhizosphere_{ph} - Bulk_{ph}$$

with $Rhizosphere_{ph}$ and $Bulk_{ph}$ representing the values measured in the rhizosphere and the bulk soil, respectively.

Second, we calculated the proportion of the rhizosphere across the root map for each tile *iph*, with *i* as the tile identity (i.e., the XYZ coordinate), *p* as the pit identity and *h* as the associated horizon sample for soil analysis (0–0.3, 0.3–0.5, 0.5–1.0 or 1.0–1.5 m) as follows:

$$(Equation\ 2)\ Proportion\ of\ rhizosphere_{\ iph}\left(g\ rhizo\ soil\ g\ soil^{-1}\right) = \frac{Till\ RLD_{iph}\ (cm\ cm^{-3})}{Rhizosphere\ sample\ RLD_{ph}\ (cm\ g^{-1}) \times fine\ soil\ bulk\ density_{ph}(g\ cm^{-3})}$$

For the analysis of the fine soil bulk density, we used data previously collected from each block of the site by Siegwart et al. (2022).

Third, we combined the results from Equation 1 for SOC ($\Delta_{r-b}\ SOCcont_{ph}$) and the rhizosphere volume from Equation 2 to determine the contribution of the rhizosphere effect to the SOC stock across the root map using the following equation:

$$(Equation\ 3)\Delta_{r-b}\ SOCstock_{iph}\left(gC\ dm^{-3}\right) = \frac{\Delta_{r-b}\ SOCcont_{ph}\ \left(gC\ g_{rhizo\ soil}{}^{-1}\right) \times Proportion\ of\ rhizosphere\ soil\ _{iph}\ \left(\ g\ rhizo\ soil\ g\ soil^{-1}\right)}{fine\ soil\ bulk\ density_{ph}\ (g\ soil\ cm^{-3})} \times 10^{3}$$

Finally, the obtained values were averaged across the X and Y coordinates for each horizon of each pit and upscaled from the average g C dm^{-3} per horizon to t C ha^{-1} at the full horizon depth. For this global estimation, we used only the $\Delta_{r-b}\ SOCstock_{iph}$ of horizons where a significant difference in the SOC content between the rhizosphere and bulk soil was detected using the mixed effects model.

3. Results

3.1. Agroforestry and tree plantation systems showed different rooting patterns

The vertical and horizontal (distance from the tree) variations in the tree root length density were mapped and exhibited differences between the AF and the TP. The average tree RLD across the soil profile was higher ($p = 0.002$) in the TP (2.31 m dm-3) than in the AF (2.03 m dm-3). Horizontally, the tree rooting density was homogenously distributed in the TP, whereas in the ploughed layer of the AF, it abruptly decreased in the crop alley and was higher beneath the stump and the perennial herbaceous strip (Fig. 2A). Vertically, compared with AF, TP resulted in deeper rooting of trees. Indeed, in the upper topsoil layer (0–10 cm), the tree root length density was higher in the AF than in the TP, whereas the opposite result was observed in the subsoil (from 0.3 to 0.9 m). However, the difference was not significant below 0.9 m (Fig. 2B). Conversely, the RLD of herbaceous species was significantly higher ($p < 0.001$) in the AF (3.17 m dm-3) than in the TP (2.13 m dm-3), with this difference observed from a depth of 0–0.7 m (Fig. S2). The distribution of the RLD in each soil horizon revealed that the influence of the rhizosphere became greater for a restricted volume of soil at that depth. Indeed, most grid tiles (10 × 10 cm) of the top horizon presented values different from 0, whereas tiles with no tree roots became more abundant down the profile (Fig. 2C).

3.2. In the deepest mineral horizon, rhizosphere SOC enrichment was associated with increased microbial abundance, simple C degradation and specific taxa

A significant interaction between horizon and rhizosphere factors was observed for the SOC content (Table S1). Specifically, the rhizosphere exhibited no significant change at 0.0–0.3 m, a negative effect at 0.3–0.5 m, and a positive effect at 0.5–1 m and 1 m–1.5 m (Fig. 3B). In addition, this observed effect of roots only below 0.3 m was further supported by the fact that the RLD associated with the rhizosphere decreased with depth (Table 1). This result indicated that for the same root length, the adhering soil (rhizosphere) mass collected was greater for samples from the deepest soil horizons (i.e., the rhizosphere effect was more diluted in the samples collected at depth). PCA of the $\Delta_{r\text{-}b}$ values of the soil and microbial properties (Fig. 3C–E) showed that most of the changes that occurred in the rhizosphere was tightly linked to variation in the $\Delta_{r\text{-}b}$ SOC with depth. The $\Delta_{r\text{-}b}$ SOC was one of the two most important variables of the PCA Axis 1, capturing 33 % of the global variation in all $\Delta_{r\text{-}b}$. On the one hand, we observed positive coupling between the $\Delta_{r\text{-}b}$ of the SOC and the $\Delta_{r\text{-}b}$ of soil C:N, hydrolase activities, the hydrolase/oxydase ratio, microbial abundance (16S and 18S copy numbers and molecular biomass), fungal richness and the relative abundances of Proteobacteria, Bacteroidota, Verrucomicrobiota, Mortierellomycota, Kickxellomycota, and saprotrophic fungi along Axis 1. On the other hand, the $\Delta_{r\text{-}b}$ of the relative abundance of GAL15, Methylomirabilota, Basidiomycota, and ectomycorrhizal fungi and the $\Delta_{r\text{-}b}$ of the fungal-to-bacteria ratio (18S:16S) decreased when the SOC content increased in the rhizosphere (Fig. 3D). The variations in the rhizosphere effect with depth for these variables were also confirmed by analysing them individually (Table S1, Fig. S3–6).

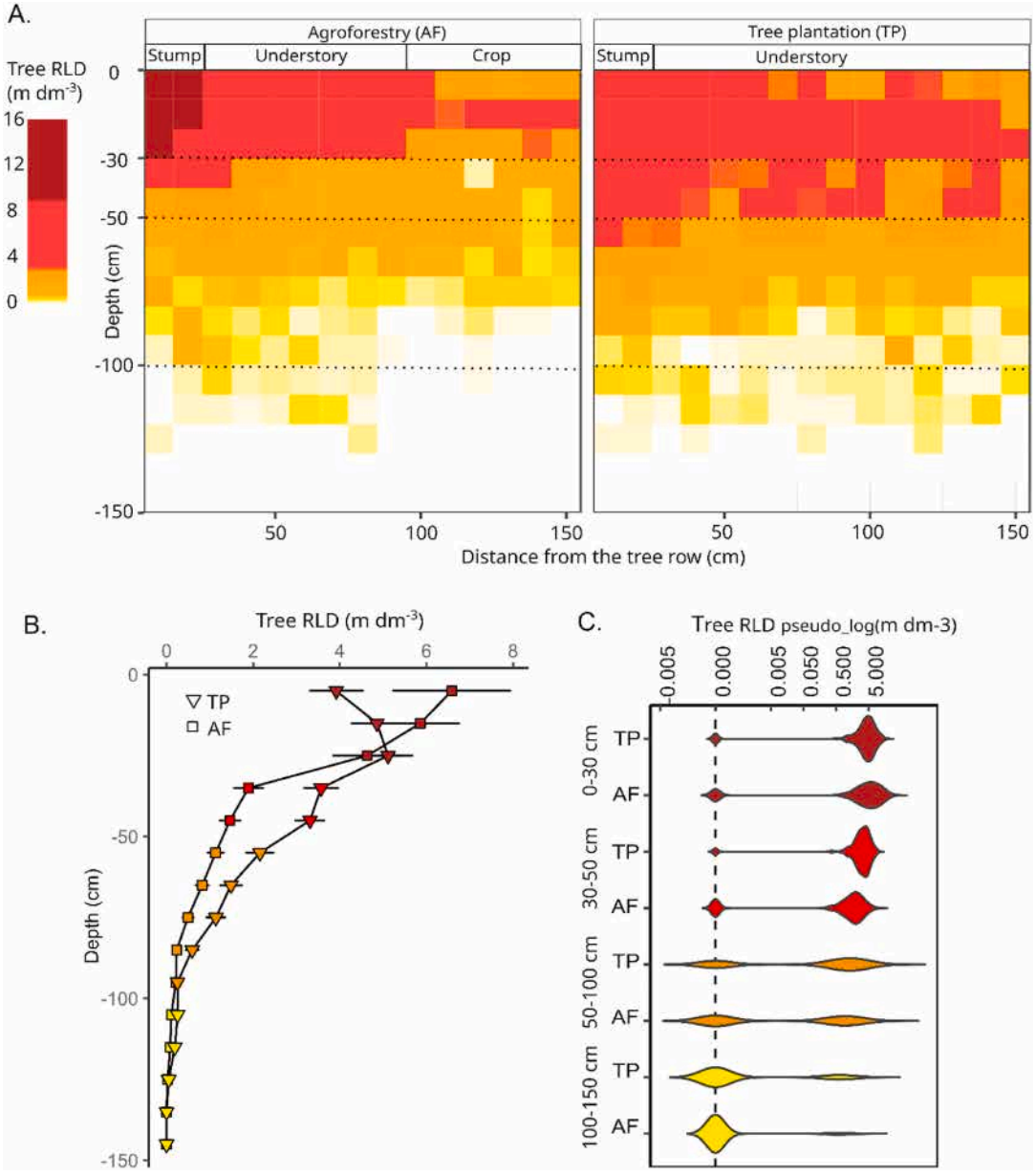

Fig. 2. Fine tree root distribution under agroforestry (AF) and tree plantation (TP) systems. (A) Tree fine root length density (RLD) across depth and distance from the tree row. (B) Vertical distribution of the tree RLD, with error bars representing 95 % confidence intervals. (C) Violin plot presenting the distribution of the RLD across the tiles of the root mapping grid at different horizons, with a violin inflation of approximately 0 indicating the proportion of 10 × 10 cm tiles with no tree roots (cold spot).

Furthermore, the general rhizosphere effects across the soil profile on the $\Delta_{r\text{-}b}$ for certain variables appeared along Axis 2 of the PCA. Across all depths, we observed consistent increases in soil N pools (Soil-N, TDN, and NH4) and the DOC content, as well as decreases in soil moisture and oxidase activity (Table S1; Figs. S3 and S6) in the rhizosphere. The analysis of the individual responses of the different microbial groups (Figure S4 and Figure S6) also showed a general association with the rhizosphere for Bacteroidota ($p < 0.001$) and Glomeromycota ($p = 0.002$) (AMF) and with the bulk soil for Basidiomycota ($p = 0.025$) and Mucoromycota ($p = 0.03$) across the whole profile (Figure S4 and Figure S6), however, these associations were not clearly reflected in the PCA plot.

The substantial variation in soil properties was also driven by depth (Table S1), with a general decrease in the SOC content (Fig. 3A), soil N content, microbial abundance (DNA quantity and 16S and 18S copy numbers), hydrolytic activity (Fig. S3) and microbial diversity (bacterial, fungal and AMF richness, Fig. S5). The depth gradient also exhibited decreases in the soil C:N ratio, fungal/bacterial ratio and β-1,4-glucosidase: phenol oxidase ratio, along with increased enzyme activity per unit of microbial DNA (increases in the hydrolase/DNA and oxydase/DNA ratios) (Fig. S3). The relative abundances of most bacterial and fungal phyla changed with soil depth (Fig. S4, Table S2). The relative abundances of the dominant bacterial phyla were reduced by approximately half (Bacteroidota, Proteobacteria) or one-quarter (Actinobacteria) from the shallower to the deeper horizon in the bulk soil, becoming outperformed by rare phyla such as Methylomirabilota, Latescibacterota, Nitrospirota, Desulfobacteria, GAL15 and Dadabacteria in deep horizons. The two latter phyla were observed only at depths below 0.5 m. Among the fungi, we observed a clear shift from a dominance of Ascomycota and saprotrophs in the topsoil to a dominance of Basidiomycota and ectomycorrhizal fungi in the subsoil (Figure S4 and Figure S6). The abundances of Glomeromycota, Mortierellomycota and

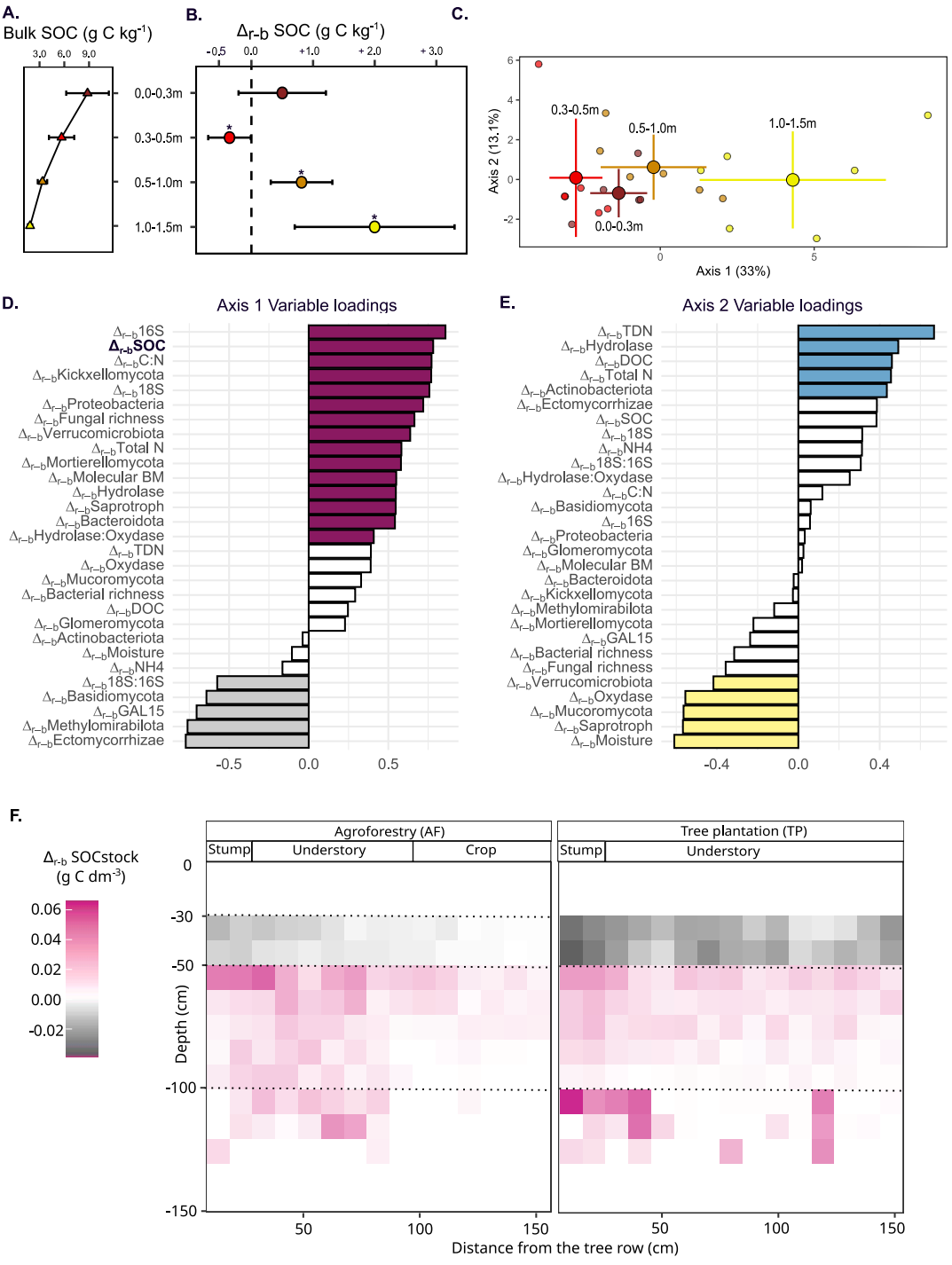

Fig. 3. Changes in soil organic carbon (SOC) contents in tree-based systems coupled with soil and microbial properties. (A) Variation in the SOC content in bulk soil across horizons. (B) Changes in the SOC content in the tree rhizosphere (Δ_{rb}), with asterisks indicating a significant effect of the rhizosphere. (C–E) PCA plot presenting the $\Delta_{r\text{-}b}$ of soil and microbial properties capturing their coupling in the response to the rhizosphere, with (C) the positions of rhizosphere samples along the 2 main axes of the PCA and (D–E) the contributions of the (Δrb) to Axes 1 and 2, with variables significantly correlated with the axis ($p < 0.05$) shown in colour and nonsignificant correlations shown in white. Points (A–C) represent the average values of each horizon, and the error bars represent the 95 % confidence intervals for the normal distribution. Yellow points (horizon 0.0–0.3 m), orange points (horizon 0.3–0.5 m), light red points (horizon 0.5–1.0 m), and dark red points (horizon 1.0–1.5 m). The values of the TP and AF land uses were aggregated (N = 6 for each horizon), as no significant interaction between land use and rhizosphere effects was detected in the mixed effects model for these variables (Table S1). (F) Distribution of the changes in SOC contents associated with the rhizosphere effect across soil depth and distance from the tree row, estimated based on the changes in the SOC content in each horizon (Fig. 3B) and root distribution (Fig. 2A) in the AF and TP systems calculated using Equations (2) and (3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Comparison of the root density in the rhizosphere samples. Mean values and standard deviations of the root mass density (RMD) and root length density (RLD) of the rhizospheres sampled from the different soil horizons. Agroforestry and tree plantations were combined (N = 6). P values indicate the difference between horizons, and different letters represent significantly different root densities between horizons.

Horizon (m)	RMD_{rhizo} (mg dry roots g dry $soil^{-1}$)			RLD_{rhizo} (mm roots g dry $soil^{-1}$)		
p value	0.468			<0.001		
0.0–0.3	1.24	±0.15	a	79.8	±12.78	c
0.3–0.5	0.73	±0.15	a	54.6	±12.11	bc
0.5–1.0	0.89	±0.17	a	36.4	±7.47	ab
1.0–1.5	1.35	±0.56	a	12.5	±1.81	a

fungal parasites also decreased with soil depth in the bulk soil, whereas the abundances of rare phyla such as Calcarisporiellomycota and Monoblepharomycota increased. In contrast, the soil properties of the two land use types exhibited minimal variations. Compared with the AF system, the TP presented slightly higher pH and lower available P (resin P) and minor differences in the enzyme and fungal community responses to the rhizosphere (Table S1, Fig. S5).

Point-biserial correlation coefficients were calculated to detect the habitat preference at the microbial genus level. The habitat preferences of the bacterial genera were structured mainly by depth, with the greatest number of indicator genera observed in the topsoil, without specificity for the bulk soil or rhizosphere (Table S3–S4). A few bacterial genera were also associated with the bulk soil or rhizosphere at specific depths. Among these, five genera were associated with the rhizosphere in the subsoil, of which four belonged to Proteobacteria. Six genera were associated with the subsoil in the bulk soil, with four of these annotated as unknown genera. Conversely, only one bacterial genus from Bacteroidota showed a habitat preference for the rhizosphere, with no specificity for soil depth or bulk soil. Fewer habitat preferences were detected for fungal genera (Table S3). Only one Calcarisporiellomycota genus showed a general preference for the subsoil, 4 genera for the subsoil in the bulk soil (all belonging to Basidiomycota) and none for the subsoil in the rhizosphere.

3.3. Changes in root-associated carbon stocks with depth under the agroforestry system and tree plantation

Based on the root length density patterns (Fig. 2A), the tree root C stock and tree rhizosphere volume (%) in the 0–30 cm horizon of the AF were 1.85- and 1.64-fold higher than those in the TP, respectively (Table 2). At greater depths, from 0.3 to 1.5 m, the opposite trend was observed, with 2.3-fold more tree root-derived C and 1.5-fold more % tree rhizosphere in the TP than in the AF. However, this quantity did not differ significantly at this scale when blocks (N = 3) were used as a replicates. When roots (Fig. 2) and the rhizosphere effect (Fig. 3B) were combined to calculate the effect of the tree rhizosphere on the SOC content ($\Delta_{r\text{-}b}$ SOC) across the soil profile (Fig. 3F), the estimated $\Delta_{r\text{-}b}$ of the SOC stock by horizon ranged from −36.4 kg C ha-1 to +42.4 kg C ha-1 below 0.3 m. In the 0.0–0.3 m horizon, no calculation of the $\Delta_{r\text{-}b}$ of the SOC stock was performed due to the lack of a statistically significant difference in the SOC concentration between the rhizosphere and bulk soil at this depth (Fig. 3B).

3.4. The more favourable $\Delta_{r\text{-}b}$ SOC in deep mineral horizons was related to limited saprotrophic activity

The $\Delta_{r\text{-}b}$ SOC was significantly correlated with some stoichiometric and microbial properties of the bulk soil (i.e., lacking roots) related to nutritional constraints on saprotrophs (Fig. 4). The $\Delta_{r\text{-}b}$ SOC became more positive in subsoil horizons characterized by a low C:N ratio and with microorganisms showing high investment in enzyme activities, especially for the degradation of complex substrates (high mass-specific activities and a low hydrolase/oxidase ratio). These subsoil horizons, with positive $\Delta_{r\text{-}b}$ SOC values, also presented lower bacterial and fungal richness and a lower relative abundance of saprotrophs and arbuscular mycorrhizal fungi (AMF) but a higher relative abundance of ectomycorrhizal fungi (Fig. 4).

4. Discussion

4.1. Quantifying rhizosphere SOC enrichment and depletion across soil profiles

In a 5-year-old agroforestry system and a tree plantation with black locust (*Robinia pseudoacacia*), this study documented changes in the SOC content in the tree rhizosphere across the whole soil profile. No significant difference between rhizosphere and bulk soil samples was detected in the topsoil horizon (0.0–0.3 m). Below the topsoil, the rhizosphere was first associated with a depletion of the SOC content in the 0.3–0.5 m horizon, indicating the dominance of a positive rhizosphere priming effect (Kuzyakov, 2002; Dijkstra et al., 2020). In contrast, in the deepest horizons of the subsoil, at 0.5–1.0 and 1.0–1.5 m, the rhizosphere showed an SOC enrichment of up to 2-fold, indicating conditions favourable for root-derived C sequestration (Rasse et al., 2005; Dijkstra et al., 2020). These observations were consistent with our hypothesis that the effect of roots on the rhizosphere SOC content increased with depth. However, such data also reveal *in situ* that the net balance following root-derived C inputs can be positive or negative (Dijkstra et al., 2020) or null. Previous studies have suggested that the mineral and structural properties of the subsoil, usually defined as the horizon below the plough layer, could be either favourable (Rasse et al., 2005; Button et al., 2022) or unfavourable (Fontaine et al., 2007; Henneron et al., 2022) for SOC sequestration. Our *in situ* observations suggest that

Table 2
Carbon stock distribution in tree roots and the rhizosphere. Averages ± standard errors of the tree rhizosphere (% soil mass) and the stocks (kg C ha-1) associated with tree roots and the rhizosphere across horizons and land uses (agroforestry (AF) system and tree plantation (TP)). The $\Delta_{r\text{-}b}$ SOC was calculated based on the product of the difference in the SOC content between the tree rhizosphere and bulk soils (Equation (1)) and the proportion of the rhizosphere in each horizon (Equation (2)) and then upscaled per ha using the fine soil density and horizon height (see Equation (3) and the associated text). The $\Delta_{r\text{-}b}$ SOC of the 0.0–0.3 m horizon was not calculated because the difference between the rhizosphere and bulk soil in this horizon was not significant (Fig. 3B). Lowercase letters indicate significant differences among soil depths and land-use types (p value < 0.05).

Horizon	Land use	Tree rhizosphere (% of soil mass)				Tree root C (kg C ha^{-1})				$\Delta_{r\text{-}b}$ SOC (kg C ha^{-1})			
0.0–0.3 m	AF	7.6	±	1.9	b	882.3	±	146.6	c		–		
	TP	4.6	±	0.6	ab	486.0	±	63.8	b		–		
0.3–0.5 m	AF	3.1	±	1.6	ab	66.0	±	3.3	a	−10.2	±	8.7	a
	TP	4.6	±	0.5	ab	201.5	±	22.4	a	−36.4	±	26.4	a
0.5–1.0 m	AF	1.4	±	0.5	a	62.8	±	3.0	a	56.2	±	41.3	a
	TP	2.0	±	0.3	a	91.8	±	17.4	a	44.7	±	12.6	a
1.0–1.5 m	AF	0.5	±	0.4	a	8.4	±	5.3	a	26.3	±	22.8	a
	TP	0.8	±	0.3	a	25.7	±	12.7	a	42.4	±	22.4	a

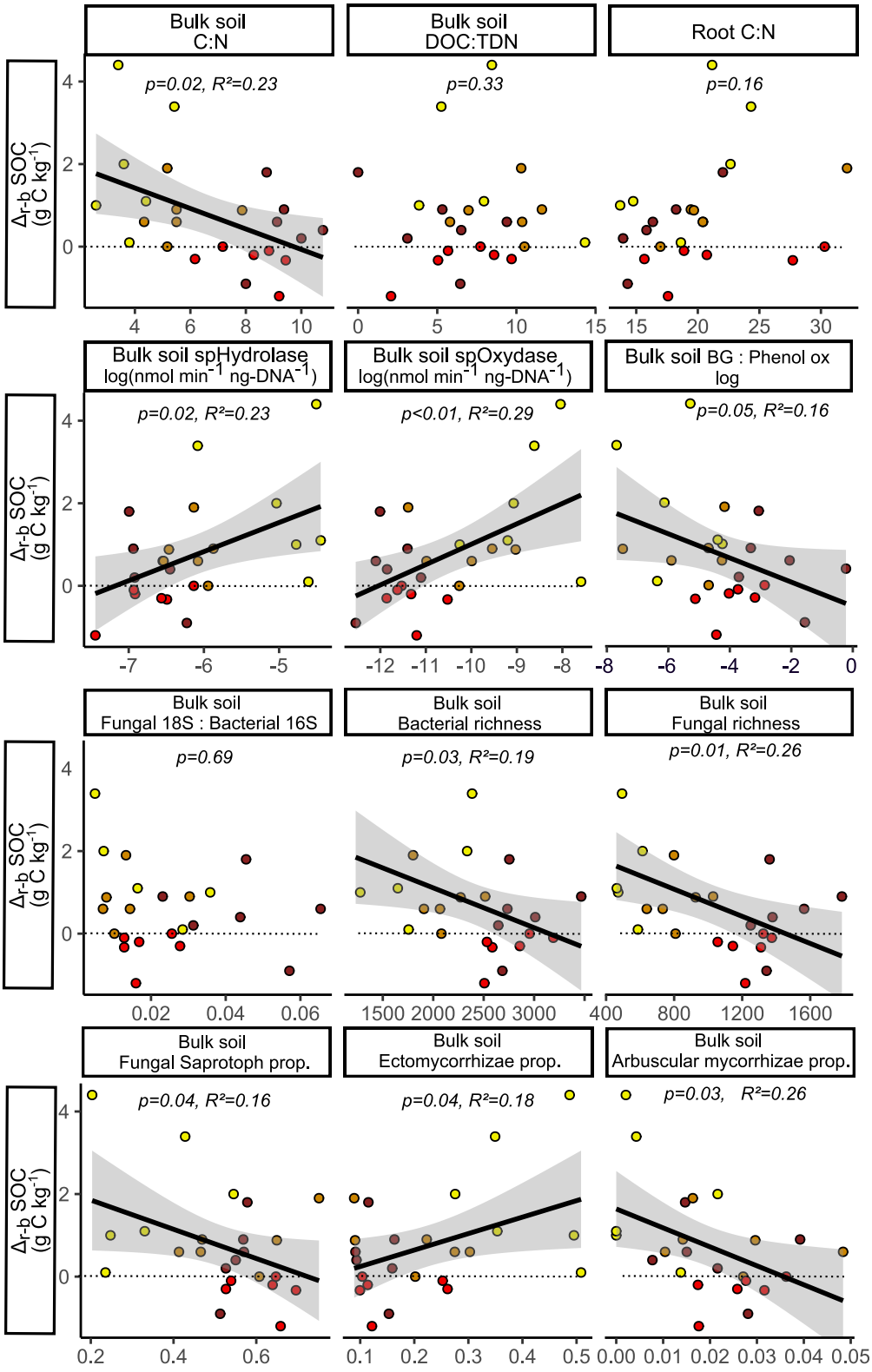

Fig. 4. Correlations between the $\Delta_{r\text{-}b}$ SOC (difference between the rhizosphere and bulk soil for each horizon of each pit) and the conditions of the bulk soil where roots were not present. Variables were selected a priori for these correlations based on their associations with organic matter stoichiometry, the chemical complexity of substrates preferentially decomposed by microorganisms, microbial community diversity and functional composition. Yellow points (horizon 0.0–0.3 m), orange points (horizon 0.3–0.5 m), light red points (horizon 0.5–1.0 m), and dark red points (horizon 1.0–1.5 m). The black line represents the slope of the correlation, and grey shading represents the confidence interval, which is presented only the variables that were significantly correlated with the $\Delta_{r\text{-}b}$ SOC (see the associated p value and R^2 on the plots). Prop. = proportion of the guild in the fungal community, BG: phenol ox = ratio of betaglucosidase: phenol oxidase activities. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

both favourable and unfavourable conditions for SOC sequestration can occur, even within the same soil profile, when different subsoil horizons are considered. To date, only one previous study has documented such variation in the effect of trees with depth in temperate agroforestry systems (Upson and Burgess, 2013). Overall, these results highlight the importance of integrating deep soil horizons and their rhizospheres when studying SOC in ecosystems integrating trees (Cardinael et al., 2025) or deep-rooted crops (Thorup-Kristensen et al., 2020).

The comparability of rhizosphere effects observed under different conditions or in different studies remains a key issue in rhizosphere research (Phillips and Fahey, 2006; Finzi et al., 2015). In our case, we measured the root mass density (RMD_{rhizo}, g roots per g of rhizosphere) and the root length density (RLD_{rhizo}, mm roots per g of rhizosphere) between horizons within the rhizosphere samples to facilitate these comparisons. The decrease in RLD_{rhizo} with depth indicated that the rhizosphere effect was more diluted in rhizosphere samples at depth compared with in the topsoil. This result strengthens our findings described above. Even though more diluted, greater rhizosphere effects were observed at depth. We argue that beyond the present study, systematically measuring and reporting such observations in rhizosphere studies represents a pathway to improve the comparability and scalability of rhizosphere research.

Measuring the root density in rhizosphere samples facilitates the upscaling of rhizosphere effects to the soil profile level, a critical advancement for assessing the net consequences of trees on climate change mitigation (Finzi et al., 2015). This study proposes an approach to upscale rhizosphere effects based on simple measurements of root density in rhizosphere samples and across the soil profile. With this rhizosphere sampling strategy, the effects of relatively young trees on the SOC stock within the subsoil rhizosphere were successfully detected and quantified. Agroforestry studies have generally compared bulk soil SOC stocks under agroforestry and control crop fields and demonstrate SOC sequestration in the topsoil but not in the subsoil (Cardinael et al., 2017). Taken together with our results, these findings indicate that changes in the SOC content in the topsoil under agroforestry trees are widespread, as opposed to a restricted response to the rhizosphere in the subsoil. This result can be attributed to the substantial C input received in the topsoil from both aboveground and belowground C inputs (Cardinael et al., 2018) combined with increased root and biological activity that homogenizes the localized root influence. In the subsoil, C inputs are primarily derived from roots, while C that moves from the topsoil through lixiviation or bioturbation can also be of varying importance (Kaiser and Kalbitz, 2012; Braakhekke et al., 2013). These root-derived C inputs decrease sharply with decreasing root biomass at depth, and their influence becomes highly diluted and challenging to detect when only the bulk soil is considered; however, these inputs result in a significant increase in the SOC content in the rhizosphere (Fig. 3). Indeed, our estimates indicated that the tree rhizosphere volumes (operationally defined by our sampling process) were restricted to 3–4.6 % of the total soil volume in the 0.3–0.5 m layer, 1.3–2 % in the 0.5–1.0 m layer and less than 1 % in the deepest horizon at a depth range of 1.0–1.5 m (Table 2). In these small fractions of the soil, estimated SOC balances ranged from -36 kg C ha^{-1} to $+56$ kg C ha^{-1} in the different soil horizons. These findings supported our hypothesis that the lower root biomass in the deepest horizon was compensated by the greater enrichment of SOC in its rhizosphere.

Upon scaling to the level of the whole soil profile, rhizosphere SOC enrichment represented an average net balance of $+50.6$ kg C ha^{-1} in the tree plantation and $+72.4$ kg C ha^{-1} in the tree row in the agroforestry system, but these SOC balances did not differ significantly. These values may appear relatively modest compared with the SOC sequestration rates estimated for the bulk topsoil of agroforestry systems. For instance, Cardinael et al. (2017) estimated SOC sequestration rates between 200 and 2000 kgC/ha/yr across 5 agroforestry sites in France. While lower than SOC sequestration in the topsoil, SOC sequestration in the subsoil is expected to persist for a longer time (Button et al., 2022). In addition, the agroforestry system studied here is composed of young (5 years old) trees. The deep root system of the trees was not fully developed, with an average fine root-derived C biomass in the subsoil horizons of 218.5 kg C ha^{-1}. The biomass of black locust roots in the subsoil can increase over several decades (Guo et al., 2022), reaching several tons of roots per ha according to former reports in the subsoil of deep loess, which is highly favourable for deep rooting (Lihui et al., 2021; Ma et al., 2024). In the same pedoclimatic area as the site examined in this study, a 13 years older agroforestry system planted with walnuts presented three times greater fine root biomass at 30–150 cm below the tree row (Cardinael et al., 2018). Hence, it is reasonable to anticipate that the SOC stock associated with the subsoil rhizosphere in this study will increase with tree age. It should be noted that our study focused on the rhizosphere of the trees and did not consider the rhizosphere of the herbaceous perennial and annual species growing in the understory vegetation strip. These herbaceous plants have a shallower rooting distribution, but their rooting density below 0.3 m (0.72 m dm^{-3}) remains on a similar order as that of the trees (0.945 m dm^{-3}). It can even be larger, as reported by Battie-Laclau et al. (2020), in a slightly older agroforestry system with walnut trees. For a comprehensive assessment of the ecosystems, characterization of the rhizosphere of the understory vegetation strip and the crops would have also been necessary. Overall, our study presents an innovative approach capable of providing an early estimate of SOC enrichment in the rhizosphere of young trees.

A discussion of the timing of SOC enrichment observed in the rhizosphere at depth might also be interesting. While this parameter cannot be definitively resolved with our sampling, preliminary calculations suggest that it did not occur only during the lifespan of the sampled roots, especially for the values observed in the 1.0–1.5 m horizon. Indeed, the SOC enrichment in the rhizosphere at the 0.50–1.0 m soil horizon is approximately the same as that of the tree root-derived C (Table 2), whereas it is 2.4-fold (TP) and 3-fold (AF) greater at depths of 1.0–1.5 m. These values are 2–6 times greater than those reported in the literature on a yearly basis. From labelling experiments, the average net C rhizodeposition (i.e., the rhizodeposited C not respired and remaining in the soil) has been estimated to be 0.5 times the root-derived C production for trees (Pausch and Kuzyakov, 2018). When black locust exudates were collected and incubated, a net C exudation between 0.3 and 0.4 times the root C content was obtained by Uselman et al. (2000), but these estimates did not include processes beyond exudation (e.g., root cap and border cells, mucilage, root hair, etc.) that are also involved in rhizodeposition (Brunn et al., 2025). Although the rhizodeposition rate can change with tree species, soil depth and pedoclimatic conditions (Brunn et al., 2022), a reasonable hypothesis is that the high degree of SOC enrichment that we observed around roots in the deepest horizons also included the C that accumulated before the present-day roots. Thus, the multiple uses of the same biopores by roots in the highly dense deep soil horizons are likely (see Fig. 1D and (Kuzyakov and Razavi, 2019)), is associated with the sequential release of C from multiple roots through their rhizodeposition and mortality. Moreover, a recent study indicated that recurrent root C inputs and associated SOC enrichment in subsoil can result in a decrease in the sensitivity of deep SOC to the priming effect (Schiedung et al., 2023). This phenomenon may have occurred in the deep biopores and could have promoted rhizosphere SOC enrichment. The large rhizosphere hotspots observed at depth were thus likely the cumulative effect of past and present-day rhizospheres.

4.2. *Deeper tree rooting and rhizosphere SOC in tree plantation systems than in agroforestry systems*

Differences in rooting depth in mixed species systems are frequently attributed to plant–plant competition for water and nutrients (Berendse, 1979). In the same Mediterranean pedoclimatic zone, a previous study reported shallower rooting in a tree plantation of walnuts than in agroforestry, a response attributed by the authors to intense competition

with winter wheat and reduced competition with perennial herbaceous species found in the tree plantation (Cardinael et al., 2015). While our hypothesis predicted a comparable response, the observed results revealed the opposite, with shallower rooting for the trees in the AF plots than for those in the TP plots. Competition for water and nutrients between trees might provide a potential explanation. The TP had a higher tree density at the plot scale, which could lead to deeper rooting in some contexts (Puri et al., 1994; Postma et al., 2020; Feng et al., 2025; Li et al., 2025). The higher tree density at our site than that reported by Cardinael et al. (2015) might explain the stronger role of tree–tree interactions and could be reinforced by agricultural practices in the crop alley that can promote shallower rooting in the AF system. Fertilization provides an additional source of nutrients, nitrogen in the present case, and the discontinuous vegetation cover allowed water and nutrients to be available for part of the year such that trees can use them instead of foraging in deeper layers. Tillage operations also cut some of the tree roots in the topsoil, thereby stimulating their reiteration in the surface layer (Schroth, 1995). Such differences in rooting patterns can have consequences for SOC when different levels of enrichment and depletion of rhizosphere SOC occur depending on the soil horizon, as in the present study (Table 2). Our study thus revealed that differences in rooting depth and the associated rhizosphere SOC content between trees in agroforestry and plantation systems are system dependent.

4.3. Constraints on saprotrophs explained the rhizosphere effect on the SOC content

Consistent with our hypothesis, the higher SOC content in the rhizosphere of subsoil horizons coincided with more favourable substrate stoichiometry and chemical complexity, promoting the dominance of copiotrophic microbial communities. The general improvement in resource availability for microorganisms is first evident from the multiplication by 7 (fungi) and 5 (bacteria) in terms of their abundances in the deepest rhizosphere (qPCR), while the SOC content increased only 2-fold relative to that in the bulk soil. Reduced constraints on saprotrophs were also supported by the ratio of hydrolase to oxidase enzymatic activities, which decreased with soil depth but increased in the rhizosphere at depth. This ratio is an indicator of the change in the chemical complexity of the organic C available for microorganisms (Sinsabaugh and Shah, 2011), with lower values in the subsoil indicating that microorganisms must invest more in oxidative enzymes that target complex molecules rather than targeting energy-rich polysaccharides through hydrolases (Takriti et al., 2018). Our observations in the subsoil indicated that roots shifted this ratio towards greater acquisition of energy-rich polysaccharides derived from plants, thereby indicating more favourable conditions for microbial C acquisition. This finding aligns with the variation in the soil C:N ratio and with the increased abundance of saprotrophic fungi compared to ectomycorrhizal fungi. In the bulk soil at deep horizons, we indeed observed a low C:N ratio and low abundances of saprotrophs relative to those of ectomycorrhizal fungi, which is indicative of highly microbially processed organic matter, with poor quality for saprotrophs (Shahzad et al., 2018; Chang et al., 2024), hence favouring mycorrhizae that can obtain C directly from their host plant tissues (Carteron et al., 2021). In the rhizosphere of the subsoil, an increase in soil C:N and saprotroph abundance reflects the release of easily degradable C-rich compounds by roots. Along with the increase in the rhizosphere C:N ratio, these inputs of fresh compounds can increase microbial carbon use efficiency (Manzoni et al., 2012; Takriti et al., 2018), which is a key parameter for the SOC balance (Tao et al., 2023). Hence, roots indirectly promote a positive SOC balance in the subsoil through their positive effect on the accumulation of microbial biomass and further stabilization of minerals (Liang et al., 2017; Sokol et al., 2022). Such a positive balance indicates a limited rhizosphere priming effect at depth, a response that we also observed in incubations of the same soil as long as nutrients remain limiting (Siegwart et al., 2023). The available N and P contents were low at depth, although the soil C:N ratio was lower (Fig. S3–4), indicating the limitation of both C and nutrients in this subsoil (Siegwart et al., 2023). In summary, rhizosphere priming did not appear dominant in the deepest horizons, characterized by high clay content, low nutrient availability, chemically complex native SOC, and a very low abundance of native saprotrophs. In these deep horizons, the microbial community around roots became largely dominated by taxa using fresh carbon inputs rather than native SOC (Figs. 3 and 4). In the upper part of the soil profile, the neutral or negative SOC balance seems to be explained by a higher root density and more easily degradable SOC combined with higher abundances of saprotrophs, increasing the sensitivity to priming. Collectively, our results suggest that the variation in soil nutrient contents, the degradability of the substrates available for microorganisms and the associated functional composition of the microbial community with depth strongly influence the rhizosphere SOC balance.

4.4. Rhizosphere- and profile-scale SOC gradients were strongly associated with specific microbial taxa and guilds

The observed effect of roots on SOC quantity and the shift in the complexity of substrates preferentially decomposed by microorganisms were also strongly associated with the selection of specific taxa among bacteria and fungi. The rhizosphere induced a more pronounced shift in the microbial community composition at greater depths, which is consistent with the response of the SOC quantity, enzymatic activities and microbial abundance described above. We observed that bacterial phyla and genera known for their preference for high C and nutrient availability (copiotrophs) were predominantly associated with the rhizosphere, whereas oligotrophs adapted to resource-limited conditions dominated the bulk soil, especially at depth (Fierer et al., 2007).

Among bacteria, the strongest association with root presence was observed for Proteobacteria, a phylum that is often associated with copiotrophic strategies and the rhizosphere (Fierer et al., 2007; Ho et al., 2017), including the rhizosphere of black locust (Fan et al., 2023). Of the six bacterial genera that exhibited a clear preference for the rhizosphere in the subsoil, 4 belonged to Gammaproteobacteria (Massilia, Variovorax, Azoarcus, and Xanthomonadales), all of which are known to be associated with plants (Han et al., 2011; Ofek et al., 2012; Fernández et al., 2014; Kwaśna et al., 2021). Opposite patterns were observed for the GAL15 and Methylomirabilota phyla, which appeared to be favoured when roots were absent. The increase in the abundance of the phylum Methylomirabilota with depth was driven by one OTU belonging to the Rokubacteria class. It represented 1 % of all 16S sequences in the topsoil and up to 10 % in the deepest soil horizon. Rokubacteria and GAL15 are both understudied groups of bacteria that were recently added to the bacterial tree of life and appeared to be characteristic taxa of oligotrophic deep soil environments (Becraft et al., 2017; Brewer et al., 2019; Frey et al., 2021; Feng et al., 2025) that were thus adapted to survive through the use of organic matter characterized by high chemical complexity.

With respect to fungi, the response of the fungal community to root presence seemed to be strongly related to their guild. The abundance of the phyla Mortirellomycota (96 % of the genus *Mortierella*) and Kickxellomycota (83 % of the genus *Ramicandelaber*) decreased with depth but increased in the rhizosphere, particularly in the subsoil. Both groups are recognized as saprotrophitic fungi associated with fresh organic matter (Poll et al., 2010; Hellequin et al., 2018; Ozimek and Hanaka, 2020). *Ramicandelaber* has previously been linked to an increase in the SOC content at a 0.1–0.4 m depth, which is consistent with our observations, a pattern that authors linked to higher fungal necromass C (Yang et al., 2022). In contrast, Basidiomycota showed the opposite pattern, increasing in abundance with depth but decreasing in abundance in the rhizosphere. This pattern was largely driven by the ectomycorrhizal genus *Tylospora*, which represented half of the sequences in this phylum. Since black locust is considered a nonectomycorrhizal species (Tedersoo and Brundrett, 2017), these ectomycorrhizal fungi

might result from legacy effects of past colonization events and the persistence of dormant ectomycorrhizal spores, which are known to remain viable for decades (Nguyen et al., 2012; Louise et al., 2024). However, ectomycorrhizal fungi may associate with black locust roots through the formation of ectendo- or ectomycorrhizal structures under certain conditions (Harley and Harley, 1987; Bratek et al., 1996). Recent evidence suggests that the boundaries between fungal guilds are more porous than previously thought (Selosse et al., 2018). Notably, although the relative abundance of ectomycorrhizal Basidiomycota in the deep rhizosphere decreased by ~50 %, the total fungal abundance (measured as 18S rRNA gene copies) increased fivefold. In other words, even though the absolute abundance of ectomycorrhizal fungi increased, their relative proportion decreased because saprotrophic fungi responded even more strongly to root-derived carbon inputs, shifting the overall community composition. These findings indicate that the subsoil rhizosphere functions as a microbial hotspot that favours most microorganisms, particularly copiotrophic saprotrophs. In contrast, bulk subsoil—characterized by a low organic matter content and chemically complex substrates for microorganisms —represents a harsh environment that mainly sustains dormant or oligotrophic microorganisms. Overall, our results provide rare observations informing on the microbial taxa and guilds closely associated with SOC gradients at the rhizosphere and soil profile scales.

5. Conclusions

Increased SOC sequestration in the subsoil through the deep roots of trees is increasingly advocated as a potential strategy to mitigate climate change. However, field studies that investigate the rhizosphere at depth and quantify its role for SOC storage are lacking. This study presents an innovative approach integrating standardized rhizosphere sampling and whole-profile mapping of root density in a Mediterranean agroforestry system and tree plantation. Deeper tree roots and rhizosphere distributions were observed in tree plantation compared to agroforestry system, contrasting observation in other temperate system. Subsequently, SOC balances were estimated at the scale of the rhizosphere, the soil horizon and the whole soil profile. Across depths, both the depletion and enrichment of SOC were observed in the rhizosphere, stressing the need to understand the mechanisms driving SOC dynamics. The most substantial SOC enrichment in rhizosphere was observed in the deepest horizon, supporting their higher potential for root-derived C sequestration. Finally, microbial analyses revealed that root presence in the deepest horizons triggered a substantial shift in community structure: abundant copiotrophic communities reliant on root-derived C inputs outcompeted native and rare oligotrophic microbial populations adapted to chemically complex substrates. This shift ultimately favoured SOC enrichment over depletion. This study presents a scalable approach to assess the potential of deep roots for SOC sequestration. The principles outlined here are applicable to a wide range of soil types and have the potential to facilitate rhizosphere research providing more *in situ*, comparative and quantitative insights.

CRediT authorship contribution statement

Gabin Piton: Writing – original draft, Visualization, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Elisa Taschen:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Clara Ducrocq:** Writing – review & editing, Investigation, Formal analysis. **Soline Martin-Blangy:** Writing – review & editing, Supervision, Methodology, Investigation, Formal analysis, Conceptualization. **Laurie Amenc:** Writing – review & editing, Supervision, Resources, Methodology, Investigation. **Pauline Castel:** Writing – review & editing, Investigation. **Damien Dezette:** Writing – review & editing, Resources, Methodology, Investigation. **Rémi Dugué:** Writing – review & editing, Resources, Methodology, Investigation. **Marion Forest:** Writing – review & editing, Resources, Methodology, Investigation. **Philippe Hinsinger:** Writing – review & editing, Funding acquisition. **Benoit Marie:** Writing – review & editing, Investigation. **Aline Personne:** Writing – review & editing, Resources, Methodology, Investigation, Conceptualization. **Manoël Seignon:** Writing – review & editing, Investigation, Formal analysis. **Jerôme Ngao:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Christophe Jourdan:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization. **Isabelle Bertrand:** Writing – review & editing, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2026.110103.

Data availability

Data presented in this study, and sequence accession numbers, are available on github: https://github.com/GabinPiton/Subsoil-rhizosphere-carbon-enrichment-and-depletion-processes-and-scaling-in-tree-based-systems.

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