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Role of trees and herbaceous vegetation beneath trees in maintaining arbuscular mycorrhizal communities in temperate alley cropping systems

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Abstract

Background & Aims Better understanding of below-ground interactions in agroforestry systems is crucial for the success of plant co-existence. Beyond root competition, associated arbuscular mycorrhizal (AM) fungi can also be involved in plant to plant interactions. Thus far, the contribution of each agroforestry component (trees, herbaceous vegetation beneath trees -HVbT-

and crops) in the establishment and maintenance of AM communities is poorly documented, particularly in temperate areas. This study investigates the spatio-temporal dynamics of both roots and AM fungi in two alley-cropping sites located in southwestern France.

Methods Over a one-year period, (i) root length density, production and distribution, (ii) AM activity (root mycorrhization rate and extra-radical hyphal production) and (iii) AM diversity (metabarcoding) were assessed at different distances from tree rows in two agroforestry systems.

Results The mycorrhization rate and hyphal production increased at the interface between tree rows and cultivated alleys, showing a positive effect of the presence of a perennial system (tree and HVbT) and of plant diversity. Compared to HVbT, tree roots colonized farther into superficial layers of the cultivated alleys. However, due to higher root densities and well-established AM fungi observed throughout all the year, HVbT appeared to be more relevant in maintaining an active source of AM inoculum for newly developing crop roots in winter.

Conclusion The spatial proximity of roots and common AM fungi provides new perspectives in deciphering the significance of arbuscular mycorrhizal communities in crop nutrition and yield in agroforestry systems.

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Abbreviations

AM   Arbuscular mycorrhiza
AC   Alley cropping
HVbT   Herbaceous vegetation beneath trees

Introduction

In recent decades, alley cropping (AC) - the integration of trees and crops - has attracted increasing interest as an ecologically advantageous promising land use strategy relative to current agricultural practices (Shukla et al. 2012; Wolz and De Lucia 2018). Adding tree rows and other perennial plants into the agricultural landscape can increase overall biomass productivity of the site (measured by the land equivalent ratio) while reducing soil erosion, nitrogen leaching, and biodiversity loss (Baah-Acheamfour et al. 2014; Rivest et al. 2013; Wilson and Lovell 2016). However, trees in agroforestry systems can also compete with crops for natural resources (light, water and/or nutrients), which can sometimes lead to a reduction in crop yield (Gao et al. 2013). Large scale adoption of alley cropping by farmers faces management constraints, such as accessibility of agricultural machinery, interactions with pathogens/pests and competition for water and nutrients. Improving our knowledge regarding the complex interspecific interactions is crucial to move AC forward. Biogeochemical mechanisms underpinning belowground interactions remain poorly studied, especially in temperate regions where agriculture is highly dependent on fertilization (Quinkenstein et al. 2009).

Maximization of facilitative effects and minimization of competition between tree rows and cultivated alleys, particularly regarding limited resources such as nutrients and water, are critical for increasing AC performances (Jose et al. 2004; Schoeneberger et al. 2012). The productivity of plurispecific cropping systems is the net result of positive and negative interactions among species. The coexistence of species with contrasting functional traits can promote the emergence of positive interactions between species making it possible to increase resource use efficiencies (Isbell et al. 2015). AC components (i.e., trees, herbaceous vegetation beneath trees, −abbreviated as HVbT-, and crops) can occupy different spatial and temporal niches. Some studies show that growth resources can be exploited more completely and can be shared between species, allowing increased economic and ecological benefits over separately cultivated species (Davis et al. 2012). Spatial niche complementarities (e.g., contrasted vertical root system distribution between intercropped species) as well as facilitative processes in the rhizosphere (Hinsinger et al. 2011) can result through root system activities and indirectly through their associated microbial communities.

Among these communities, arbuscular mycorrhizal (AM) symbiosis plays a central role in nutrient acquisition of plants and biogeochemical cycles (Barea et al. 2005). Thanks to the increased volume of soil explored by extra-radical mycelia, AM symbiosis facilitates the mobilization of resources for plants and contributes to the maintenance of soil structure and biodiversity (Smith and Read 2008). AM fungi form symbioses with most terrestrial plants including cultivated species, and are strongly involved in interactions between plants (Bever et al. 2010; Wipf et al. 2019). These interactions can rely on changes in inoculum density and diversity or more direct interactions triggered by the formation of common mycorrhizal networks (Giovannetti et al. 2004) when neighbouring plants are colonized by the same fungi (Jakobsen and Hammer 2015). This connection can either lead to facilitation or competition through differential access to the nutrient pool from the common hyphal network (Jakobsen and Hammer 2015; van der Heijden and Horton 2009) and eventual plant-to-plant nutrient transfers (He et al. 2009).

The roots of trees associated with crops generally compete for water and nutrients within the same rooting zone. In response to crop competition, a few studies have shown that tree roots preferentially explore soil areas inaccessible to crop roots, particularly at depth, but also in the cultivated alley, thus leaving topsoil horizons free for the propagation of crop roots (Dupraz and Liagre 2008; Mulia and Dupraz 2006). However, other studies have shown that the exclusion of tree roots from the topsoil in the alley was not as clear and that coexistence between roots of trees and crops occurred in both upper and deep soil layers (Cardinael et al. 2015), allowing possible interactions through mycorrhizal fungi. Fungal abundance and diversity are generally higher in multispecies ecosystems than in monospecific stands, benefiting from aboveground diversity through high root densities and increasing chances of host matches (Burrows and Pfleger 2002). In AC systems, the coexistence of different plant components may enhance AM diversity. Furthermore, the presence of a perennial

system (trees and HVbT) may support the maintenance of an active mycorrhizal inoculum, enhancing root colonization of the annual crop and being favourable for crop development and facilitative mechanisms between trees and crops (Bainard et al. 2011, 2012; Ingleby et al. 2007; Upson and Burgess 2013). In modern AC systems, spontaneous or sown vegetation in the tree rows creates permanent herbaceous understory strips that could also play an important role in the maintenance of AM communities. However, the impacts of trees and HVbT on AM fungal dynamics and their implications related to crop nutrition and yields are poorly documented in the temperate zone (Cardoso et al. 2003). Studies of AM fungi conducted at the community level in temperate AC systems are limited to those of Bainard et al. (2011, 2012), Chifflot et al. (2009) and Lacombe et al. (2009) and Furze et al. (2017), who showed a positive effect of trees (or hedgerows in Holden et al. (2019)) on AM abundance and diversity compared to conventional mono-cropping fields. However, the role of HVbT on the activity and diversity of AM communities remains poorly understood.

As symbiotic processes operate at relatively short distances from roots, AM-mediated interactions between plants of the AC system are conditioned to the proximity of fine roots, AM colonization synchronism and overlapping community similarity. Our study aimed to verify these prerequisites by examining the spatio-temporal distribution of trees, HVbT and crop root systems and their associated AM fungi in terms of abundance (root mycorrhization rate and extra-radical hyphal production) and diversity (metabarcoding). We hypothesized that (1) compared to HVbT, trees produce longer lateral roots, creating a larger common rooting coexistence zone with crops, (2) AM diversity and abundance are higher close to the tree row, affecting AM colonization of crop roots, and (3) trees, HVbT and crops share a common AM community. These hypotheses were tested in two different alley-cropping systems (different soils and tree ages) to identify general trends in AM community activities depending on their distance from the tree row.

Materials and methods

Study sites

The study was conducted over a one-year period (from December 2016 to December 2017) in two alley-cropping systems aged 5 (Pamiers, Ariège) and 11 (Noilhan, Gers) years, both located in southwestern France. The climate is semi oceanic temperate (Cfb, Köppen and Geiger) with a mean annual rainfall of 751 mm and a mean annual temperature of 12.4 °C (1982–2012 average). Both tree stands comprise a mixed planting of black walnut (*Juglans nigra* L.) and other high value hardwood tree species (*Fraxinus excelsior* L., *Sorbus torminalis* L., *Sorbus domestica* L., *Prunus avium* L., *Acer pseudoplatanus* L.). The study focused on walnut trees because their black roots are easily recognizable among the roots of other plant species. Furthermore, walnuts are the predominant tree species in current European agroforestry systems (Wolz and De Lucia 2018). Both fields were surface tilled to a depth of approximately 10 cm in September 2016, and soft wheat, a traditional cereal in this region, was sown in December 2016. No fertilizer or phytosanitary treatments were applied in 2017. The field characteristics and soil properties are presented in Tables 1 and S1, respectively.

Field sampling design

To examine spatial variation in root and hyphal densities, sampling zones consisted of 5 (at Pamiers) to 6 (at Noilhan) positions from the trunk of five walnuts (five replicates) chosen at random in tree rows. In each zone, measurements were performed along two transect lines perpendicular to the tree row at a distance of:

- 0.1 m (called the "*Tree*" position), 1 m (called the "*Tree-crop*" position), 3 m (called the "*Crop*" position at Pamiers and "*3 m from tree*" at Noilhan) and 11 m (called the "*Crop*" position only at Noilhan) from the walnut trunk to the middle of the inter-row (along the tree-crop transect),
- 0.1 m (called the "*HVbT*" position) and 1 m (called the "*HVbT-crop*" position) from halfway to the neighbour tree to the middle of the inter-row (along the HVbT-crop transect) (Fig. 1).

Positions at 3 m from the walnut trunk at Pamiers and at 11 m from the walnut trunk at Noilhan were considered as the "Crop" position because the distance from the tree was sufficient to avoid tree root influence on crop development. For each position, roots and hyphae were collected in two soil layers (0–10 cm and 20–

Table 1 Pamiers and Noilhan field characteristics

	Pamiers	Noilhan
Location	43°07′28.89″N 1°38′25.35″E	43°31′18.49″N 0°56′02.27″E
Elevation (m)	294	205
Field area (ha)	2.2	5.7
Stand age (years)	5	11
Mean walnut tree height (m)	2.21	6.16
Mean walnut tree circumference* (cm)	9.4	18.6
Inter-row spacing (m)	14	22
Intra-row tree spacing** (m)	9	6 to 8
Uncropped row width (m)	1.5	1.5
Dominant herbaceous species in uncropped rows (frequency > 90%)	*Digitaria sanguinalis* L. Scop.	*Festuca arundinacea* Schreb.
Tree rows orientation	east-west	north-south
Previous crop	*Medicago sativa L. subsp.*	*Trifolium repens L.*

*tree trunk circumference was measured at 130 cm height

**distance between two adjacent trees, in the tree row

30 cm) on four dates (December 2016, March, July and October 2017 corresponding to the beginning of winter, spring, summer and autumn respectively).

During the experiment, soil temperature was monitored with 18 thermocron sensors (DS 1922 L iButton thermochron, Maxim Integrated, San Jose, CA-USA) installed at both sites, in three positions (0.1 m, 1 m and 3 m from three walnut trunks) and two soil depths (5 and 25 cm).

Root and hyphal sampling and processing

Standing walnut, HVbT and wheat fine root length density (FRLD; cm cm^{-3} soil; diameter < 2 mm) and production (FRLP; cm cm^{-3} soil month^{-1}; diameter < 2 mm) were estimated by sequential soil coring and ingrowth core methods, respectively (Jourdan et al. 2008) for each site, date, position and soil layer. Soil cores were taken at Pamiers (4 dates × 5 positions × 2 soil layers × 5 replicates = 200 soil cores) and at Noilhan (4 dates × 6 positions × 2 soil layers × 5 replicates = 240 soil cores) using a root auger (8-cm diameter, 10-cm deep). All living roots were removed from each soil core and were stored in plastic bags at 4 °C until they were processed (within one week). Each root sample was washed free of soil and separated into walnut (black, highly branched and hard), HVbT (light brown to red, difficult to break) and wheat (white, poorly branched and soft) sub-samples. All sub-samples were scanned

and analysed for their total length using Analyra software (CIRAD, Montpellier, France). The fine root length density was calculated by dividing the root length by the soil core volume (502.4 cm^3). Three (out of 5) replicate sub-samples were then divided into two portions: one (a random subset of twelve 1-cm-long root segments) was submerged in 60% ethanol and stored at 4 °C before being coloured for AM fungal colonization determination, and the other was stored at −20 °C until DNA extraction and subsequent molecular analyses of AM fungal communities (Jalonen et al. 2013).

Immediately after removing the root fractions from each soil core, root ingrowth mesh bags made of 4 mm polyethylene mesh of approximately 500 cm^3 (Ø 8 cm and 10 cm long) were filled with the fresh root-free soil fraction, reinstalled in the corresponding sampling hole and marked with plastic sticks for easy location. All bags were retrieved after two to four months of ingrowth (in March, July, October and December 2017), placed in plastic bags and stored at 4 °C until analysis. New, freshly established core holes were dug the same day close to previous sampling holes, and new bags were installed according to the method described above. The retrieved bags were expected to reflect growth effects directly related to the 2–4 months' growth phase considered. All roots were collected from each bag (no fine root necromass was observed in the ingrowth cores), washed thoroughly in distilled water, separated by species and scanned as described above to determine FRLP.
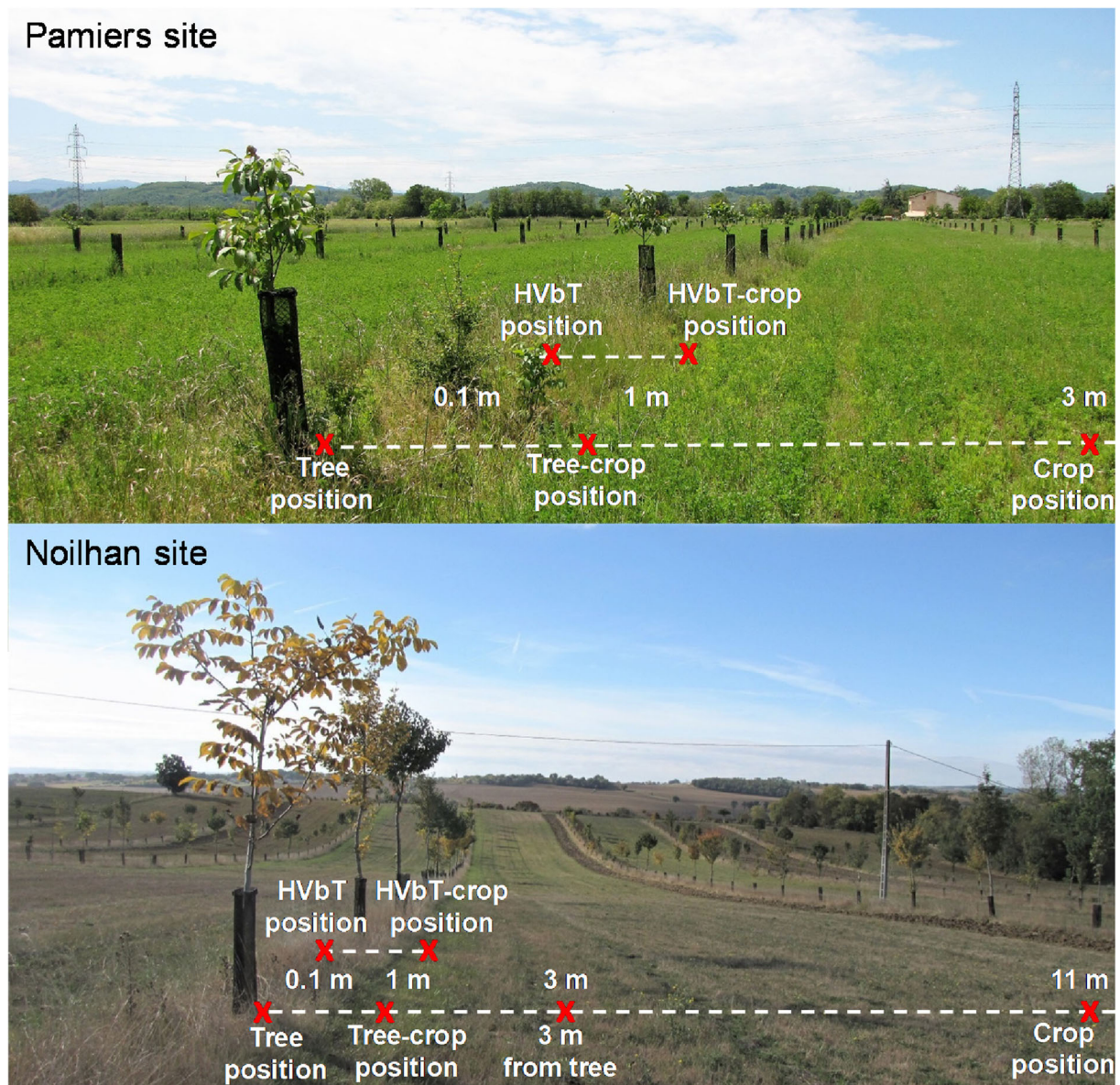
Fig. 1 Sampling positions along two transects in the Pamiers (5 positions per tree) and Noilhan (6 positions per tree) alley-cropping systems

The production of extra-radical AM hyphae (hyphal length production, HLP) was determined using hyphal ingrowth bags made from 30 μm nylon mesh (Buisine, Clermont de l'Oise, France) adapted from Olsson and Wilhelmsson (2000) and Wallander et al. (2001). The 30-μm- porosity allows only hyphal ingrowth but excludes roots and is in line with the range of 25–50 μm most commonly used (Ekblad et al. 2013). Each bag was filled with 120 g of acid-washed quartz sand to minimize organic matter and thus saprotrophic hyphal growth into the bag (Bakker et al. 2015) and sealed

using a sewing machine. The contact area with the soil of each individual ingrowth bag was approximately 140 cm^2 (10 cm long × 7 cm wide). Field installation of the nylon mesh bags was performed on the same occasions and locations as root-sampling campaigns (December 2016, March, July and October 2017) in core holes dug at a distance of 10–20 cm from holes where root core samples were taken. Two hyphal mesh bags were installed vertically in each hole, in both the 0–10 cm and in the 20–30 cm soil layer. All hyphal mesh bags were collected by hand at the same time as root

ingrowth mesh bags (in March, July, October and December 2017), wrapped in plastic bags and kept intact at 4 °C until sample processing. New hyphal mesh bags were immediately placed in the field in the same manner as in the previous mesh bag installation, giving a total of 200 hyphal mesh bags at Pamiers (4 dates × 5 positions × 2 soil layers × 5 replicates) and 240 bags at Noilhan (4 dates × 6 positions × 2 soil layers × 5 replicates). Hyphal length was assessed by a modified line intersection method (Wallander et al. 2004). For each hyphal mesh bag, a 30 g sand aliquot was placed in a vial with 80 ml of deionized water and shaken for 1 min. Then, 4 ml of the solution was transferred to a Petri-dish, and hyphal lengths were observed with the aid of a binocular microscope (60–160X magnification) and an optical monocular glass with a regular grid. Intersections of the grid (interline distance of 1 mm) were used to calculate HLP (expressed in cm cm^{-3} of sand month^{-1}) as described by Tennant (1975).

Determination of AM fungal colonization

Sampled root fragments (3 replicates of 12 fragments for each date, site, position, soil layer and species) were cleared in 10% KOH at room temperature for one night and rinsed with distilled water. Highly pigmented walnut roots were additionally cleared in 70 °C in a water bath for 40 min with 3% w/v H_2O_2 (10 volumes) and rinsed with distilled water. Root fragments were then stained with Schaeffer black ink as described in Vierheilig et al. (1998). Excess colour was removed by immersing the samples in lactoglycerol. Thereafter, the samples were immersed in a mixture of 50% glycerol and 50% water. Roots were mounted onto microscope slides and examined under 200-800X magnification. The number of sections where mycorrhizal arbuscules, vesicles or hyphae were observed was noted separately for each structure type. For each sample, the frequency of mycorrhiza in the root system (F%), root mycorrhization rate (M%), and arbuscular (A%) and vesicular (V%) abundance in colonized root sections were evaluated according to Trouvelot et al. (1986) using the MYCOCALC (http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html) programme.

Arbuscules are considered as the main interface for nutrient transfer between the mycorrhizal fungus and the host plant (Bonfante and Genre 2010; Smith and Smith 1990).

Assessment of walnut, HVbT and wheat fine root distributions

At Noilhan, pits (5.5 m long × 1 m wide × 1.3 m deep) were dug around three walnuts (3 replicates) perpendicular to the tree-row in July 2017 after crop harvest. Fine roots of walnut, HVbT and wheat were observed on each 5.0 m × 1.0 m pit wall that extended from the tree trunk to the middle of the cultivated alley using the root intersect method (Battie-Laclau and Laclau 2009). Roots were exposed using a knife to remove surrounding soil. Each vertical trench wall was divided into 5 cm × 5 cm grid cells in which the number of fine root intersects of each species with the vertical plane was counted.

Molecular analysis of AM fungal communities

AM fungal communities were analysed on roots of the three AC components (walnut, wheat and HVbT) sampled in soil cores taken at 10 cm depth, on transects across three and four positions at Pamiers and Noilhan, respectively, along the tree-crop transect: "Tree", "Tree-crop", "Crop" (3 m and 11 m from the walnut tree at Pamiers and Noilhan) and "3 m from tree" at Noilhan). To follow seasonal changes of AM communities, analyses were performed on two sampling dates (March and July), with three repetitions per AC component x position x site and sampling date.

DNA extraction and sequencing

For each sample, roots were ground in liquid nitrogen and DNA extraction was conducted on 100 mg using the MP DNA kit for plants (MP Biomedicals, Europe), according to the manufacturer's protocol. To cope with roots with high polyphenol contents, such as walnuts, a purification step with insoluble polyvinylpolypyrrolidone (PVPP, ref. P6755, Sigma Aldrich) was added before step 4 of the manufacturer's protocol. One ml of the supernatant free of proteins (step 3) was mixed with 70 mg of PVPP and shaken end-over-end (23 rpm, 20 min) at room temperature. The solution was centrifuged (14,000 g, 10 min), and the colourless supernatant was transferred into a new tube for DNA binding (step 4). DNA concentrations were measured by fluorescence using PicoGreenTM (Molecular Probes, Carlsbad, New Mexico).

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Amplicon libraries were constructed following a two-step PCR protocol adapted from the Illumina 16S Metagenomic Sequencing Library Preparation Guide (Ref. 15,044,223 Rev. B). Briefly, a first round of PCR was performed with locus-specific primers with 5′ nucleotide overhangs. These extensions aimed at anchoring a second round of PCR that introduced indices and completed Illumina adapters. The first round of PCR was carried out as follows: for each sample, two replicated dilutions of 7.5 ng.μl^{-1} were used for PCR amplification of the nuclear 18S rRNA gene primers NS31 [5′- TTGGAGGGCAAGTCTGGTGCC -3′] (Simon et al. 1992) and AML2 [5'GAACCCAAACACTT TGGTTTCC -3′] (Lee et al. 2008) with overhangs. PCRs were performed in a total volume of 25 μl with 2 μL DNA, 1.25 μL of each specific primers (10 μM), 12.5 μL PCR MasterMix Platinum Super-Fi (Thermo Fisher, Massachusetts, Etats-Unis), 1 μl BSA, 7 μl of DNase-free water (BioRad, California) with the following cycling conditions: 98 °C for 4 min; 30 cycles of 98 °C for 30 s, 66 °C for 30 s, 72 °C for 1 min, and a final elongation step at 72 °C for 10 min. After magnetic bead purification (Clean PCR, Proteigene, France), the second round of PCR was performed using a Nextera® XT Index Kit (Illumina, San Diego, USA) following the manufacturer's instructions. After a purification with magnetic beads, these final PCR products were multiplexed and sequenced on a MiSeq Illumina sequencer using MiSeq Reagent Kit v3 (600-cycle; Illumina).

Sequencing data processing

The pipeline used to analyse the sequences was developed following Taudière et al. (2018). Having too short an overlap between R1 and R2 sequences, only R1 sequences were used for the analyses. Briefly, quality was filtered using sickle (the command *single se*, with a -*q* threshold of 33), each sequence was reassigned to its sample, and primers and tags were suppressed (fastx; *fastx_trimmer*) before the data set was dereplicated using SWARM. Chimeras were deleted using UCHIME (*uchime_ref,* Edgar et al. 2011) and the Glomeromycotina specific database, MAARJAM (Öpik et al. 2010). From 10,046,446 R1 sequences retained after the quality filtering, 1,024,097 unique sequences were obtained (as well as 174,026 chimaeras). Sequences were grouped into operational taxonomic units (OTUs, or "molecular species") at 97%

similarity using Uparse (*cluster_otus*), and taxonomy was assigned using the MAARJAM database (using qiime; assign_taxonomy.py; Caporaso et al. 2010).

Plant sampling

Crop aerial biomass and plant height were measured at harvest (in July 2017) at three positions (*Tree-crop*, *HVbT-crop* and *Crop*) at both sites with three replicates. At each position, three consecutive strips of wheat of 1 m long each were cut by hand at the soil surface. Plant samples were air-dried at 65 °C to constant weight. In addition, the plant height was measured, and the grain was threshed and weighed. Yield values were reported on a per hectare basis.

Statistical analyses

Statistical analyses were performed in SAS for the entire experimental period (from December 2016 to December 2017). A general linear model procedure was used in three-way analysis of variance (ANOVA) to test for differences due to site, date, position and their interaction in FRLD, FRLP and root AM colonization parameters (M%, A%, V%). Individual three-way ANOVAs were performed for each AC component (wheat, HVbT and walnut) and each soil layer (0–10 cm and 20–30 cm). In addition, one-way ANOVAs were used for each soil layer to test differences in total FRLD (sum of wheat, HVbT and walnut FRLD) between positions at each date. One-way ANOVAs were also used for each soil layer to test differences in total FRLD between dates at each position, as well as to test differences in the mycorrhization rate (M%) and arbuscular abundance (A%) of walnut, HVbT and wheat fine roots between positions at each site, for each sampling date and each soil layer. Moreover, differences in M% and A% between AC components (walnut, HVbT and wheat) at each position in each soil layer of each site were also tested using one-way ANOVAs. For each soil layer at each site, one-way ANOVAs were used to test differences in hyphal length production (HLP) between positions in each season, as well as between seasons at each position. Homogeneity of variances on each date was tested by Levene's test. Normality was tested with the Shapiro-Wilk W test. For FRLD and FRLP, a log-transformation was used to normalize the data and variances. When the assumption of normality was not met, the nonparametric Kruskal-Wallis test was used. The

probability level used to determine significance was $P < 0.05$.

Sequencing data were analysed using R 3.4.3 (R Core Team 2013) and the Phyloseq 1.12.2 packages (McMurdie and Holmes 2013). Alpha-diversity (i.e., local diversity) was evaluated by Hill numbers ('vegan' package 2.3–4, Oksanen et al. 2016): qH with q = 0 (species richness), q = 1 (exponential of Shannon's index) and q = 2 (inverse of Simpson's concentration index). Hill's diversity series is an intuitive measure of diversity in which the q order of diversity indicates its sensitivity to species abundance. The effect of variables (site, date, position, or vegetation type) on diversity (Hill indices) was tested by ANOVA. To take into account differences induced by the number of sequences per sample, the square root of the number of sequences was introduced as the first factor in the linear models of diversity measures (Bálint et al. 2015). Hill indices were compared between modalities (sites, dates, positions, and vegetation type) by Tukey post hoc tests. Community composition between samples was compared by calculating Bray-Curtis dissimilarity indices, which were used to produce a two-dimensional non-metric representation (NMDS) of communities ('vegan' 2.3–4 package; Oksanen et al. 2016). To test for differences in community composition between plant species and position at each sampling site, permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis matrices was performed using the vegan package ('vegan' package 2.3–4, Oksanen et al. 2016). To test for specific differences between communities of plant species or positions, pairwise PERMANOVA analyses were performed using the pairwise Adonis package (Martinez-Arbizu 2019).

Results

Seasonal and spatial variations in root length density (FRLD) and production (FRLP)

At both sites, regardless of dates, positions and AC components (i.e., wheat, HVbT and walnut), FRLDs were globally higher in the surface (0–10 cm) than in the deeper (20–30 cm) soil layer (Fig. 2). Independent of the date and soil layer, significant increases ($P < 0.05$) with increasing distance from the tree row for wheat FRLD were observed at both sites (Fig. 2 and Table 2). Lateral roots of HVbT colonized the cultivated alley up

to 1 m from the middle of the uncropped row (*Tree-crop* and *HVbT-crop* positions), whereas walnut roots reached 1 m from the trunk (*Tree-crop* position) at Pamiers (the young site, Fig. 2a, b) and 3 m from the trunk (*3 m from tree* position) at Noilhan (the mature site, Fig. 2c, d). No walnut roots were found in the *HVbT* position at either sites, but a few colonized the *HVbT-crop* position in October at Noilhan (Fig. 2c, d). During the crop-growing season, the three AC components were simultaneously present only in the *Tree-crop* position at both Pamiers and Noilhan. At Noilhan, *Tree* and *HVbT* positions presented the highest total FRLDs (total wheat, HVbT and walnut FRLDs) due to high HVbT FRLD registered throughout the year (reaching 2.4 cm cm^{-3} and accounting for more than 80% of total FRLD; Fig. 2c and d). At Pamiers, the same trend was observed in December and October, but in March and July, the highest total FRLDs were registered in *Tree-crop* and *HVbT-crop* positions due to high wheat FRLDs. HVbT root production occurred during all years (with a peak FRLP in autumn at Pamiers and in spring at Noilhan), whereas roots only grew in winter and spring for wheat plants and in spring and summer for walnut trees (Fig. 2, Fig. S1).

Root distribution down to a depth of 1 m

At the crop harvest at Noilhan, independent of AC components, roots were concentrated in the surface soil layers along the 1-m-deep vertical trench walls (Fig. 3): 72%, 67% and 64% of the total wheat, HVbT and walnut root intersects, respectively, were observed in the 0–30 cm layer. The maximum lateral distance from the tree trunk reached by walnut and HVbT roots on the three trench walls was 3.75 m and 2.60 m respectively. Only a few wheat roots were observed in the tree row and nearby tree row/inter-row interface.

Seasonal and spatial variations in root AM fungal colonization

Roots of all AC components were highly colonized by AM fungi, wheat, HVbT and walnut M% and A% reaching 76%, 72%, 70% and 89%, 78%, 66%, respectively. Wheat M% was strongly influenced by position at Noilhan in the upper soil layer (Fig. 4). The same trend was observed in the lower soil layer and at Pamiers (Table 2, Fig. S2). Indeed, independent of the date and soil layer, wheat M% significantly increased at the row/
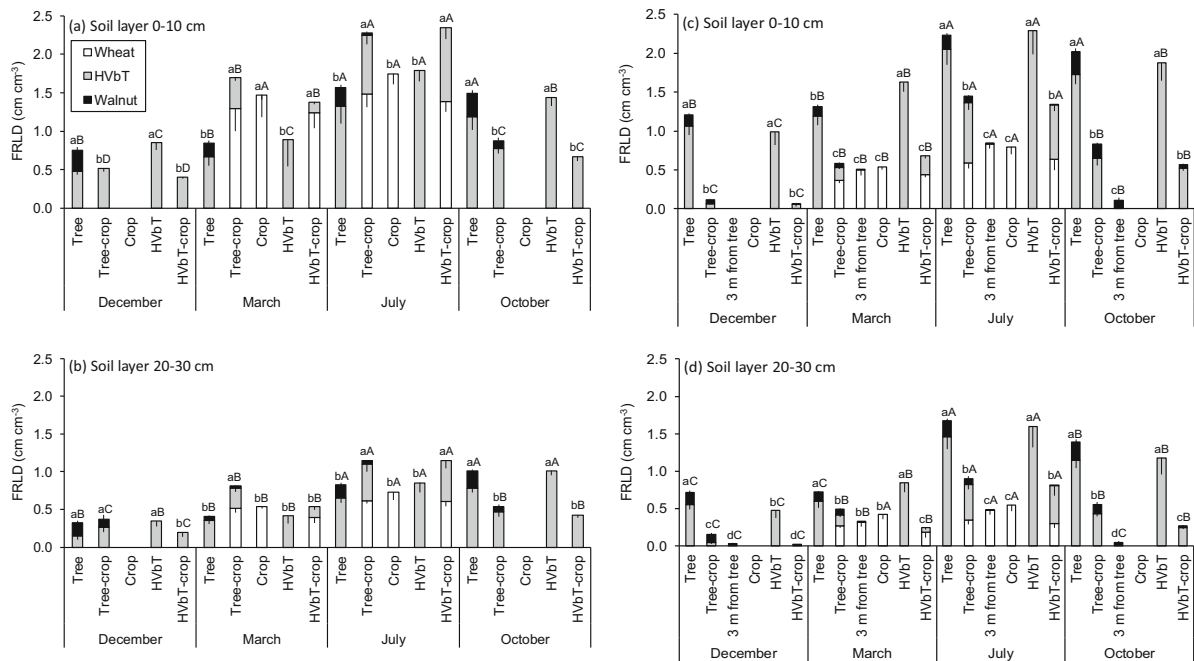
Fig. 2 Seasonal variations in fine root length density (FRLD) of wheat (crop), HVbT and walnut at different positions and soil depths at Pamiers (**a**, **b**) and at Noilhan (**c**, **d**). Vertical bars indicate standard errors of samples ($n = 5$). Lower cases indicate significant differences in total FRLD (sum of wheat, HVbT and walnut FRLD at each position) between positions ($P < 0.05$) on each date. Upper cases indicate significant differences in total FRLD between dates ($P < 0.05$) at each position. Roots of the few wheat and weed plants that grew spontaneously in the cropped alley after the harvest in summer are not presented

inter-row interface (in *Tree-crop* and *HVbT-crop* positions) compared to *3 m from tree* and *Crop* positions at both Noilhan (Fig. 4, Fig. S2C, Fig. S2D) and Pamiers (Fig. S2A, Fig. S2B). At both sites, in the *Tree-crop* position, roots of HVbT presented higher values of M% than walnut roots throughout the year (P-values not shown). The same trend was observed for A% in December and March. Indeed, the development of arbuscules in walnut roots occurred only from July onwards (after the budburst of walnut trees, Fig. 2, Fig. S2). Whereas HVbT and walnut A% tended to increase in July and October compared to December and March (Table 2, Fig. 2, Fig. S2), walnut V% decreased and HVbT V% remained relatively constant (Fig. S2).

Seasonal and spatial variations in extra-radical hyphal length production (HLP)

At both sites, HLP was influenced by season and position (Table 2, Fig. 5). Irrespective of the position, HLP was globally higher in spring and autumn than in winter and summer. Over the entire experimental period at both

sites, HLP varied between 0.5 and 8 cm cm^{-3} of sand month^{-1} in the 0–10 cm soil layer and between 0.3 and 5 cm cm^{-3} of sand month^{-1} in the 20–30 cm soil layer. Globally, the *Crop* and *3 m from tree* positions were the least colonized positions by extra-radical hyphae. For each season, there was a positive linear correlation between HLP and total young roots produced and colonized by AM (data not shown). It must although be noted that the much lower nutrient availability inside the ingrowth bags than in the surrounding soil might bias the total estimations of total AM hyphal production (Hodge et al. 2001).

AM fungal communities

In total, 9,393,852 sequences could be assigned to the taxonomy rank of the family, classified into 155 OTUs (represented by at least 5 sequences). The mean sequence number per sample was 121,998.1 (sd 32,210.85), and one sample was suppressed because of low sequencing depth (<50,000). The thirty most abundant OTUs included 91.8% of all sequences but all 155 were included in the following analyses to consider the

Table 2 *P*-values for the effects of site (S, Pamiers, Noilhan), date (D, December, March, July, October), position (P, Tree, Tree-crop, 3 m from tree, crop, HVbT, HVbT-crop), interaction between site and date (S x D), site and position (S x P), and date and position (D x P) on wheat, HVbT and walnut fine root length densities (FRLD), mycorrhizal rate (M%), arbuscular (A%) and vesicular (V%) abundance of colonized root sections, and fine root length production (FRLP) in the 0–10 cm and 20–30 cm soil layers

	FRLD			M%			A%			V%			FRLP		
	Wheat	HVbT	Walnut	Wheat	HVbT	Walnut	Wheat	HVbT	Walnut	Wheat	HVbT	Walnut	Wheat	HVbT	Walnut
0–10 cm soil layer															
Site	***	ns	***	***	ns	***	***	***	ns	ns	***	ns	***	ns	***
Date	***	***	***	***	*	*	**	***	***	***	ns	***	***	***	***
Position	**	***	***	***	***	*	***	***	ns	ns	ns	**	ns	***	***
S x D	ns	***	*	***	ns	ns	***	***	ns	ns	ns	ns	***	***	***
S x P	ns	***	***	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	*	ns
D x P	ns	**	*	*	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	***
20–30 cm soil layer															
Site	***	ns	***	***	ns	***	***	***	ns	ns	***	***	***	*	***
Date	***	***	**	*	***	ns	***	***	***	***	ns	***	***	***	***
Position	***	***	***	***	***	*	***	*	ns	ns	ns	ns	**	***	***
S x D	ns	*	ns	***	ns	ns	**	**	ns	ns	ns	**	ns	***	***
S x P	ns	***	**	ns	ns	**	ns	ns	ns	ns	ns	ns	ns	ns	ns
D x P	ns	*	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns	***	***

FRLD are shown in Fig. 2, M%, A%, V% in Fig. 5 and Fig. A.1, A.2, A.3, FRLP in Fig. 3. As no roots grew (1) in "December", "October" and at the "Tree", "HVbT" positions for wheat, (2) at the "3 m from tree", "Crop" positions for HVbT and (3) at the "HVbT", "Crop" positions for walnut, these dates and positions for respective species were not considered for data analysis

P-values are indicated by asterisks (*** $P \leq 0.001$, ** $P \leq 0.01$, * $P \leq 0.05$) and *ns* ($P > 0.05$)

broad diversity. The taxonomic assignment of OTUs revealed a main affiliation with Glomeraceae followed by Claroideoglomeraceae, Paraglomeraceae, Diversisporaceae, Archaesporaceae, Gigasporaceae and Ambisporaceae (Fig. S3). Glomeraceae was the most abundant family (in terms of sequence number) on all vegetation types, followed by Paraglomeraceae on wheat and HVbT, and low abundance on walnut roots. Among these 155 OTUs, 101 were shared by the three vegetation types and 128 OTUs by at least two of them, particularly between wheat and the HVbT (sharing respectively 83.5 and 94.5% of their OTUs, respectively). The community composition differed between sites as shown on the NMDS based on Bray-Curtis dissimilarities (Fig. 6). At both sites, AM community composition was significantly different between vegetation components but not sampling positions (PERMANOVA, Table S4). More specifically, sampling position did not impact the wheat AM community composition at any

site (Fig. 6; PERMANOVA *p* value = 0.511). OTU richness and diversity were affected by the site, the sampling date and position (Hill indices, Fig. 7, Table S2). Species richness (H0) was significantly higher at Noilhan than at Pamiers (Table S2, post hoc Tukey test p value <0.05). Sampling date affected all three Hill's indices, with higher values in spring than in summer (Table S2, post-hoc Tukey tests p value <0.01). Considering all AC components, AM fungal species richness was significantly affected by sampling position, with higher diversity at the *Crop* position than at the *Tree* or *Tree-crop* position (Fig. 7). A special focus on wheat showed that AM species richness was not significantly impacted by position (Table S3, Fig. S4). AM communities of wheat were particularly affected by the sampling date, with a significant drop in species richness in summer (ANOVA p value <0.005;
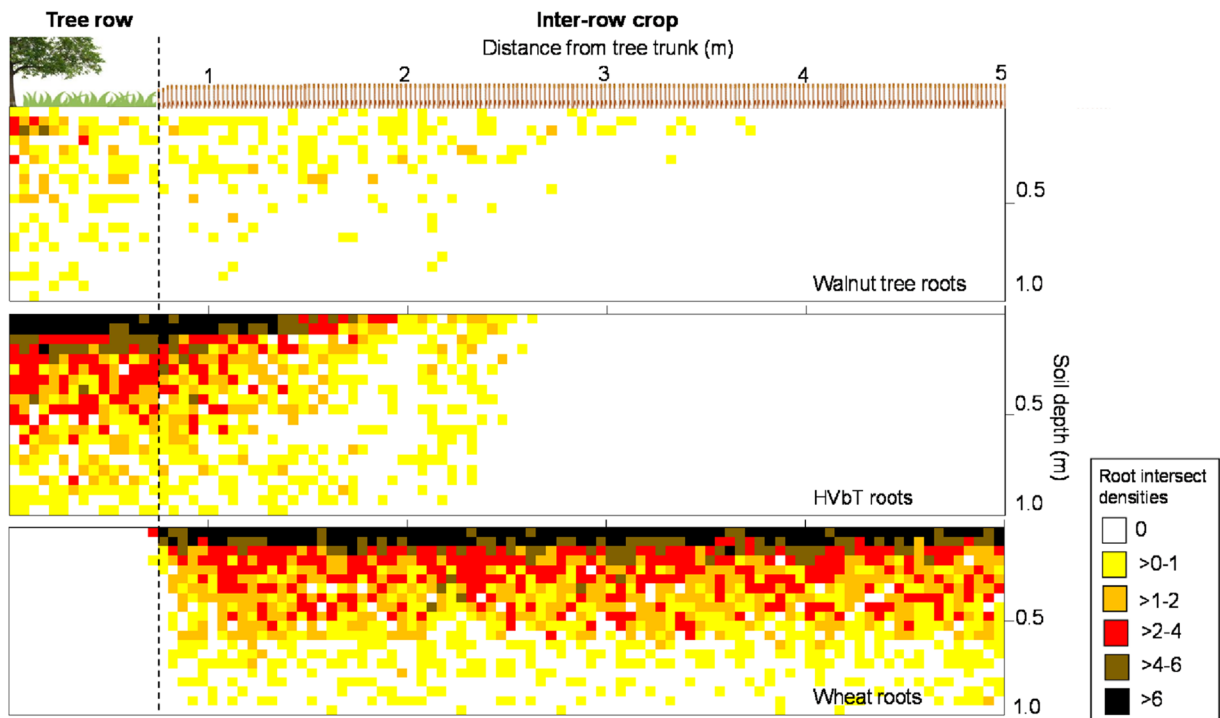
Fig. 3 Mean number of fine root intersects ($n = 3$) of roots of walnut, HVbT and wheat in each grid cell of 25 cm2 on a vertical trench wall in the agroforestry plot of Noilhan one week after crop harvest in July 2017

Table S3), whereas communities of HVbT and walnut were not affected significantly by the season when considered separately (Table S3).

Variations in soil temperature, plant height, biomass production and grain yield

Seasonal variations in soil temperature were similar at both sites over the entire experimental period (from December 2016 to October 2017) with a minimum registered in January (0.5 °C at Pamiers and 1 °C at Noilhan) and a maximum in June (26.5 °C at Pamiers and 30 °C at Noilhan) (data not shown). The soil depth affected the soil temperature: the surface layer and the 20–30 cm layer differed by 1.5 °C, the surface layer being warmer in summer and colder in winter. At both sites and independently of soil depth, the soil temperature at the *Crop* and *Tree-crop* positions was one degree higher than at the *Tree* position ($P < 0.05$).

Wheat plant height, biomass and yield measured at the *Tree-crop* position significantly decreased by 14%, 8% and 19%, respectively, at Pamiers and by 10%, 17% and 19% at Noilhan compared to the Crop position ($P < 0.05$). No significant difference was found between Tree-crop and HVbT-crop positions (data not shown).

Discussion

Influence of tree row on spatio-temporal root distributions in a walnut/wheat AC system

Regardless of AC components, positions and soil characteristics in both our agroforestry systems, vertical fine root profiles *were affected by soil depth following the usual pattern of steadily* decreasing in root densities along a vertical soil profile (Cardinael et al. 2018; de Kroon et al. 2012; White and Kirkegaard 2010; Zhang et al. 2015; Duan et al. 2017; Hodgkinson et al. 2017). Walnut and wheat FRLDs were within the range of values reported for temperate AC systems (e.g. Duan et al. 2017; Mulia and Dupraz 2006; Zhang et al. 2015), but HVbT was not included in these previous studies. As expected, a significant influence of tree rows on the horizontal distribution of companion crop roots was also observed at both sites. Our data corroborate previous studies conducted in temperate AC systems in which the amount of wheat roots increased with the distance from the tree row due to the decrease in belowground inter-specific competition (Duan et al. 2017; Mulia and Dupraz 2006; Zhang et al. 2015). Indeed, despite annual soil tillage and root destruction in the top 10 cm layer,
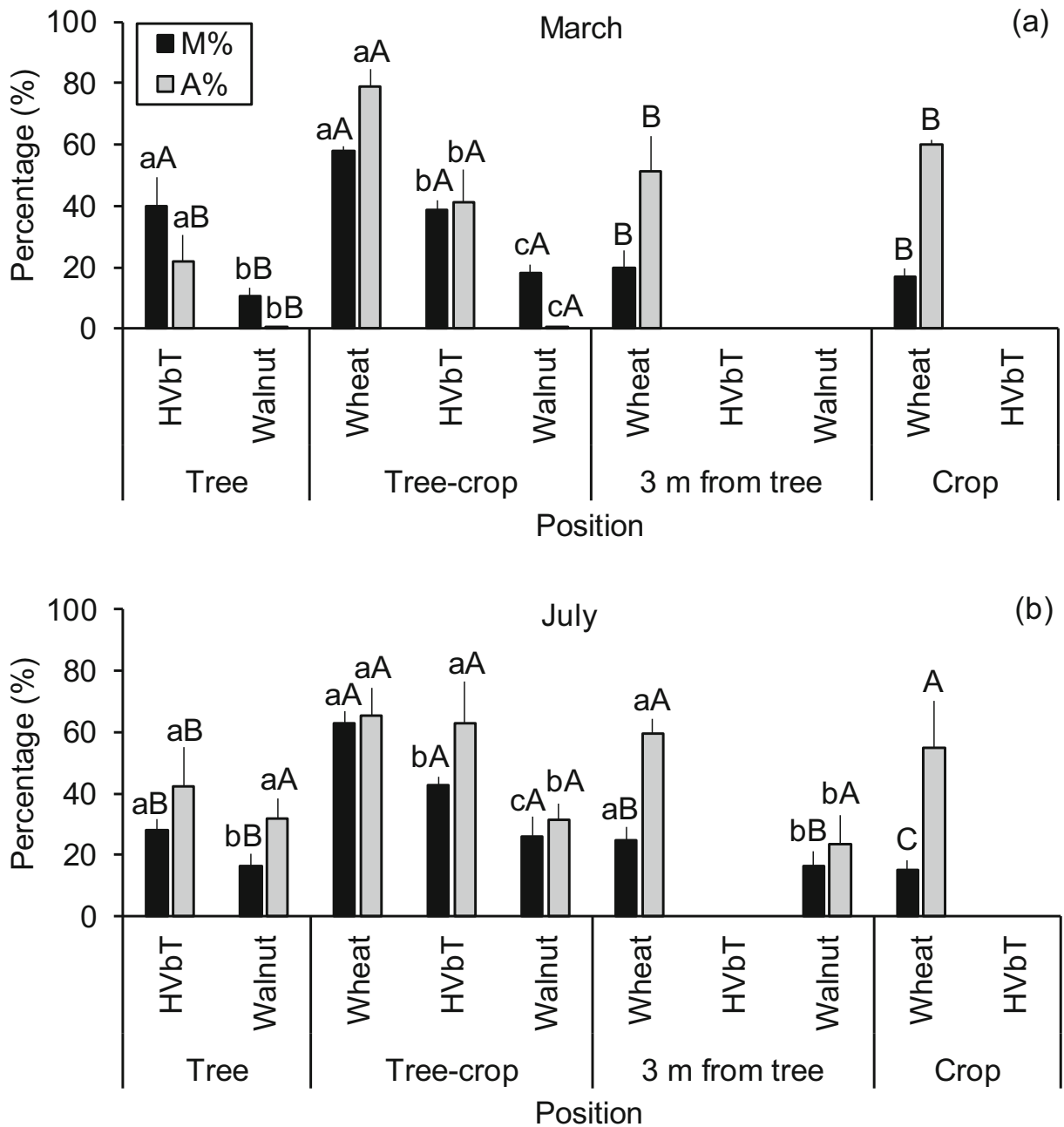
Fig. 4 Mycorrhization rate (M%) and arbuscular abundance (A%) of walnut, HVbT and wheat fine roots extracted from soil cores at different positions at Noilhan in the 0–10 cm soil layer in March (**a**) and July (**b**). Vertical bars indicate standard errors of root samples (n = 3). Lower cases indicate significant differences in M% and A% between AC components (walnut, HVbT and wheat) (P < 0.05) at each position. Upper cases indicate significant differences in M% and A% between positions (P < 0.05) for each AC component

6 m tall trees (at Noilhan) and to a lesser extent HVbT rapidly colonized the first metres of cropped alleys creating a large zone of coexistence between AC components along the row/inter-row interface. At Pamiers, lateral root extension of the 2 m tall trees was limited to

the first metre from the tree trunk due to their lesser stage of development. However, tree and HVbT roots did not spread underneath the wheat rooting zone as described in the literature (Dupraz and Liagre 2008; Mulia and Dupraz 2006) but intermingled with crop
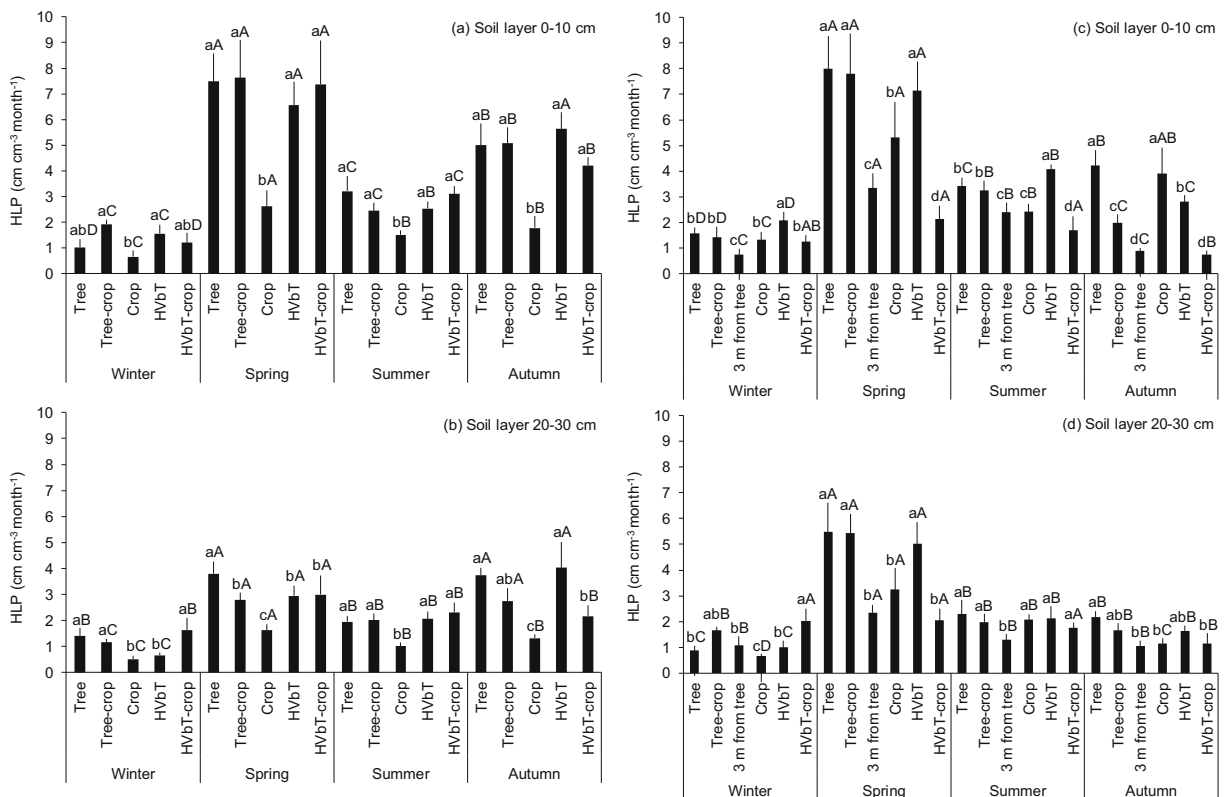
Fig. 5 Seasonal variation in extra-radical hyphal length production (HLP) at different positions and soil layers at Pamiers (**a**, **b**) and at Noilhan (**c**, **d**). *Winter*, *Spring*, *Summer* and *Autumn* refer to the December–February, March–June, July–September and October–November periods, respectively. Vertical bars indicate standard errors of samples ($n = 5$). Lower cases indicate significant differences in HLP between positions ($P < 0.05$) in each season. Upper cases indicate significant differences in HLP between seasons ($P < 0.05$) at each position

roots in the upper soil layers. Although sole tree systems tend to show denser, larger and shallower root systems compared to intercropped trees (Cardinael et al. 2015; Mulia and Dupraz 2006; Zhang et al. 2015), a significant proportion of our 6 m tall walnut tree roots was found in the topsoil rooted by the wheat crop. In contrast, intercropped trees of a similar stage of growth (7 m tall walnut trees) showed a high degree of plasticity, extending their root system laterally below the wheat crop zone (Mulia and Dupraz 2006). These contrasting findings may be due to differences in sampling date, tilling depth, crop FRLDs, and soil and climate conditions, and further work is needed to gain insight into plant plastic responses to intercropping. Interestingly, no walnut roots could be found in the top 30 cm soil layer at *HVbT* positions, while trees exhibited superficial roots at the same distance from the tree trunk at the *HVbT-crop* position at the end of the walnut root growing period (in October, at Noilhan). This spatial distribution suggests a relative plasticity of walnut tree roots driven by the root density of neighbouring plants forcing trees to extend their lateral root system into less competitive zones. Indeed, the total FRLDs observed at the *HVbT* position were 2 to 3 times higher than those at the *HVbT-crop* position.

Unlike walnut and HVbT, wheat roots were strongly limited laterally along the row/inter-row interface following the crop root profiles described by Duan et al. (2017) and Mulia and Dupraz (2006). Indeed, as observed by Cardinael et al. (2018) in a 18-year-old agroforestry system, tree rows remained free of intercropped wheat roots over the entire crop-growing period at both sites. The intense rooting systems developed by HVbT and trees within tree rows induced a strong and permanent belowground competition preventing colonization and resource access of other species from adjacent habitats, such as annual crops. However, contrary to the general trend of a shallower distribution of intercropped crop roots compared with sole-cropping (Duan et al. 2017; Farooq et al. 2009; Zhang et al. 2015), the vertical
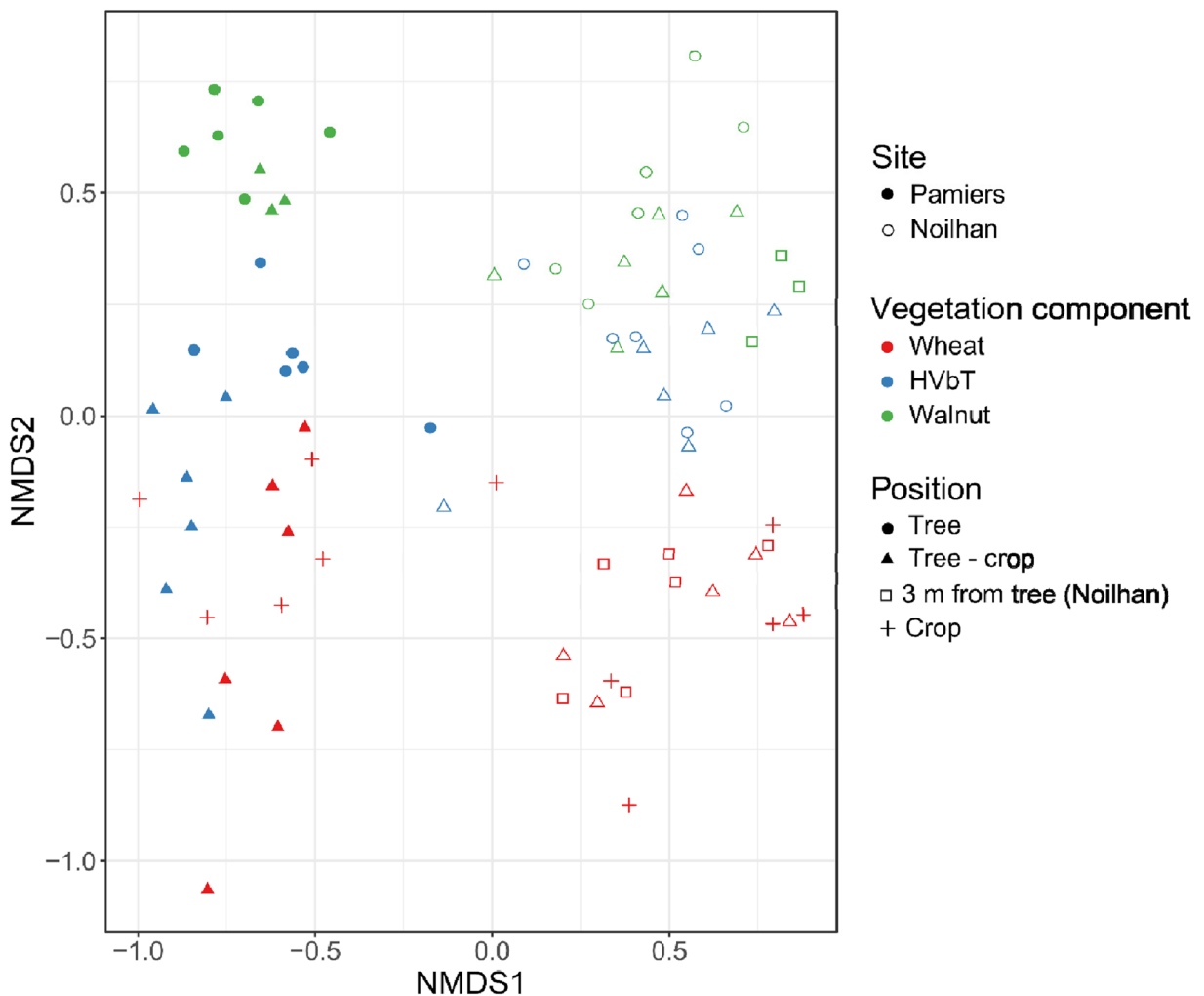
Fig. 6 Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities of the community composition of AM on roots of the three different AC components of alley cropping systems (tree: walnut, HVbT: various herbaceous plants; crop: wheat)

distribution of wheat roots remained relatively uniform irrespective of the horizontal position, including within the root zone of co-occurring species (from 1 m to 2.6 m from the tree trunk at Noilhan). The absence of spatial niche segregation between the three AC components may partly explain the negative impact on wheat development (height biomass and yield) measured up to 2 m from tree rows at both sites compared to the *Crop* position. Although these reductions could also be due to the negative effect of tree shading on the quantity and quality of light at the tillering stage, as reported by other authors (Bouttier et al. 2014; Kohli and Saini 2003; Sparkes et al. 1998), it did not seem to be the case as budburst of walnut occurs only in May and young trees still had a small canopy. The high degree of root intermingling between the three AC components rather

than underground niche differentiation suggests strong root competition and could better explain the decline of crop yield near tree rows (Cardinael et al. 2018; de Parseval et al. 2017).

In contrast to our expectations, root competition induced by tree rows on the crop appeared predominantly due to HVbT at both sites, producing two to eight times more roots than walnut trees at the *Tree* position. Although 6 m tall trees (at Noilhan) developed longer lateral roots (up to 3.75 m from the tree trunk) than HVbT (up to 2.60 from the tree trunk), walnut FRLDs were probably too low to affect the wheat rooting system. Indeed, no significant difference in wheat FRLDs was found between the *Tree-crop* position (colonized by walnut roots) and the *HVbT-crop* position (free of walnut roots) at both sites and soil depths. Likewise, no
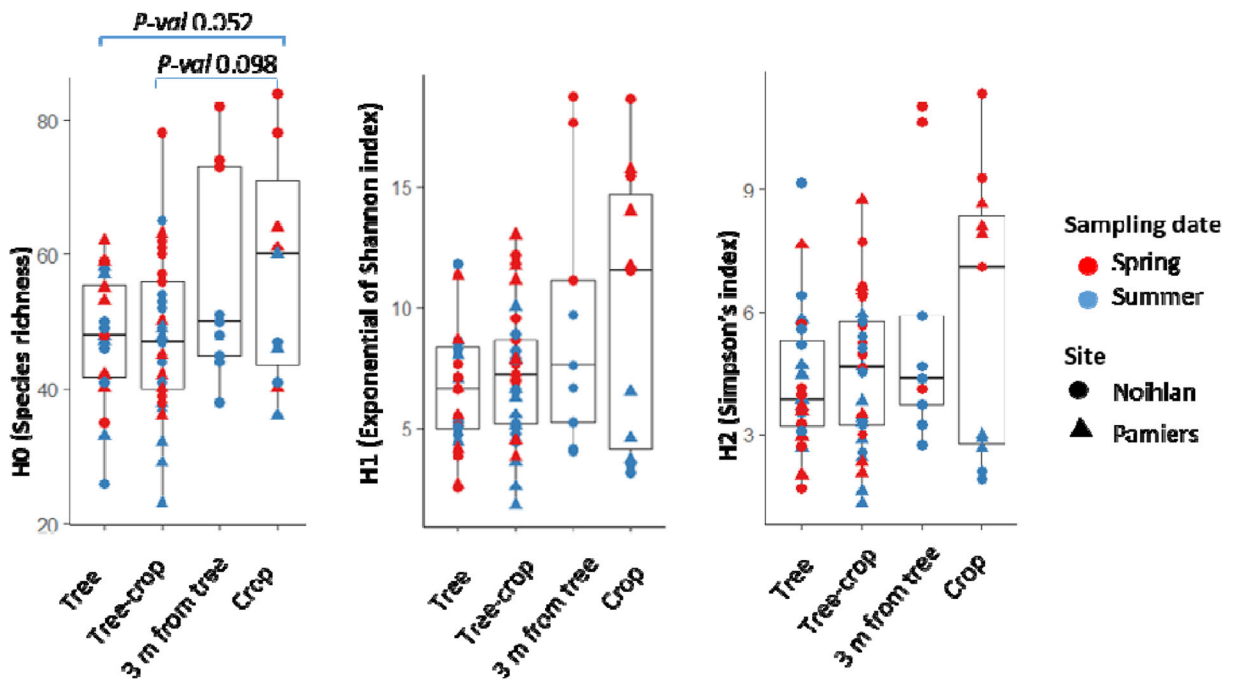
Fig. 7 Local diversity assessed by Hill's indices according to sampling positions at the two sites and two sampling dates. Sampling position had a significant effect on species richness (p-values of Tukey's post hoc tests are indicated on the graph)

significant difference in wheat FRLDs was found between the *3 m from tree* position (colonized by walnut roots) and *Crop* position (free of walnut roots) at the oldest site. Wheat was not affected by the presence of walnut roots. Furthermore, the growing season of wheat (from sowing in December to harvest in July) was synchronously associated with the dormancy of walnut (from leaf fall in October to leaf budburst in May). The same trend was observed by Germon et al. (2016) in a 20-year-old Mediterranean walnut/cereal AC system where walnut trees tended to invest in shallow roots only in spring and summer. Compared to the short period of growth of superficial walnut roots, HVbT presented a faster and denser development of fine roots throughout the year affecting crop growth more severely. These original results question the role of trees in AC systems over the first stages of tree development and show the importance of considering all AC components, including the HVbT, over space and time to better evaluate the mechanisms of interspecific interactions. However, our oldest site is only 11 years old, and the influence of trees is expected to increase further in the coming years of intercropping due to the development of a larger and denser root system.

Influence of tree rows on spatio-temporal AM colonization in a walnut/wheat AC system

In agreement with our second hypothesis, tree rows did impact AM fungi, in terms of abundance (root mycorrhization rate and extra-radical hyphal production), diversity and community composition. The crop root mycorrhization rate and extra-radical hyphal production were significantly reduced with increasing distance from tree rows. Agroforestry systems are known to induce strong spatial heterogeneity in soil functioning (Guillot et al. 2019). Although soil disturbances are known to reduce AM viability, richness and infectivity (Kabir 2005), no difference was found in extra-radical hyphal production between *Tree* (untilled area) and *Tree-crop* (tilled area) positions in the surface soil layer. Quick recovery of hyphal networks in the *Tree-crop* position was probably due to the proximity of dense perennial root systems rapidly recolonizing large soil volumes despite annual destruction by tillage (Fig. 2, Fig. 5). As observed in tropical AC systems (Hailemariam et al. 2013), intensive root systems overlapping in the first cropped metres coupled with high levels of AM root mycorrhization enhanced the production of extra-radical hyphae enabling the maintenance of high levels of AM fungal inoculum nearby tree rows.

Indeed, wheat roots were highly colonized close to tree rows, both at the *Tree-crop* position and *HVbT-crop* position.

However, our hypothesis on higher AM diversity close to tree rows linked to higher plant diversity was not confirmed. Conversely, even when considering all AC components, AM diversity was significantly higher in crop positions (Fig. 5). Crop rotation leading to plant diversity over time may be one explanation, as it is known to enhance AM diversity (Oehl et al. 2009), particularly after mycorrhiza dependent crops (Douds et al. 1997) such as legumes (Chalk et al. 2006), the previous culture in our study. Another explanation may be the difference in AM inoculum sources and the mode of colonization of seedling roots. Whereas hyphal networks of the most well-established AM fungi rapidly colonize new roots when close to the perennial alley, in culture positions, AM inoculum is in the form of isolated propagules (spores, disrupted hyphae and root fragments), leading to more diverse but less abundant colonization levels (Johnson et al. 2004). The potential of agroforestry systems to maintain AM diversity in the field is still confirmed when looking at the annual dynamics, the perennial alley maintaining the AM diversity through the seasons.

In addition to differences observed in root phenology, seasonal variations also differ between AC components concerning their mycorrhizal status. While HVbT and wheat exhibited well-established and functional AM associations over their growing season (marked by the presence of numerous arbuscules throughout seasons for HVbT and from March to July for wheat), walnut roots developed an active AM symbiosis only during the active tree growth period, after budburst (from May to October). Consequently, a phenological mismatch between crop and trees led to dyssynchronous AM symbiosis growth dynamics between the two root systems. In contrast, wheat and HVbT exhibited a high level of synchrony, reflected in a similar time variation of the plant growing period, active mycorrhizal root colonization and arbuscular formation.

In agreement with our third hypothesis, predominant overlapping of AM fungi was observed among the three AC components confirming the potential of common mycorrhizal network formation. However, plant species differed significantly in terms of community composition, confirming low host specificity but species preferences of AM fungal species (Vandenkoornuyse et al. 2003). For now, all we can say is that more than 94% of

the OTUs of the HVbT were also present on the wheat. Thus, following the observation of dense roots intermingling over the first two metres (Fig. 3) and similar phenology, HVbT, more than walnut trees, meets the prerogatives for sharing an active common mycorrhizal network in early stages of wheat establishment. However, in terms of crop productivity, wheat development was significantly reduced close to the alley. So, did AM fungi alleviate or enhance competition with the HVbT? It has been documented that under some field conditions, intense mycorrhization of wheat has negative rather than positive growth effects (Ryan et al. 2005). It may also be that common mycorrhizal networks enhanced competition between plants. Indeed, Merrild et al. (2013) showed that interspecific and size-asymmetric competition between plants was amplified by common mycorrhizal networks as phosphorus is transferred to large plants, providing them with the most carbon and rendering small plants phosphorus deficient. Another recent study showed that between grass species differing in mycorrhizal dependence, the common mycorrhizal network benefitted the highly dependent plant species the most (Weremijewicz et al. 2018). Studies focusing on the functional aspects of common mycorrhizal networks in the field should now be conducted, such as experiments comparing the effects of maintained or disrupted networks. Once we better understand the drivers of those plant-to-plant interactions, attention can be paid to the better selection of crop varieties as well as HVbT plant species adapted to AC systems, reducing competition or even favouring facilitation between perennial species and crop alleys.

Conclusion

To improve our understanding of the belowground spatial heterogeneity induced by agroforestry systems, the strength of our study was to couple exhaustive analysis of roots and AM fungal distribution according to different positions within stands, including HVbT and activity, over a one-year period. While most studies on agroforestry focused on the effect of trees, our study reveals the importance of considering the HVbT that maintained an active AM hyphal network that rapidly colonized wheat roots, contrary to walnut roots in young AC systems. Future research should focus on the effects of AM fungi functioning in crop productivity, with a special focus on the mycorrhizal driven interactions

between the crop and the perennial species growing in tree rows. These studies should help identifying suitable functional traits for the perennial species that could be sown in tree rows to optimize positive interactions for crop development.

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