RESEARCH ARTICLE

Root distribution and properties of a young alley-cropping system: effects on soil carbon storage and microbial activity

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Abstract

Purpose In agroforestry systems, the root distributions and properties of the annual and perennial vegetation are poorly known, although they are recognized for fostering soil carbon (C) stocks through annual root-derived C inputs and by altering microbial activity. This study aimed to evaluate the potential contribution of roots from the understory vegetation strip (UVS) and the crop to top- and subsoil C stocks (0–100 cm) to a 3-year-old agroforestry system of the alley-cropping type.

Methods Root biomass, chemical composition, functional traits and anatomical structure were assessed in parallel to a characterization of soil physicochemical

and microbiological properties, including enzymatic activity, at different depths and locations perpendicular to the tree line.

Results The root biomass and properties were heterogeneous in the young alley-cropping system due to the presence of different plant communities and the heterogeneity of the soil mineral N content according to the location perpendicular to the tree line. The soil microbiological properties and organic C stocks did not vary horizontally at this stage of agroforestry but should be monitored through multiple-time samples to confirm a differentiation in subsequent years suggested by the tight link between root stoichiometry and microbial extracellular enzymatic activities that we found.

Conclusions Altogether, our results suggested that increasing the root biomass in topsoil in agroforestry systems positively contributes to increasing soil organic C stocks, but in deeper soil layers, an increase in litter inputs with a high C:N ratio might accentuate microbial N limitations and limit soil C storage.

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Keywords Agroforestry · Soil organic carbon stocks · Root functional traits · Root-derived carbon inputs · Soil enzymatic stoichiometry

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Abbreviations

UVS	Understory vegetation strip
C	Carbon
N	Nitrogen
P	Phosphorus

CaCO$_3$	Calcium carbonate
NH$_4^+$	Ammonium ions
NO$_3^-$	Nitrate ions
HNO$_3$	Nitric acid
MB C and N	Microbial biomass carbon and nitrogen
BG	β-1,4 Glucosidase
CBH	Cellulobiohydrolase
NAG	β-1,4-N-acetyl-glucosaminidase
LAP	Leucine aminopeptidase
AP	Alkaline phosphate

Introduction

Agroforestry systems are increasingly recognized for their soil C sequestration potential (Kaur et al. 2000; Sharrow and Ismail 2004; Cardinael 2015). The alley-cropping agroforestry system encompasses diverse root systems due to the simultaneous presence of three types of vegetation: trees planted in lines, the understory vegetation strip in the tree lines (UVS) and the crop in the inter-rows (Torquebiau 2000; Boinot et al. 2019; D'Hervilly 2021). Three plant communities (i.e., vegetation cover type) would result from species cohabitation in these systems: UVS + tree in the tree line, crop + UVS at the interface between the crop and UVS, and the crop in the inter-row. At the community scale, these root systems might differ in their properties, encompassing root chemical quality (C, N, P, lignin, cellulose, hemicellulose, and soluble compound contents), dynamics (growth rate and turnover) and average functional traits (specific root length, root tissue density and diameter). Indeed, the trade-off between resource acquisition and conservation would control plant tissues, resulting in differences in the root systems at the community level that operate within all root types (Prieto et al. 2015). The spatial heterogeneity of root and soil properties suggest different mechanisms of soil C dynamics including soil C sequestration at the plant-community scale. However, characterization of these differences in alley-cropping systems and especially in deep soil layers is still lacking, despite their higher soil C sequestration potential (Tautges et al. 2019) with longer C turnover times (Balesdent et al. 2018) compared with the topsoil (0–30 cm), which is mainly composed of younger, fast cycling organic matter. The tree and herbaceous roots in the UVS, which is dominated by perennial species,

might have a higher root biomass density, with roots colonizing deeper soil layers than the annual crop roots (Beniston et al. 2014; Ferchaud et al. 2015; Duchene et al. 2020). Furthermore, perennials use a conservative strategy, compared to annual plants, preferring soil exploration for better resource absorption with greater specific root length (root length per root mass) and root N concentration and lower root tissue density (Roumet et al. 2006; Hummel et al. 2007; Weemstra et al. 2016).

The heterogeneity of the root systems (distribution, biomass and properties) at the communities level in alley-cropping systems would induce a heterogeneity of the root contribution to soil C storage (i) as direct inputs of root-derived C through rhizodeposition and root mortality (Bolinder et al. 1997; Pausch and Kuzyakov 2018) since root quality (recalcitrance) controls root litter decomposition kinetics (Bonanomi et al. 2021) and subsequently induces the formation of stable soil organic matter at a level 2 to 5 times greater than aerial plant parts (Balesdent and Balabane 1996; Rasse et al. 2005; Lin et al. 2020; Villarino et al. 2021). Additionally, the heterogeneous root contribution to soil C storage (ii) is due to the effect on soil microbial activity, since the plant, soil and microbial (including enzymatic) C:N:P ratios respond positively to increasing plant diversity (Chen and Chen 2021). The root system diversity in agroforestry systems also has consequences for the temporal dynamics of root-derived C inputs that occur mainly with the death of plant roots at each crop harvest (Carvalho et al. 2013; Hirte et al. 2018) and through root turnover for the perennial species of the UVS and trees (Houde et al. 2020). Alley-cropping systems have modified environmental conditions compared to conventional cropping systems (Batish et al. 2007; Garrett et al. 2021) that may induce further spatial heterogeneity of the root biomass and root properties of plant communities (Deyn et al. 2008). Perpendicular to the tree line, gradients of soil properties (Guillot 2018; D'Hervilly 2021), competition for light (Dufour et al. 2013; Querné et al. 2017; Zhang et al. 2019) and water acquisition *vs*. redistribution (Livesley et al. 2004; Bayala and Prieto 2020) have been observed. Indeed, the crop yield was often shown to be reduced near the tree or hedge rows (Singh et al. 1989; Eastham and Rose 1990; Lawson and Kang 1990; Miller and Pallardy 2001), despite the improved soil qualities at these locations (Guillot et al. 2021). Furthermore, at the UVS-crop interface,

hydraulic constraints and nutrient shortage conditions may occur due to interspecific competition (Cardinael et al. 2015; de Parseval et al. 2017; Battie-Laclau et al. 2020) induced by the colonization of the crop zone by UVS roots (Battie-Laclau et al. 2020). In response to these constraints, crops at the vicinity of the UVS could, for example, modify their root functional traits by increasing the diameter or increasing the specific root length to improve absorption capacity and soil exploration, respectively (Pradier 2016; Duan et al. 2019). However, the few studies observing differences in soil and root properties between alley-cropping and conventional cropping systems analyzed mature agroforestry systems (Udawatta et al. 2014; Cardinael et al. 2017; Dufour et al. 2020). To our knowledge, no study has assessed period required for the root horizontal gradient to be established in agroforestry systems, while few studies have shown modifications of the soil properties starting 5 years after the introduction of trees (Nyberg and Högberg 1995; Wang et al. 2005). A better understanding of the root system growth and distribution is necessary to fully understand the mechanisms underlying the spatiotemporal variation in C stocks in agroforestry.

The vertical variations in soil physical and chemical properties (Hartemink et al. 2020) also affect root development and root properties (Eissenstat et al. 2000; Konopka et al. 2009; Hodge et al. 2009; Freschet et al. 2017), and the different components of alley-cropping systems might respond differentially to this vertical variation. In the crops of the agroforestry system, vertical variations in the crop root system depend only on the crop phenotypic plasticity that might involve a response to competition with the perennial vegetation at the border of the inter-row (Duan et al. 2019; Battie-Laclau et al. 2020). On the other hand, root system variations along the soil profile in the perennial part of the agroforestry system are due to a combination of individual root system plasticity and the vertical organization of the different plant species composing the perennial community (Milchunas and Lauenroth 1989; Aerts 1999; Prieto et al. 2015). In the literature, studies analyzing the relationships between soil and root properties across the soil profile are mainly conducted on tree plantations (Makita et al. 2011; Tückmantel et al. 2017; Germon et al. 2020). Wang et al. (2016) and Prieto et al. (2015) compared topsoil roots with those found in deeper layers and showed that deep roots

had a larger diameter for better soil penetration, less N because of less available nutrients at depth, and larger and more numerous xylem vessels for better hydraulic conductivity. In contrast, the finer textured soil that is sometimes observed at depth is characterized by a smaller pore size and thus induces a smaller root diameter (Castle and Krezdorn 1979), while the soil bulk density favors thicker and denser fine roots (Freschet et al. 2017). An understanding of the vertical response of root systems in alley cropping is essential for understanding their potential for C sequestration in the subsoil.

The root systems directly (as C inputs) and indirectly (by controlling microbial decomposing activity) affect the soil C stocks (Smith et al. 2014; Poirier et al. 2018; Freschet et al. 2021). As the root properties might be heterogeneous in agroforestry systems (due to the presence of several plant communities with different strategies and the effects of soil physicochemical properties) along vertical and horizontal axes, our study aimed to investigate vertical and horizontal variations in the roots, soil and microbiological characteristics associated with CNP cycles along the rooted soil profile (up to a depth of 100 cm) in a young alley cropping agroforestry system from a Mediterranean climate to evaluate the potential contribution of roots to soil organic C stocks.

We first hypothesized that the response of root properties to depth (and the associated vertical gradient of soil physicochemical properties) differs between perennial (UVS) and annual (crop) plant communities and at the interface of the perennial-annual communities' interface in an agroforestry system. More precisely, we hypothesized that perennial UVS has a deeper root system, uses a conservative strategy (lower specific root length and a higher root C:N ratio) and exhibits a more important variation of root properties than the annual crop. We expected this depth response to be characterized by changes in root properties indicating a shift from exploitative nutrient acquisition function (high nutrient content) in the shallow, nutrient-rich layer to conservative water acquisition and transport functions in deeper, nutrient-poor layers (larger diameter and higher lignin content). For the crop, we expected the root response to depth to be more pronounced at the interface with the UVS as a response to interspecific competition.

Second, we hypothesized that the horizontal variations (i.e. under the different plant communities perpendicular to the tree line) in the root biomass and properties would (i) induce a horizontal differentiation of the soil microbial activity that would already be visible after 3 years of agroforestry and (ii) show an initial differentiation of the soil organic C stocks across the agroforestry system, with an increased microbial biomass, activity and soil C stock under perennial UVS and at the interface between the crop and UVS. Moreover, we expected these horizontal differences to be greater at depth, where roots are the principal source of soil organic matter.

We sampled and analyzed roots and soils in a 3-year-old alley-cropping system with a Mediterranean climate to test these hypotheses. The sampling design considered 3 locations in a horizontal gradient perpendicular to the tree line (UVS, crop near the UVS and crop far from the UVS) and 3 soil depths up to 100 cm. Average root properties of plant communities were assessed and related to microbial and soil properties to assess the effects of the plants on ecosystem functioning (Lavorel and Garnier 2002; Legay et al. 2014).

Materials and methods

Study site

The "Dispositif Instrumenté en Agroforesterie Méditerranéenne sous contrainte hydrique" (DIAMs) is located 10 km south of Montpellier (France) under a Mediterranean climate (43.612°N; 3.976°E). The soils are classified as Skeletic Rhodic Luvisols (IUSS Working group WRB 2014), according to the high proportions of stones (up to 60%), a pronounced red color and a layer deeper than 100 cm with accumulation of clay. The following layers were identified: Ap1: plow layer with a lumpy texture (0–20 cm), Ap 2: second plow layer (20–50 cm), Bt 1: illuvial layer with lattice clays and stones (50–80 cm), Bt2: illuvial layer with lattice clays without stones (80–125 cm) and IICk: bedrock with calcium carbonates (Fig. 1b). Before setting the agroforestry system (2017), the annual crops were cereals (wheat and alfalfa).

This agroforestry site of 5 ha is divided into three blocks separated by several hundred meters and representing independent replicates. The alley-cropping system was planted in 2017 with black locust (*Robinia*
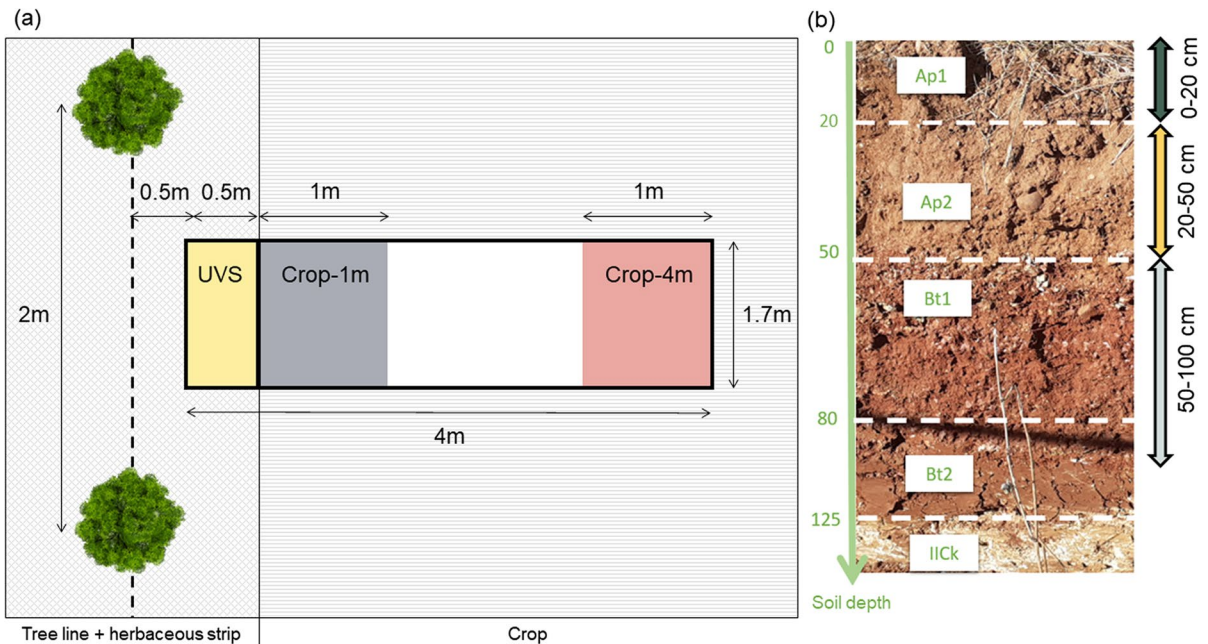

Fig. 1 Aerial view of the pit (a) with the three types of vegetation (trees, UVS and crop) and of the soil profile with the identified soil layers (Skeletic Rhodic Luvisols) according to depth (from 0 to 165 cm of depth) and the 3 studied soil depths (0–20, 20–50 and 50–100 cm)

pseudoacacia), a N-fixing species, in 2-m-wide rows covered with an understory vegetation strip (UVS). The herbaceous species sown in 2019 (*Festuca arundinacea, Dactylis glomerata, Medicago sativa* and *Trifolium pratense*), however, were only scarcely present. Instead, some of the common species found in the UVS were *Lolium arundinaceum*, *Bromus*, *Papaver rhoeas*, *Crepis sancta*, *Sonchus arvensis, Lolium perenne*: community of plurennial plants. The trees were planted every 2 m on the rows, leading to a density of 294 trees ha^{-1}, and were intercropped with 17 m crop alleys made of rotations of cereal and legume (wheat/barley/pea) crops. Beginning in October 2019, the soil was plowed to 20 to 50 cm. At the end of October 2019, durum wheat (*Triticum turgidum L.* subsp. *durum*) was sown as a winter crop with a sowing density of 140 kg ha^{-1}. A phytosanitary treatment (Atlantis Pro) was applied on January 30, 2020. Fertilization campaigns were conducted on February 6, 2020, with Nexens 46 fertilizer (60.0 kg N ha^{-1}) and on the April 11, 2020, with Smart N 46 fertilizer (80.0 kg N ha^{-1}). Crop harvest occurred on June 25, 2020, and straw was left on the topsoil.

To evaluate the homogeneity of N fertilizer spread, 3 pseudoreplicates × 3 blocks = 9 replicated cups with a diameter of 19.5 cm were set in the crop at 1.5 and at 4 m from the tree line. Immediately after fertilizer application, the granules were collected in the cup, weighed, and reported on a per m^2 basis.

Sampling strategy

Sampling started on May 19, 2020, after 3 years of agroforestry. It corresponded to the flowering stage of the wheat and herbaceous species in the UVS. The tree DBH (diameter at breast height) was 4.3 ± 1.2, 4.0 ± 1.3 and 3.9 ± 0.9 cm in blocks 1, 2 and 3 respectively. The tree height was 338 ± 65, 323 ± 72 and 327 ± 56 cm in blocks 1, 2 and 3 respectively (measured in January 2021). One pit (4 m long × 1.7 m wide × 1.5 m deep) was dug per plot (3 replicates) with a backhoe. The pits were dug between two trees (spaced by 2 m) instead of at the base of a tree, perpendicular to the herbaceous strip and starting from 0.5 m from the middle of the tree row to the intercrop to focus on the interaction between the UVS and wheat in the transition zone (Fig. 1a). All pits were oriented toward the south, i.e., the sunny side of the trees. In addition to the vertical axis with 3 soil depths (0–20,

20–50 and 50–100 cm of depth) established according to the identified soil layers (Fig. 1b), the sampling strategy also considered a horizontal axis with three locations depending on the distance to the tree (Fig. 1a): the UVS (between 0.5 and 1 m from the tree line), the Crop-1 m (between 1 and 2 m from the tree line) and the Crop-4 m (between 3.5 and 4.5 m from the tree line). In summary, the sampling strategy was defined by 3 independent plots × 3 locations according to the distance from the tree line × 3 soil depths.

Just before digging the pits, aboveground biomass was sampled on each subplot and location in a 1 m^2 area. Samples were air-dried at 50 °C for 72 h and weighed, and values were reported on a per m^2 basis.

Root characterization

Root biomass

During the excavation of the pits, a backhoe bucket was used to sample a volume of 28 dm^3 of soil at each location and depth. All living roots were sorted manually from this volume, carefully washed on a 0.5 mm sieve, air-dried at 50 °C for 72 h and weighed. Knowing the volume of the backhoe bucket, the root biomass density (g dm^{-3}) was calculated for each location and soil depth ($n = 3$). Root dry biomass was reported on a per m^2 basis (g m^{-2}), and the proportion of root biomass in each soil depth (%) was calculated as the ratio of the root biomass in the soil depth to the total root biomass in the entire sampled soil profile.

Root mapping

For each pit and at each location, 4 replicated grids with a size of 50 × 100 cm were hung against the refreshed pit soil profile (Chopart and Siband 1999). The grids were composed of elementary tiles of 10 × 10 cm (5 in width and 10 in length). The number of root intersections in each elementary tile in the grids was manually counted. At each location, the root map was then established from depths of 0 to 100 cm and the mean value of the root impact density was calculated for the 3 blocks and the 4 pseudoreplicates ($n = 12$).

The root intersect method from Chopart and Siband (1999) was applied to analyze the root isotropy (preferred orientation of the direction of root growth). Two stainless steel cubes of

$10 \times 10 \times 10$ cm (1 dm^3) with opposite orientations were placed in 2 soil profiles (pseudoreplicates) of each pit and at each location. However, because the soil compaction at 50–100 cm was too high, it was not possible to use the cubes at that depth. The cubes were then extracted as intact as possible. On each of the 3 faces (H- perpendicular to the profile and parallel to the soil surface, T- parallel to the profile and perpendicular to the soil surface and L- perpendicular to the profile and to the soil surface), the root impact density was determined as the number of root intersections with the vertical plane; see Maurice et al. (2010) for details. The A coefficient was calculated as an indicator of the root isotropy (Chopart and Siband 1999):

$$A = \sqrt{\frac{\left(RID_T - \overline{RID}\right)^2 + \left(RID_L - \overline{RID}\right)^2 + \left(RID_H - \overline{RID}\right)^2}{6 \times \overline{RID}^2}}$$

where RID_i is the root impact density on face i and $\overline{RID}$ is the average root impact density on all faces of the cube. When A = 0, there is root isotropy, no specific orientation for root growth. When A = 1, the roots grow only in a preferred direction.

In total, 72 cubes were sampled with 2 cube orientations × 2 soil depths × 3 locations × 2 pit soil profiles × 3 pits. After determining the root impact density on the 3 faces of the soil cubes, the roots inside the soil cubes were carefully sorted, washed with water on a 0.5 mm sieve and stored at 4 °C prior to analyses.

While conducting the root mapping, few medium roots (diameters between 2 and 10 mm) were observed and identified as being from the UVS plants. Because they were so rare (less than 4 roots considering all pits), they were not considered in this study, which focused on fine roots (smaller than 2 mm in diameter). Indeed, fine roots include (i) 100% of the crop roots (which are all entering the soil compartment at harvest) and (ii) the roots of the herbaceous plants that are present due to root turnover and thus play a role in soil C inputs.

Root functional traits

Roots extracted from the cube sampling were spread out in a transparent bin with deionized water and scanned at 300 dpi with a scanner (Epson Expression © 10,000 XL). The resulting images were processed with image analysis software (WinRHIZO v. 2005b

Regent, Canada©) for each cube content, allowing us to determine the total root length (cm) and the mean root diameter (mm). Knowing the volume of the cubes, the root length density (cm dm^{-3}) was calculated. After the scans, roots from the cube sampling were air-dried at 50 °C for 72 h and weighed. The specific root length (m g^{-1}) was calculated as the ratio of fresh root length to root dry mass in each cube. Because the root traits strongly depend on the soil depth and plant species (Van noordwijk and Brouwer 1991), these functional root traits were measured in a pool of roots from different communities (UVS, Crop-1 m and Crop-4 m), as average root traits using the method reported by Legay et al. (2014).

Furthermore, the root biomass density obtained in the cubes at 0–20 and 20–50 cm was compared with the root biomass density obtained from the backhoe sampling as described above. A significant difference was not observed between the two methods (p-value = 0.12, data not shown). Only the results from the backhoe method were used for root biomass quantification.

Root chemical composition

Dry roots from backhoe sampling were used for the biochemical analyses because of the larger root biomass retrieved. C fractions (soluble compounds, cellulose, hemicellulose and lignin) were determined in a 500 mg root litter subsample with a fiber analyzer (Fibretherm®, Gerhardt) using the protocol described by Van Soest (Goering and Van Soest 1970). Root C and N contents were determined from 3 mg subsamples of root litter with an automatic elemental analyzer (Flash 2000, ThermoFischer Scientific). For the analysis of the total root P content, 50 mg of litter powder were mixed with 65% HNO_3 and mineralized for 15 min at 200 °C in a Milestones Ethos Easy microwave with a standard and blank. The total P content was then quantified colorimetrically with the yellow vanadomolybdate assay (NF U42-246). The C stocks in living roots (kg of C m^{-3} soil) were calculated by multiplying the root C content (%) by the root biomass density (kg m^{-3}). Several root quality indexes were calculated: the lignin:N ratio (Melillo et al. 1982), the soluble compounds:cell wall ratio (McClaugherty et al. 1985) and the ligno-cellulosic index (LCI, Melillo et al. 1989), which was calculated using the following equation:

$$LCI = \frac{lignin}{lignin + cellulose + hemicellulose}$$

where lignin, cellulose and hemicellulose are the corresponding root C fractions (%).

Root anatomy

Just after digging the pits, living wheat roots were sampled from the pit's wall at each soil depth and at 2 locations (Crop-1 m and Crop-4 m) and immersed in a mixture of formaldehyde, acetic acid and ethanol (Kladnik 2013) kept at 4 °C pending further preparation for anatomical observation. The roots were then fixed in a 25% glutaraldehyde and 10% paraformaldehyde solution in a phosphate buffer (0.2 Na, pH 7) with 1% caffeine and stored in a 70% ethanol solution at 4 °C (Salma 2015). Alcohol baths with increasing concentrations of absolute alcohol allowed gradual dehydration. Embedding of samples in resin was performed with a Technovit® 7100 kit. Samples were then cut into 4.5 μm sections with a manual Leica RM2255 microtome and mounted on a glass slide. Sections were then successively stained in periodic acid-Schiff's reagent (to reveal insoluble carbohydrates) and in naphthol blue black to reveal proteins (Buffard-Morel et al. 1992). Screening of the glass slides was performed automatically with a Hamamatsu NDP slide scanner (Hamamatsu Nanozoomer 2.0HT, MRI). Several measurements and counts were performed on 1 section×4 pseudoreplicated roots (for each location, soil depth and plot) with ImageJ Software (version 1.53e): total root area, stele area, number of metaxylem vessels, area of each metaxylem vessel and endoderm width. For each measurement, we calculated the mean value of the pseudoreplicated roots to analyze the root anatomy for each soil depth and each location ($n=3$).

Soil characterization

During the excavation of the pits, more than 5 kg of soil was sampled at each location and soil depth ($n=3$). The soil was immediately passed through a 2 mm sieve. Subsamples were stored at 4 °C to retain their field moisture prior to analyses, other subsamples were frozen, and others were dried at 65 °C for 72 h. The soil moisture content of all samples was determined using a gravimetric method after drying for 48 h at 105 °C.

Dry soils (dried at 65 °C for 72 h) were analyzed for total C and N contents using dry combustion (Matejovic 1997). Other dry soil subsamples were analyzed by the Laboratoire d'Analyse des sols (INRAE, Arras) to determine the following parameters: soil $CaCO_3$ content (Allison 1960), cation exchange capacity using cobalt hexamine (Ciesielski and Sterckeman 1997), soil pH in a 1:5 soil–water suspension, total P content (Ciesielski et al. 1997; Ivanov et al. 2010), available P content (Olsen 1954), organic P content and soil texture of five fractions, namely, clay, silt (fine and coarse) and sand (fine and coarse). The organic C content was calculated as the difference between the total C content and inorganic C content as measured in $CaCO_3$.

Mineral N was extracted from wet soils with a 1:4 soil–1 M KCl solution. NO_3^- and NH_4^+ contents were determined using continuous flow colorimetry (Continuous Flow Analyzer, Skalar), and the sum of NO_3^- and NH_4^+ contents represented the mineral soil N content. Dissolved organic C was extracted with a 1:5 soil-distilled water solution, and the dissolved organic C content was measured with a TOC/TN analyzer (TOC-Vsch-TNM SHIMADZU). The soil MBC (microbial biomass C) and MBN (microbial biomass N) were quantified with the chloroform fumigation-extraction technique (Vance et al. 1987). A correction factor of 0.45 was included.

In frozen soils, the abundance of the 16S and 18S rRNA genes was estimated by performing real-time quantitative polymerase chain reaction (q-PCR) of ribosomal DNA (Smith and Osborn 2009) using a thermal cycler (Bio-Rad, Hercules, California). The potential activities of 5 extracellular enzymes involved in the C, N and P cycles were measured using the protocol reported by Bell et al. (2013): β-1,4-glucosidase (BG), cellulobiohydrolase (CBH), N-acetyl-glucosaminidase (NAG), leucine aminopeptidase (LAP) and alkaline phosphatase (AP). The enzymatic activities were obtained in nmol g^{-1}_{soil} min^{-1} with a fluorometric microplate reader (Victor 3, Perkin Elmer, 365 nm excitation wavelength and 450 nm emission wavelength).

The soil bulk density was measured in the topsoil (0–20 cm) using a water method as it is suitable for stony soils (Jolivet et al. 2018). An excavation was

made from 0 to 20 cm. The soil was passed through a 2 mm sieve, dried at 105 °C for 48 h and weighed. The volume was determined by placing a plastic bag in the excavation hole and measuring the volume of water that could filled the bag. At deeper soil depths (20–50 and 50–100 cm), an imaging-sensor-based measurement method was used as a convenient method for stony soils (Coulouma et al. 2021). An excavation was made in the wall of the pit at depths of 20–50 and 50–100 cm. The soil was passed through a 2 mm sieve, dried at 105 °C for 48 h and weighed. The volume of the excavation was determined by three-dimensional imaging. For all the samples from depths of 0–20, 20–50 and 50–100 cm, the gross fraction (>2 mm) was washed with water, dried and weighed to obtain the mass of stones. The stone volume was measured on several stones immerged in a burette, and the stone density was calculated as the ratio of the mass of stones to the stone volume (2.13 g cm^{-3}).

Data analyses

In the cube sampling, the 2 cubes with opposite orientations taken next to each other were spatially correlated for each soil depth and location. Furthermore, all cubes from the same soil profile, location and depth were considered as pseudoreplicates. Consequently, we considered them all as one sample and used the mean value ($n=3$).

Using the linear regression coefficient of the relationship between the root length density and the root impact density (the average over 3 dimensions) obtained in each cube, we converted the root impact density map into a root length density map using the method described by Maurice et al. (2010).

The C stocks in the living roots were calculated by multiplying the root biomass density by the root C content for each location and depth ($n=3$). For the crop (at the Crop-1 m and Crop-4 m locations), all roots entered the soil after harvest, and 100% of the C stocks in living roots were thus considered as annual root-derived C inputs to the soil. In contrast, for the perennial herbaceous species in the UVS, the annual root-derived C inputs to soil were considered 53% of the C stocks in living roots based on an annual root turnover rate of 53% in the grasslands (Gill and Jackson 2000). This estimation method is conservative, as it did not take into account the release of C through rhizodeposition, which might still represent an important part of

additional annual C inputs, as C in the rhizodeposits represents approx. 3% of the plant gross primary production (Pausch and Kuzyakov 2018).

The C-enzymatic activity was calculated as the sum of BG and CBH. The N-enzymatic activity was calculated as the sum of NAG and LAP. The P-enzymatic activity was that of AP. The C-, N- and P-enzymatic activities were integrated over time (180 min) to obtain cumulative enzymatic activities (µmol g$^{-1}_{soil}$) or divided by the MBC to obtain biomass-specific enzymatic activities (µmol g$^{-1}_{MBC}$ min^{-1}). An eco-enzymatic stoichiometry analysis was conducted as described by Fanin et al. (2016). Briefly, the relative proportion of C- versus P- acquiring enzymatic activities (C/[C+P]) and the relative proportion of C- versus N-acquiring activities (C/[C+N]) were calculated (x and y respectively). Then, the length of the vector quantifying relative C versus nutrient acquisition was calculated as the square root of the sum of squared values of x and y [Length $=$ Sqrt(x^2+y^2)]. The angle of the vector quantifying the relative P versus N acquisition was calculated as the arctangent of the point (x, y) [Angle (degrees) $=$ Degrees(Atan^2(x, y))].

The soil bulk density was calculated as follows:

$$BD_{soil} = \frac{M_{soil}}{V_{excavation}}$$

where BD_{soil} is the soil bulk density (g cm^{-3}), M_{soil} is the total dry mass of the sample (g) and $V_{excavation}$ is the volume of the excavation (cm^3).

The fine soil bulk density was calculated as:

$$BD_{finesoil} = \frac{M_{finesoil}}{(V_{excavation} - \frac{M_{stones}}{D_{stones}})}$$

where $BD_{finesoil}$ is the fine soil bulk density (g cm^{-3}), $M_{finesoil}$ is the dry mass of the fine fraction <2 mm (g), $V_{excavation}$ is the volume of the excavation (cm^3), M_{stones} is the mass of stones in the sample (g) and D_{stones} is the stone density (g cm^{-3}).

The soil organic C stocks were calculated at each plot, location and soil depth using the 'M4' method described by Poeplau et al. (2017) as recommended for stony soils:

$$C_{stock\_i} = C_{org} \times BD_{finesoil} \times (1 - Stones)$$

where C_{stock_i} is the soil organic C stock at point i (specific location×depth×plot) (kg$_C$ m$_{soil}$$^{-3}$), C_{org} is the organic C content in the fine soil measured in i (mg$_C$ g$_{soil}$$^{-1}$), $BD_{finesoil}$ is the bulk density of the fine soil in i (g cm^{-3}) and $Stones$ is the volumetric fraction of stones (%).

To analyze the effect of location as a fixed factor and the three replicated profiles as random factors on aerial and total root biomass and on the mass of spread N fertilizer, linear mixed models were fitted for each variable. For variables analyzed at different depths, the effects of soil depth, location and their interactions as fixed factors and the three replicated profiles as random factors were tested using linear mixed models: soil physical, chemical and microbiological properties, root biomass density, root properties (functional traits, chemical composition, quality index and anatomy), soil C stocks and living root C stocks. Post hoc Tukey tests were used to assess differences between soil depths and locations.

In addition to the ANOVA-like mixed effect model, a complementary set of covariance analyses (ANCOVA) mixed effect models were used to test our hypotheses. To investigate the correlation between soil physicochemical properties and root variables along the vertical gradient and if these correlations changed according to the 3 locations in the agroforestry system (Hypothesis 1), we used a model comparison approach. Only the most informative root variables associated with root quantity (biomass density), root quality (lignin content) and root anatomy (stele diameter) were investigated. The selection of the soil physicochemical properties used as covariates in these analyses was based on the principal component analysis (PCA) (Fig. 7b) and represented the main axes of variations: soil bulk density, mineral N and Olsen P contents (axis 1, Fig. 7b) and the stone volumetric content (axis 2, Fig. 7b). First, to test the interaction (if the effect of the soil physicochemical covariate on root variables varies according to the location), we compared a model including the soil physicochemical covariate, the location and their interaction (lm1) with the same model but excluding the interaction (lm2) using the anova() function in R. Second, we compared the model with both the soil physicochemical properties and the location (lm2) with a model with the location only (lm3) to test if the soil physicochemical covariate explained a part of the root

variable that was not explained by the location (the vertical variation of root variables).

We used a second set of model comparisons to investigate the correlation of root properties with microbial activity and soil C stocks along the horizontal gradient of the agroforestry system and whether these correlations changed according to the 3 depths investigated (Hypothesis 2). We selected the most informative root variables as covariates in these analyses to reveal the potential contributions of roots to soil C stocks: the living root C stocks, the root quality (informing the root decomposability), including the lignin:N and C:N ratios and the ligno-cellulosic index. First, we tested if the effect of root covariate on microbial activity or soil C stock variables changed according to depth by comparing a model including the root covariate, the depth and their interaction (lm1) with the same model but excluding the interaction (lm2) using the anova() function in R. Second, we tested whether the root covariate explained a part of the soil variable that was not explained by the depth (the horizontal variation of soil variables). We compared the model with both the root covariate and the depth (lm2) with a model with the depth only (lm3).

For all the linear mixed models and analyses of variance, the *lme4* and *car* packages were used. The normality of the residues was always verified with a Shapiro–Wilk test, and the homogeneity of the variances was verified with a Bartlett test. When necessary (p-values < 5%), logarithmic, square root, Box–Cox or Yeo Johnson transformations were applied. Simple ordinations of the root variables and the soil physicochemical and microbiological properties were conducted using PCAs with the *vegan* and *factoextra* packages.

All statistical analyses were performed with R software (version 4.0.5). For each measurement, data are presented as the mean values ± standard deviations of three independent replicates.

Results

Aerial and root biomass density

The aerial biomass and the root:shoot ratio were relatively homogenous among the 3 locations investigated in the agroforestry system (Table 1).

Table 1 Total aerial and root:shoot ratios according to location (UVS, Crop-1 m, Crop-4 m). Data are mean values ± standard deviations ($n = 3$)

	UVS	Crop-1 m	Crop-4 m	p-value
Aerial biomass (g m^{-2})	991 ± 327	1045 ± 150	1107 ± 120	0.82
Root:Shoot	0.065 ± 0.025	0.032 ± 0.021	0.045 ± 0.016	0.28

Below ground, the UVS and the crop developed their rooting system from the 0 to 100 cm depth. Horizontally, no UVS roots were found in the crop zone. No tree fine roots were found in any of the pits. The root biomass density was significantly affected by the interaction between soil depth and location (p-value $= 3.48 \times 10^{-2}$, Supplementary Table 1 in Online Resource 1). In the 20–50 and 50–100 cm soil layers, all locations had similar root biomass densities, whereas in the topsoil (0–20 cm), root biomass density was significantly reduced in Crop-1 m compared to UVS and Crop-4 m (Fig. 2). In the UVS and Crop-4 m locations, the root biomass density decreased significantly between the 0–20 cm and lower soil layers ($59.2 \pm 8.2\%$ and $50.0 \pm 18\%$ of the total root biomass, respectively, p-value $= 3.82 \times 10^{-2}$, Fig. 2). In contrast, Crop-1 m presented no significant differences between the soil depths, and the root system was mainly concentrated between 20 and 100 cm of depth ($75 \pm 9\%$ of the total root biomass Fig. 2).

The roots were isotropic at all locations and soil depths, as the average coefficient A was not significantly altered by any studied factors (Supplementary Table 1 in Online Resource 1) and was low (0.16 ± 0.09, data not shown), indicating that no direction of root growth was predominant. We also confirmed that the root impact density was not significantly different (p-value $= 0.08$) according to the H, L or T cube face (Supplementary Fig. 1 in Online Resource 1).

For each location, the linear relationships between the root length density and the root impact density exhibited high R^2 values ($> 79\%$, Supplementary Table 2 in Online Resource 1). The UVS had a higher coefficient (4.24) than Crop-1 m and Crop-4 m (2.80 and 2.63, respectively), indicating a higher root tortuosity or branching rate in the UVS location than in the others.

Root functional traits, chemical composition and anatomy

The root elemental composition, particularly the P content, was affected by soil depth (Supplementary Table 1 in Online Resource 1). The root C content tended to increase with depth (p-value $= 0.06$, Fig. 3a) and the root P content decreased significantly from the 0–20 to 50–100 cm soil layers (p-value $= 1.53 \times 10^{-2}$, Fig. 3c), consequently resulting in lower root C:P and N:P ratios in the topsoil than in deeper layers at all locations (data not shown). In parallel, the specific root length was not significantly altered by the soil depth or the location (Supplementary Table 1 in Online Resource 1), but it tended to increase from the 0–20 to 20–50 cm soil layers (Fig. 3h).

Conversely, the average root diameter showed a significant response to the location (p-value $= 6.13 \times 10^{-3}$), with lower values in the UVS than in the crop locations, and SRL tended to be higher in Crop-1 m than in Crop-4 m and UVS at all depths (Fig. 3g-h). Even if the effects of the location and soil depth on the root quality indexes (lignin:N ratio, ligno-cellulosic index, soluble compounds:cell wall ratios, Supplementary Table 1 in Online Resource 1) were not significant according to the mixed effect models, the mean root lignin content at 0–20 cm was more than twice as high in Crop-1 m ($13.3 \pm 1.1\%$) and in the UVS ($9.4 \pm 0.9\%$) than in Crop-4 m ($5.5 \pm 0.8\%$, Fig. 4). The lignocellulosic index presented the same tendency as the root lignin content (Fig. 3d), and it was also particularly high at depth for UVS roots.

The root anatomical analysis (Supplementary Fig. 2 in Online Resource 1) in Crop-1 m and Crop-4 m did not show any significant results (Supplementary Table 3 in Online Resource 1), except for the endoderm width, which tended to decrease with increasing soil depth (Supplementary Fig. 3a in Online Resource 1), and the stele area, which was significantly higher in the topsoil than at other soil depths in all locations (Supplementary Fig. 3b in Online Resource 1).

Soil properties

Most of the soil properties changed with depth (Supplementary Table 4 in Online Resource 1).

Table 2 Soil physical, chemical and microbiological properties according to depth (0–20, 20–50 and 50–100 cm). Data are mean values (Mean) and standard deviations (Sd) and were merged for all locations ($n=6$). The lowercase letters indicate significant differences between the soil depths (p-value <0.05)

		0–20 cm			20–50 cm			50–100 cm		
		Mean	Sd		Mean	Sd		Mean	Sd	
Physical properties										
Bulk density	g cm^{-3}	1.17	0.05	a	1.69	0.01	b	1.87	0.10	c
Bulk density on fine particles	g cm^{-3}	1.01	0.15	a	1.56	0.10	b	1.63	0.08	b
Stones volumetric proportion	%	14	9	a	20	14	a	47	19	b
Clay content	g kg^{-1}	192.00	18.59	a	271.33	75.22	a	468.78	71.19	b
Silt content	g kg^{-1}	327.78	12.32	b	322.56	23.09	b	263.78	56.68	a
Sand content	g kg^{-1}	480.22	18.67	c	406.11	91.14	b	267.44	24.91	a
Moisture	%	37	6	a	38	4	a	37	6	a
Chemical properties										
Organic C content	g kg^{-1}	8.76	1.91	c	5.07	1.79	b	3.53	0.93	a
Organic C stocks	kgC m^{-3}	7.41	1.21	c	5.88	0.88	b	3.00	1.22	a
Total N content	g kg^{-1}	0.90	0.20	b	0.68	0.10	a	0.63	0.10	a
Total P content	g kg^{-1}	0.55	0.13	c	0.45	0.13	b	0.35	0.05	b
Soil C:N		9.74	0.71	c	7.25	1.68	b	5.59	1.00	a
Soil N:P		1.67	0.33	a	1.59	0.25	a	1.78	0.22	a
Soil C:P		16.19	2.74	b	11.22	1.93	a	10.04	2.37	a
Dissolved organic C content	mg kg^{-1}	64.24	14.11	b	40.96	14.80	a	42.94	11.31	a
Olsen P content	mg kg^{-1}	40.19	13.69	c	24.67	20.68	b	8.08	4.31	a
CEC	cmol + kg^{-1}	8.35	1.93	a	10.38	0.87	a	17.97	3.62	b
pH		6.82	0.64	a	7.14	0.32	ab	7.33	0.12	b
Microbiological properties										
MBC	mg kg^{-1}	103.40	43.30	b	60.21	20.91	a	43.31	20.97	a
MBN	mg kg^{-1}	7.14	3.70	a	4.04	2.97	a	3.39	3.58	a
MBC:N		18.05	10.94	a	29.62	33.51	a	19.93	12.11	a
16S	copy numbers g^{-1}	1.99×10^9	8.76×10^8	c	1.01×10^9	4.57×10^8	b	2.50×10^8	1.34×10^8	a
18S	copy numbers g^{-1}	1.79×10^9	7.05×10^8	c	7.56×10^8	1.16×10^9	c	8.78×10^7	7.23×10^7	a
16S:18S		1.12	0.29	a	2.79	1.77	a	3.69	1.81	b
C-enzymatic activity	µmol g$^{-1}_{soil}$	0.34	0.09	b	0.09	0.07	a	0.03	0.02	a
N-enzymatic activity	µmol g$^{-1}_{soil}$	0.31	0.14	b	0.23	0.12	ab	0.16	0.14	a
P-enzymatic activity	µmol g$^{-1}_{soil}$	0.83	0.17	b	0.45	0.13	a	0.35	0.11	a
C-enzymatic specific activity	µmol g$^{-1}_{MBC}$ min^{-1}	19.39	4.31	b	7.98	3.52	a	3.65	1.57	a
N-enzymatic specific activity	µmol g$^{-1}_{MBC}$ min^{-1}	16.63	5.45	a	20.62	9.56	a	21.34	16.90	a

Table 2 (continued)

		0–20 cm			20–50 cm			50–100 cm		
		Mean	Sd		Mean	Sd		Mean	Sd	
P-enzymatic specific activity	$\mu mol\ g^{-1}_{MBC}\ min^{-1}$	54.28	28.96	a	45.76	20.41	a	52.35	22.24	a
Vector length		0.62	0.08	b	0.33	0.08	a	0.20	0.14	a
Vector angle	radians	1.07	0.17	a	1.05	0.23	a	1.16	0.19	a
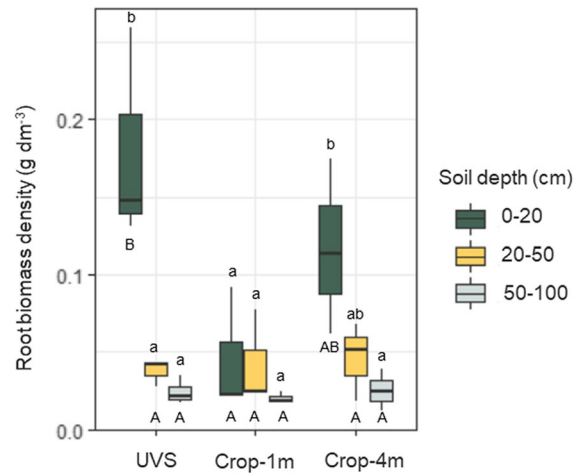
Fig. 2 Root biomass density (g dm^{-3}) according to soil depth (0–20, 20–50 and 50–100 cm) and location (UVS, Crop-1 m, Crop-4 m). Data are mean values, and error bars represent the standard deviations ($n=3$). The uppercase letters indicate significant differences between the locations within each soil depth, and the lowercase letters indicate significant differences between the soil depths within each location (p-value < 0.05)

For all locations, soil organic C, total N, mineral N, total P and Olsen P significantly decreased with depth, while the bulk density and the clay content increased with increasing depth (Table 2). In contrast, the soil moisture content was not affected by the soil depth (p-value $= 0.95$) or the location (p-value $= 0.58$, Supplementary Table 4 in Online Resource 1). The location significantly affected only the soil mineral N content (Supplementary Table 4 in Online Resource 1). Crop-4 m had a significantly higher soil mineral N content than the UVS, and that of the Crop-1 m location was in between those of the other locations (p-value $= 1.18 \times 10^{-2}$, Fig. 5), in agreement with the lower quantity of spread fertilizer (p-value $= 2.65 \times 10^{-3}$) in Crop-1 m (2.42 ± 1.52 g m^{-2}) than in Crop-4 m (5.11 ± 2.66 g m^{-2}, data not shown).

Regarding the microbial properties, the MBC, 16S and 18S copy numbers and enzymatic activities decreased with soil depth, indicating lower microbial biomass and activity at deeper soil depths. In parallel, the 16S:18S ratio increased from 1.1 ± 0.3 at 0–20 cm to 3.7 ± 1.8 at 50–100 cm and, although the difference was not significant, it tended to be higher in the UVS than in Crop-1 m and Crop-4 m, especially in the

Fig. 3 Root elemental composition (C content (**a**), N content (**b**) P content (**c**)) according to soil depth (0–20, 20–50 and 50–100 cm and merged for all locations (*n*=6), quality index (lignin:N (**d**), lignin-cellulosic index (**e**) soluble compounds:cells (**f**)) and functional traits (diameter (**g**) and specific root length (**h**) according to soil depth (0–20, 20–50 and 50–100 cm) and location (UVS, Crop-1 m, Crop-4 m, *n*=3). The uppercase letters indicate significant differences between the locations, and the lowercase letters indicate significant differences between the soil depths (*p*-value < 0.05)
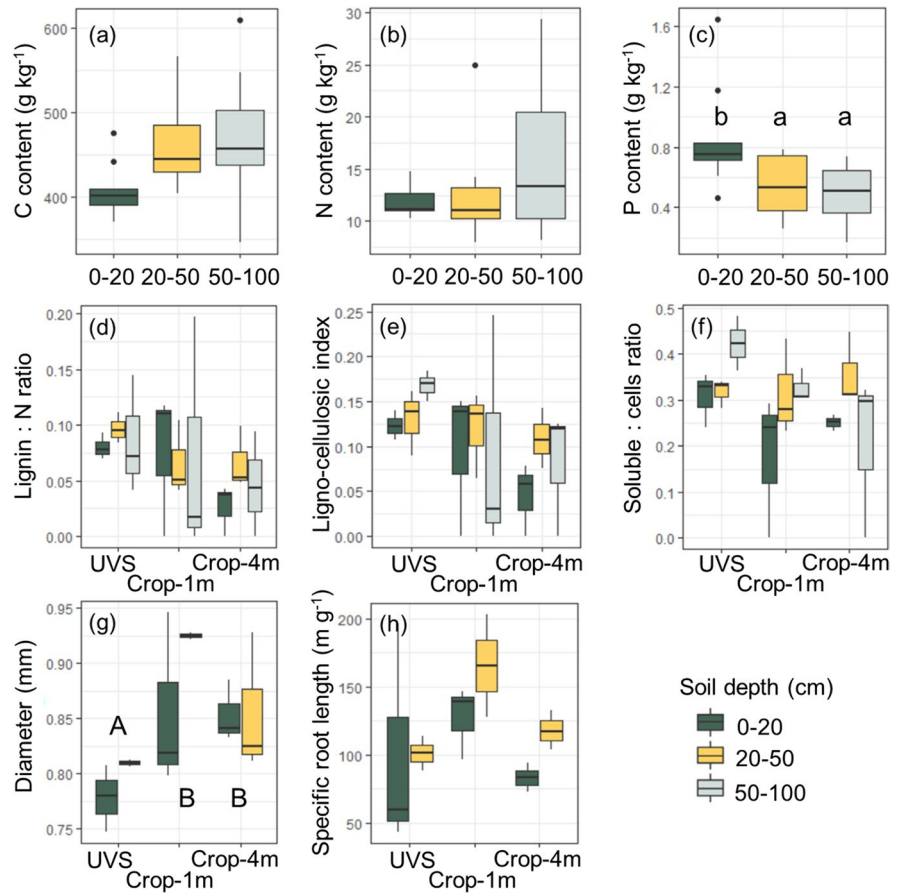
Fig. 4 Root C fractions (lignin, cellulose, hemi-cellulose and soluble compound contents, *n*=3) according to soil depth (0–20, 20–50 and 50–100 cm) and location (UVS, Crop-1 m, Crop-4 m)
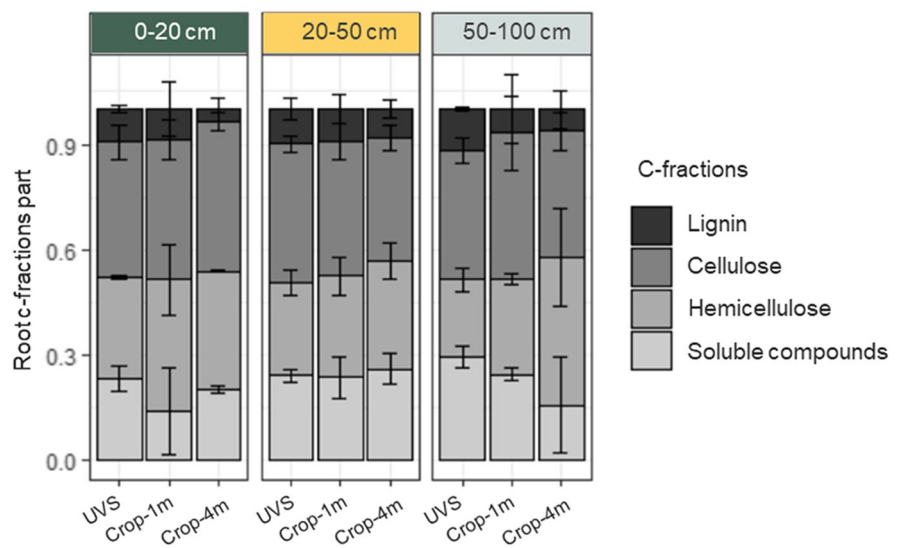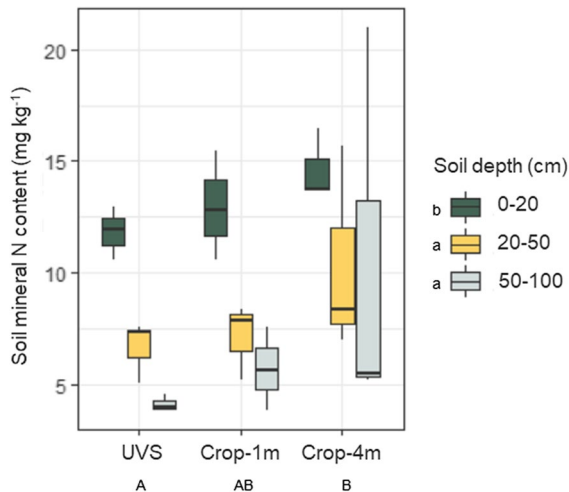
Fig. 5 Soil mineral N content according to depth (0–20, 20–50 and 50–100 cm) and location (UVS, Crop-1 m, Crop-4 m). The uppercase letters indicate significant differences between the locations, and the lowercase letters indicate significant differences between the soil depths (p-value < 0.05, $n = 3$)

deeper soil layers (Supplementary Fig. 5 in Online Resource 1). The C-enzyme (BG + CBH) specific activities (per unit of MBC) decreased with depth (Table 2, p-value = 3.21×10^{-7}, Supplementary Table 4 in Online Resource 1) but not the specific activity for N (NAG + LAP, p-value = 0.59) or P (p-value = 0.72). Confirming a decrease in C acquisition with depth, the eco-enzymatic stoichiometry analysis showed a lower relative microbial investment in C compared with nutrient acquisition (vector length) in the subsoil than in the 0–20 cm layer (Supplementary Fig. 6 in Online Resource 1, p-value = 1.31×10^{-6}, Table 2). The P vs. N root acquisition (vector angle) was not significantly affected by location or depth (Supplementary Table 4 in Online Resource 1).

Annual root-derived C inputs and soil organic C stocks

The soil organic C stock changed significantly between the 0 and 100 cm depth (Supplementary Table 4 in Online Resource 1), and 58.6% of the organic C was located below 20 cm (Fig. 6). The estimated annual root-derived C inputs followed the root length density map, decreasing with soil depth, especially in UVS and Crop-4 m (Fig. 6). In Crop-4 m,

where all crop roots decayed into the soil after harvest, the annual root-derived C inputs were equivalent to 0.59%, 0.34% and 0.25% of the soil organic C stock at 0–20 cm, 20–50 cm and 50–100 cm, respectively. The inputs in Crop-1 m represented 0.28%, 0.35% and 0.21%, respectively. In the UVS, based on an annual root turnover rate of 53% (Gill and Jackson 2000), the annual root-derived C inputs would be 0.49%, 0.14% and 0.12%, respectively.

Covariations in root, soil and microbial properties across soil depth and location

The first axes of the PCA of the root variables (Fig. 7a) captured 28.2% of the variations and showed a gradient of root biomass tightly linked to an increase in root P contents and anatomical changes with increasing diameter (bigger stele and endoderm) mainly present through the ellipse representing the root system at 0–20 cm of depth in the UVS. The second axis (18.7% of the total variations) grouped the lignin-rich roots in the UVS and in Crop-1 m differently than the roots with higher concentrations of cellulose and hemicellulose in Crop-4 m.

The soil physicochemical properties (Fig. 7b) were better separated along the horizontal axis (53.3% of the variations) according to the soil depths, with higher C, N and P contents at 0–20 cm and higher CEC, clay content and bulk density at depth.

The first axes (40.5% of the variations) of the PCA of the soil microbiological properties (Fig. 7c) separated the topsoils from the two deeper soil layers with higher microbial biomass and enzymatic activities, and differences between the locations were not noteworthy.

The comparison of ANCOVA-like mixed effect models showed that root properties were correlated with soil properties along the vertical gradient. At all locations, the root stele diameter decreased in parallel with the bulk density (p-value = 6.53×10^{-7}, Supplementary Table 5 in Online Resource 1, Fig. 8d) and with the stone volumetric content (Supplementary Fig. 7a in Online Resource 1). For other root properties, the correlations with soil physicochemical properties were different between locations in the agroforestry system. The root biomass density decreased with increasing soil bulk density (Fig. 8a) and increased with increasing soil mineral N content (Fig. 8b) with a steeper slope in the UVS than in Crop-1 m and Crop-4 m (p-value = 2.38×10^{-3} and 1.87×10^{-2}, respectively,
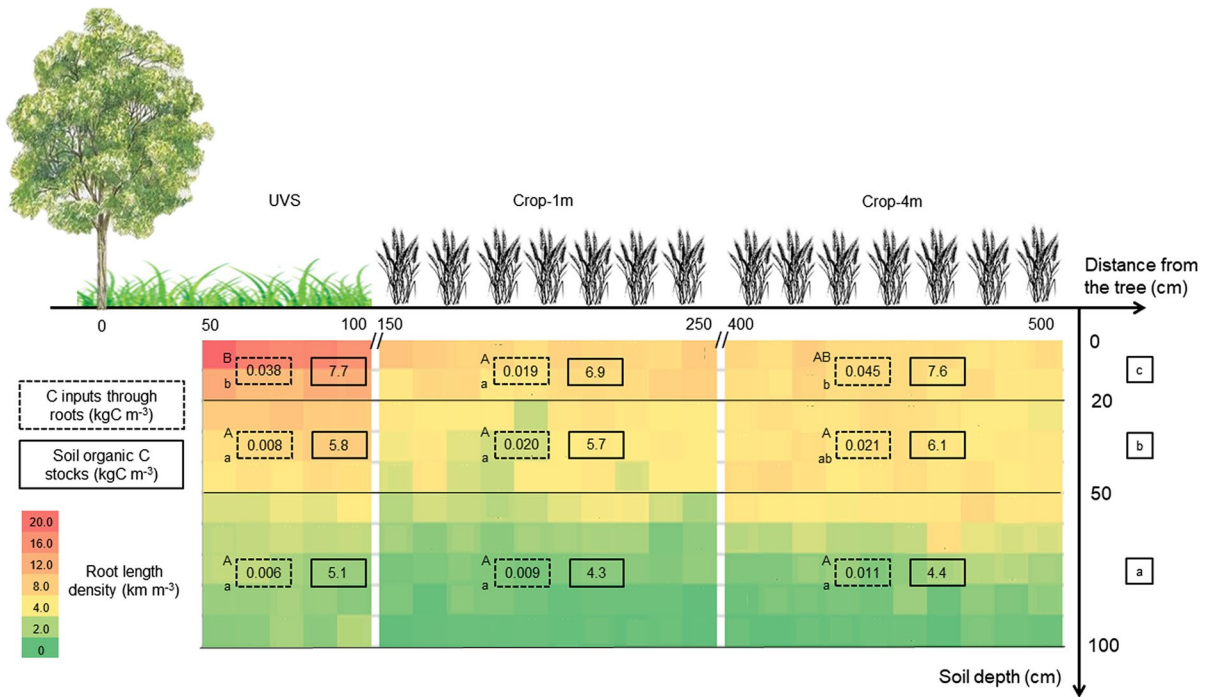
Fig. 6 Soil organic C stocks and annual root C inputs in the studied agroforestry system. Data are mean values ($n = 3$). Colors in the background represent root length density from 0 to 100 cm of depth and from 50 to 500 cm from the tree line. The uppercase letters indicate significant differences in annual root C inputs between the locations within each soil depth, and the lowercase letters indicate significant differences in annual root C inputs between the soil depths within each location. The framed lowercase letters indicate significant differences in soil organic C stocks between the soil depths for all locations (p-value < 0.05)

Supplementary Table 5 in Online Resource 1). The effect of the stone volumetric content on the root lignin content tended to be different according to the location (p-value $= 7.58 \times 10^{-2}$, Supplementary Table 5 in Online Resource 1), as it was negative in Crop-1 m and missing at the other locations (Fig. 8c).

The comparison of ANCOVA-like mixed effect models also showed that microbial activity and soil C stocks were correlated with root properties along the horizontal gradient of the agroforestry system. Some properties associated with microbial activity and C stocks were correlated with root properties with no interaction with depth (Supplementary Table 6 in Online Resource 1). The soil C stocks tended to increase with the root lignin:N ratio (p-value $= 0.09$, Supplementary Fig. 7c in Online Resource 1) and with the root C:N ratio (p-value $= 0.06$, Fig. 8f). The MBC strongly increased with increasing root C stocks (Fig. 8g), but it was not related to any tested root quality variable (p-value $= 0.23, 0.30, 0.38$ for the root lignin:N, C:N and ligno-cellulosic index, respectively). Conversely, some correlations of root properties with microbial activity and soil C stocks differed between soil depths (Supplementary Table 6 in Online Resource 1). The soil organic C stocks were positively related to the root C stocks between 0 and 50 cm of depth, whereas this correlation was reversed at 50–100 cm (Fig. 8e). The specific N-enzymatic activity presented different responses to an increasing lignin:N ratio according to the soil depths (p-value $= 1.80 \times 10^{-2}$, Supplementary Table 6 in Online Resource 1): it decreased at a depth of 0–20 cm and increased between 20 and 100 cm (Supplementary Fig. 7f in Online Resource 1). The response of the specific N enzymatic activity to the root C:N ratio also differed according to the soil depths (p-value $= 3.21 \times 10^{-2}$, Supplementary Table 6 in Online Resource 1): it increased more substantially at a depth of 50–100 cm than at the other soil depths (Fig. 8h).

Fig. 7 Principal component analysis of the root (**a**), soil physicochemical (**b**) and soil microbiological (**c**) variables according to location (UVS, Crop-1 m and Crop-4 m) and soil depth (0–20, 20–50 and 50–100 cm)
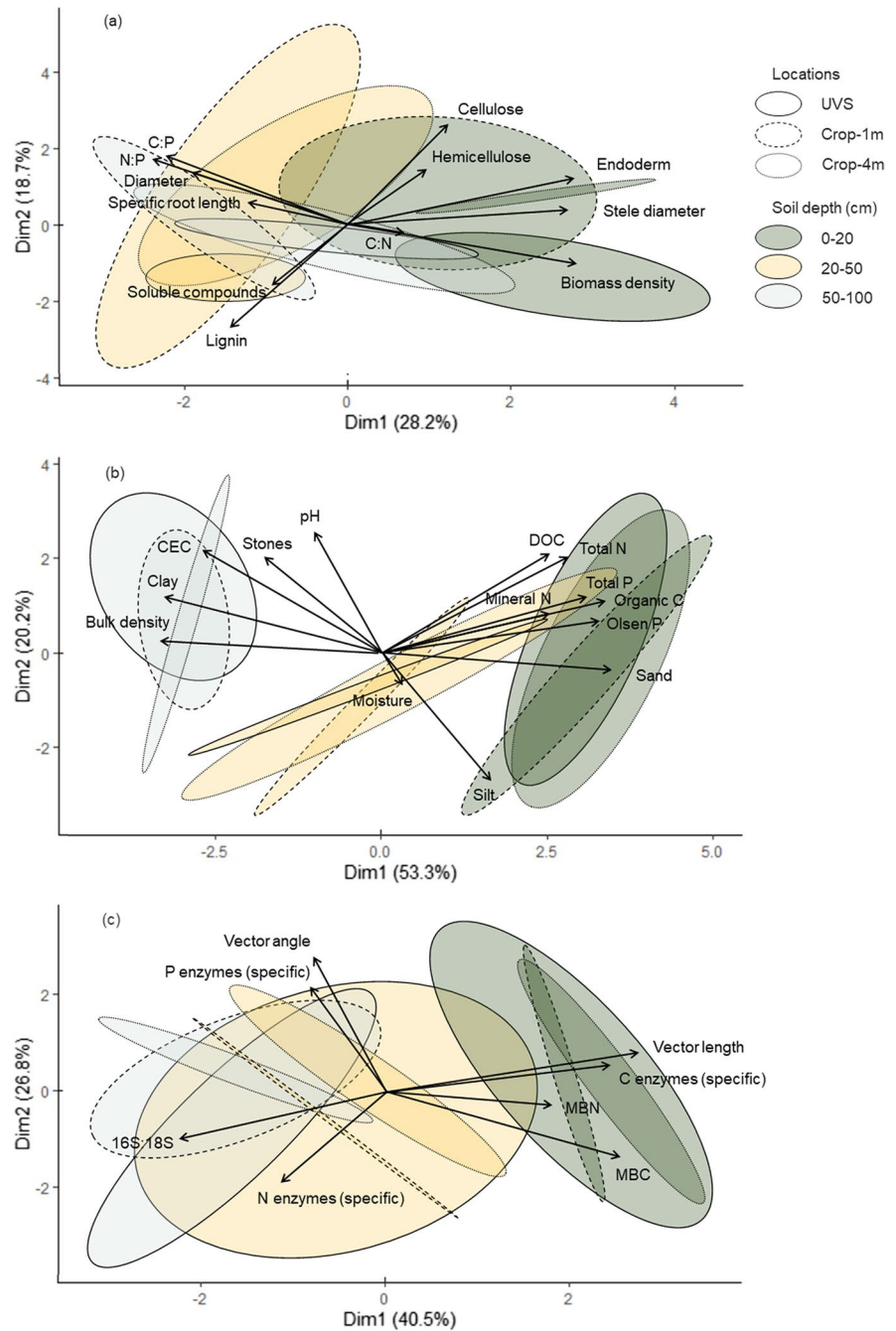
Discussion

Quantitative and qualitative assessments of the root systems and the soil properties

The root properties varied according to the soil depth. Some of these variations were common for all locations. For example, the anatomical structures of roots showed higher stele area in the topsoils than at depth. This difference was expected as uptakes from the entire soil profile needed to pass through this zone to reach the plant aerial parts; however, opposite patterns were reported by Wang et al. (2016).
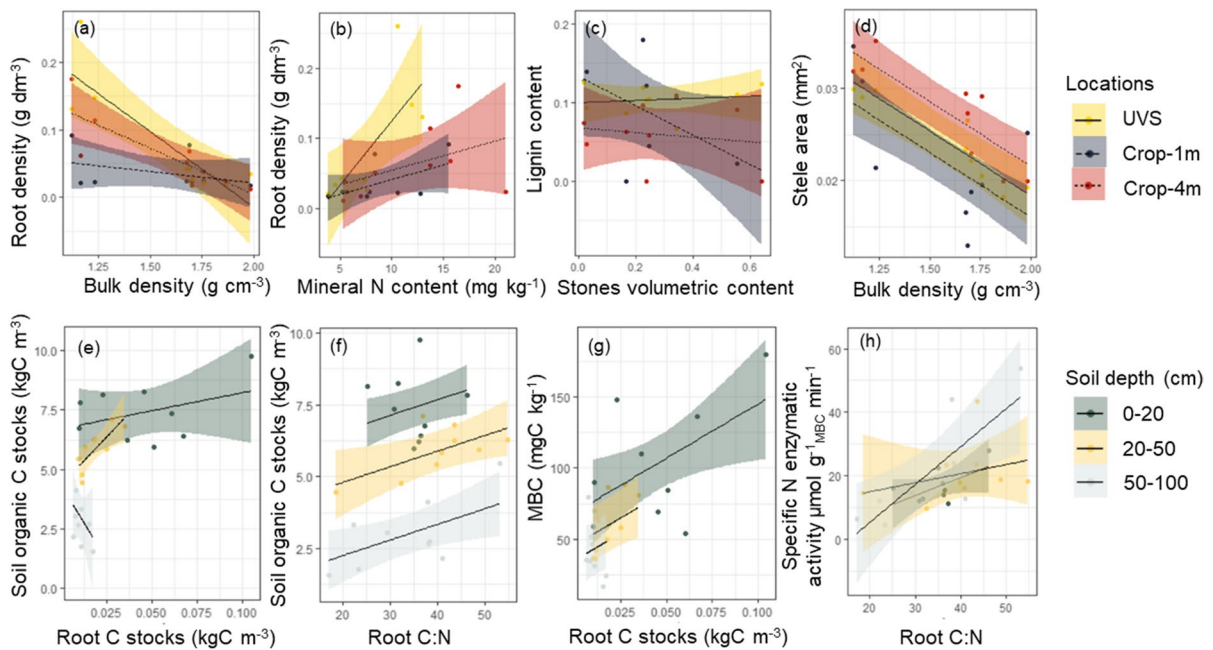
Fig. 8 Correlations between roots and soil variables at different locations (UVS, Crop-1 m and Crop-4 m) to test the H1 hypothesis (**a**, **b**, **c** and **d**) and at different soil depths (0–20, 20–50 and 50–100 cm) to test the H2 hypothesis (**e**, **f**, **g** and **h**)

In addition, the root properties differed between the 3 studied locations despite the absence of major differences in the soil properties in the horizontal gradient considered in this young Mediterranean agroforestry system, and compared to mature systems (Guillot 2018; D'Hervilly 2021). On the one hand, differences occurred between UVS and the crop. The UVS roots showed a higher tortuosity or branching rate and a lower specific root length, suggesting lower root respiration rates, absorption and elongation efficiency, which were indicators of a conservative strategy compared to the acquisitive strategy of the annual crop (Roumet et al. 2006; Freschet et al. 2010; Caplan et al. 2019). Moreover, UVS roots tended to have higher lignin contents than crop roots, suggesting a longer residence time due to the microbial and enzymatic cost needed for lignin degradation (Moorhead et al. 2013). On the other hand, several differences between the Crop-1 m and Crop- 4 m root systems were noticed in the topsoil, despite equivalent R:S ratios, confirming our hypothesis that horizontal heterogeneity of root systems might also occur even in the same component of the agroforestry system (i.e., the crop). In Crop-1 m, we observed a threefold

reduction in root biomass density and a longer specific root length, which suggested an exploratory strategy (de Britto Costa et al. 2021) and a faster return of investment (Freschet et al. 2017). Furthermore, higher lignin contents suggested that the Crop-1 m roots were more differentiated than the Crop-4 m roots. Because they were more differentiated, these roots would be more likely to show a loss of their cortical parenchyma (Gardner and Sarrantonio 2012), consistent with the decreasing lignin content with increasing soil stone content in Crop-1 m, in contrast to the absence of such a relationship in the other locations. The lower soil mineral N content observed near the UVS, likely due to lower N fertilizer inputs, might explain these differences between Crop-1 m and Crop-4 m roots (a lower biomass density, longer specific root length and more differentiation) by limiting root growth (Fageria and Moreira 2011). One alternative explanation may be competition for nutrients induced by the UVS at Crop-1 m but (i) the absence of UVS root colonization under Crop-1 m, in contrast to the reported findings (Battie-Laclau et al. 2020) for older agroforestry sites (5 and 11 years) tilled to 10 cm only (*vs*. up to 30 cm in our experimental site) and (ii)

the root isotropy found at all depths and all locations supported the absence of a preferential vertical direction of the crop roots and thus the absence of any root competition (Bolte and Villanueva 2006; Cardinael et al. 2015). Indeed, the global fine root isotropy identified in our study reveals a rather stable root foraging strategy to explore soil in all directions, regardless of the soil depth and distance to UVS. These features seem to be quite common in other plantations, such as maize (Chopart and Siband 1999), sugarcane (Chopart et al. 2008) and eucalyptus (Maurice et al. 2010). Another potential explanation for differences between Crop-1 m and Crop-4 m is the competition for light induced by the tree shade, but the absence of shade from the young trees on the sunny side of the tree line, i.e., where the pits were dug, refute this hypothesis. In conclusion, the functional root properties and biomass differed between the agroforestry compartments due to the presence of different plant communities and the heterogeneity of the soil mineral N levels. This result highlighted the importance of also considering heterogeneity in fertilization practices that lead to changes in N availability to explain the usually observed decrease in crop growth along the tree lines in agroforestry systems (Singh et al. 1989; Lawson and Kang 1990; Miller and Pallardy 2001).

Because of the N-fixing trees, in the next years, N contents might increase near the UVS due to greater litter aerial and belowground N inputs (Danso et al. 1995; Uselman et al. 2000; Von Holle et al. 2013) and different microclimatic conditions near the tree (Guillot et al. 2021; D'Hervilly et al. 2022). This changes might eliminate part of the heterogeneity related to the fertilization distribution across the agroforestry system and limit the observed differences in the crop root systems according to the distance to the tree.

The effects of the soil properties on the root systems along the vertical gradient (soil depth) differed between the locations. This result was confirmed for the root biomass, as it was mostly concentrated at 0–20 cm of depth in the UVS and Crop-4 m (59.2 and 50.0%, respectively) but not for the crop near the UVS (Crop-1 m: 25% only). The correlation between roots and soil properties along the soil profile showed that the UVS roots rapidly increased their biomass with increasing soil mineral N contents, while Crop-1 m and Crop-4 m root biomasses increased less rapidly.

These results suggested that UVS plants, as globally plurennial species, increased their root systems in the nutrient-rich soil patches (Hodge 2009) or that the soil N-shortage conditions limited their growth (Cuevas and Medina 1988), whereas crop roots were less linked to this vertical nutrient heterogeneity, as they were sown only few month before sampling. Consequently, the root biomass density of the UVS was not as high as that reported in other studies at depth (Battie-Laclau et al. 2020). This pattern was associated with herbaceous plants of the 3-year-old UVS, as no tree roots were found in any of the 3 pits, even at 1 m from the nearest tree. Taken altogether, our observations showed that the agroforestry system components presented similar responses of root properties to depth (in the UVS, Crop-1 m and Crop-4 m) but different root biomass responses to soil chemical property gradients along the soil profile because they belonged to different plant communities (UVS *vs.* crops).

Relationships between root-derived carbon inputs and soil carbon stocks

At all locations, our estimation of annual root-derived C inputs represented a small part of the soil organic C stocks (less than 0.6%), even in the topsoil, where the root biomass was the highest but the aerial biomass also contributes C inputs from the crop residues in Crop-1 m and Crop-4 m and from the aboveground mortality in the UVS (Fig. 6). However, annual root-derived C inputs are renewed each year, and this quantification did not consider the release of C through rhizodeposition, which might also represent an important part of additional annual C inputs (Pausch and Kuzyakov 2018). The same turnover rate was used for each soil layers to estimate the root-derived C inputs in soil from the UVS because of a lack of reference quantifying herbaceous plant root turnover at depth under similar pedo-climatic conditions. However, root turnover was observed to decrease with soil depth (Gill et al. 2002; Houde et al. 2020) and our estimations of root-derived C inputs might be over-estimated for the UVS at depths of 20–50 and 50–100 cm. The UVS presented equivalent root-derived C inputs to Crop-4 m, but it did not receive any fertilization. Integrating perennial vegetation in the crop system might allow equivalent C inputs from the roots, with less nutrients inputs. Furthermore,

we observed that the soil C stocks increased significantly with increasing C stocks in living roots at depths of 0–20 cm and 20–50 cm (Fig. 8e). Although a regression analysis does not imply causal effects, this observation is potentially consistent with the hypothesis that a higher root biomass contributes to SOM accumulation (Balesdent and Balabane 1996; Rasse et al. 2005; Kätterer et al. 2011). Conversely, at depths of 50–100 cm, a similar negative relationship was observed, suggesting that the deep soil C stocks resulted from older C, i.e., a longer time of accumulation than surface soil C (Balesdent et al. 2018; Abbas et al. 2020). At depth, increased root-derived C inputs might stimulate more C-release processes such as the priming effect, than C stabilization processes (Fontaine et al. 2007; de Graaff et al. 2014; Karhu et al. 2016; Cardinael et al. 2018). Indeed, subsoil C is more sensitive to the priming effect than topsoil (Fontaine et al. 2007). However, studies specifically designed to investigate these processes (e.g., isotopic labeling) in our system will be necessary to validate this hypothesis. These results stress that roots might exert different effects on the soil C budget in shallow and deep soil layers and suggest the need for more investigations of these differences to fully appreciate the potential of deep roots to increase C sequestration in agricultural soils (Bertrand et al. 2019).

Both the root quantity and the root quality seemed to affect soil C storage as a major factor controlling root decomposition (Bonanomi et al. 2021). In this young agroforestry system, the roots in Crop-1 m and in the UVS were richer in lignin than those in Crop-4 m. Furthermore, we observed that the soil C stocks increased with an increasing root lignin:N ratio, suggesting that root chemical recalcitrance increases soil C storage because of the slower root litter decomposition rate (Moorhead et al. 2014; Bonanomi et al. 2021) in UVS than in Crop-4 m (Abbasi Surki et al. 2021).

Relationships between the root properties and the soil microbial properties

Soil C stock evolution in response to root-derived C input depends on soil microbial activities (Zechmeister-Boltenstern et al. 2015; Tate 2020), which are known to respond more rapidly to vegetation and management changes than the total soil C stock. This study was performed at a single sampling date to establish the relationships between the roots and the microbial properties at the community scale of the agroforestry ecosystem. Therefore, it does not take into account the intra-annual effects due to seasons and physiological development of the biomass. Nevertheless, according to several studies, spatial differences in microbial activities (and root properties) are maintained across the seasons (Picon-Cochard et al. 2012; Baldrian et al. 2013; Ali et al. 2015). Despite the spatial heterogeneity of the root systems described above and the lower mineral N content in and near the UVS compared to far from it, the measured soil microbial properties did not differ between the three studied locations. The MBC and the soil C stocks were more strongly affected by the root C stocks than the root quality variables. In contrast, the N enzymatic activities were strongly related to the root stoichiometry (C:N ratio), the lignin:N ratios and the ligno-cellulosic index, which are all usually negatively related to decomposition (Bonanomi et al. 2021). The specific N enzymatic activity increased with increasing root C:N ratios, consistent with the study by Zechmeister-Boltenstern et al. (2015) suggesting that soil microorganisms adapt their investment in enzymes to target limiting resources from the root litter. Furthermore, the negative relationships between the specific N enzymatic activity and the root stoichiometry (C:N and lignin:N ratios) were stronger below than above a depth of 20 cm. Indeed, at depth, the eco-enzymatic stoichiometric analysis suggested nutrient shortage conditions (Sinsabaugh et al. 2009; Cui et al. 2018) compared to those in topsoil, where additional amendments occurred through tree aerial litter inputs in the UVS that were enriched in N due to N fixation, and with N chemical fertilization in the intercropped area (Sun et al. 2020). Our observations in the deeper soil layers suggested other mechanisms by which specific stoichiometric constraints of subsoil might alter the effect of roots on soil C contents (Bertrand et al. 2019): increasing root biomass density at depth would increase the input of litter with high a C:N ratio, which might accentuate the microbial N limitation (Zechmeister-Boltenstern et al. 2015) and reduce the potential entombing effect on C sequestration (Cotrufo et al. 2013; Liang et al. 2017). This mechanism might have contributed to the negative relationship observed between living root C stocks and soil organic C stocks in the subsoil. These hypotheses suggest that the mechanisms for soil C sequestration in the subsequent years of the agroforestry systems would be

differentiated in the 3 communities: higher availability of N in the UVS due to increasing UVS and N-fixing tree root litter inputs, lower availability of N in the crop zone near the UVS due to lower fertilization and crop far from the tree line hypothesized as without any effect of the tree, behaving as pure crop regarding soil C sequestration mechanisms. Our findings stress the importance of studying plant-microborganisms stoichiometric relationships to elucidate the potential of deep rooting to increase C sequestration in agricultural soils (Bertrand et al. 2019) and the strong sensitivity of subsoils to C inputs compared to topsoils (Loeppmann et al. 2019).

Conclusions

After 3 years of agroforestry, this study assesses the quantitative and qualitative properties of annual and perennial root systems. Under the tree line, roots from the UVS played a major role in determining C inputs (still shallow at this stage but more recalcitrant), as no tree roots were present. The root biomass under the crop near the UVS was reduced by 3-fold in the topsoils compared to the crop far from the UVS. The contributions of root biomass at flowering stage to soil organic C stocks were estimated along the rooted profile (a depth of 100 cm) for each community (UVS, Crop-1 m and Crop-4 m) and showed horizontal and vertical heterogeneity. These different plant communities have not yet imprinted the soil C stock of this young system and soil depth was still the most important factor explaining the soil organic C stock. However, the heterogeneity of the quantity and quality of the root-derived C inputs and their relationships with microbial activity across the agroforestry system should be monitored through multiple-time samples to evaluate the dynamics of distance differentiation of the soil properties to tree, depending on soil depth. The relationships between root and microbial properties suggested that an increase in litter inputs with high C:N ratios, such as roots, might accentuate microbial N limitations and limit soil C storage at depth. Further investigations of the microbial response to fresh organic matter input below the plow layer are needed to evaluate the benefit that increasing root biomass could provide for soil C storage.

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