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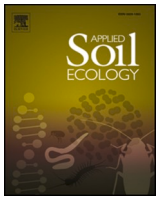

Research paper

The effect of trees on arbuscular mycorrhizal fungi and grassland root biomass: Case study of two temperate silvopastoral systems

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ABSTRACT

Despite their importance for plant nutrient acquisition and inter-species interactions, the role of arbuscular mycorrhizal fungi (AMF) in tree-crop interactions in temperate silvopastoral systems has not been studied. The aim of this study was to investigate the effect of trees on AMF biomass and grassland root colonisation in relation with root biomass and root nutrient stocks of the grassland plant community (GPC) in temperate permanent grazed silvopastures. Samples were collected at two soil depths (0–20 cm and 20–60 cm) in two paired sites on commercial farms, each combining an apple (*Malus domestica*)-based silvopasture adjacent to a grassland managed identically excepting the presence of trees. Soil chemical and physical properties were determined. AMF biomass was measured by extracting and quantifying Neutral Lipid Fatty Acids (NLFA). GPC roots were isolated from the soil samples and their colonisation by AMF, biomass, and N and P concentrations were measured. Our results showed that apple trees had a consistent negative effect on AMF biomass and AMF colonisation of the GPC at both sites. This was despite site-specific effects of trees on soil organic carbon (SOC) stocks and soil water content. Generally, we could not identify strong correlations between AMF and measured soil properties in the topsoil, while AMF biomass was correlated with SOC stocks, pH and Olsen P in the subsoil. We hypothesize that the promotion of competitive microbial communities by trees in the topsoil at the expense of AMF is a possible mechanism explaining these results. We also found a consistent negative effect of trees on GPC root biomass, likely resulting from competition for resources. The lack of correlations between AMF biomass/arbuscular colonisation and soil properties, GPC root biomass and the N:P ratio in the topsoil, may also suggest that AMF were not a mediator between trees and the GPC, but rather that both AMF and the GPC were affected by plants through different mechanisms. Our approach capitalising on collaboration with farmers to characterise tree-crop interactions in two commercial farms provides realistic observations of a negative effect of apple trees on AMF biomass and colonisation. Mechanisms can, however, only be speculative. Our results call for more observations at different sites and additional mechanistic studies to confirm these results and understand the role that AMF play in tree-crop interactions in silvopastoral systems.

1. Introduction

The high-input, resource-intensive farming systems that dominate modern agriculture have contributed to a global reduction of hunger and extreme poverty, but at a serious cost: that of soil degradation, mass deforestation, water scarcity, and high levels of greenhouse gas emissions (FAO, 2017). Today, agriculture faces the unprecedented challenge of feeding the world's growing population without negative environmental and social effects. Transitioning towards agricultural approaches which consider social, economic and ecological aspects of farming is increasingly seen as necessary to deliver sustainable agricultural production (FAO, 2017; Tittonell et al., 2020; Wilson and Lovell, 2016; Moinet et al., 2023). At the same time, the agricultural sector is immediately affected by changes in climate (FAO, 2022; Ghazali et al., 2021). This further complexifies the challenge, adding the need for adaptation measures to ensure the resilience of farming systems to climate change.

Agroforestry is increasingly recognised as a promising farm management approach in this context (Centeno-Alvarado et al., 2023; Kletty et al., 2023; Ntawuruhunga et al., 2023; Wilson and Lovell, 2016).

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Agroforestry involves the combination of trees with agricultural crops and/or animals on the same land in a way that promotes ecological and economic interactions between the trees and other agricultural components (Sinclair, 1999). With the appropriate design and management, agroforestry systems have been shown to be equally or more productive than systems without trees (Sollen-Norrlin et al., 2020). Moreover, agroforestry systems can improve the major soil metrics that define soil health (Dollinger and Jose, 2018). For example, compared to monocropping systems, agroforestry can increase soil organic carbon (SOC) stocks, suggesting potential for climate change mitigation (Terasaki Hart et al., 2023). Agroforestry systems have also been shown to improve soil nutrient availability by improving nutrient cycling, increase the diversity of microbial communities (Wilson and Lovell, 2016), and improve water cycling processes and hydrological ecosystem services (Cardinael et al., 2020).

Agroforestry systems are deliberately designed to optimise the use of spatial and temporal resources above- and belowground (Jose et al., 2000). The aim for management is to maximise positive interactions between the trees, crops and/or animals in the system, leading to facilitation, and limit negative interactions which result in competition. The potential competitive interactions in agroforestry systems led Cannell et al. (1996) to define the central biophysical hypothesis for agroforestry as: "benefits of growing trees with crops will occur only when the trees are able to acquire resources of water, light and nutrients that the crops would not otherwise acquire". Research to date, however, shows that both competition and facilitation can occur in agroforestry systems and suggests critical roles of rooting systems (Bayala and Prieto, 2020) and microbial communities. Rooting systems, particularly root system morphology and fine root distribution, have been shown to have a strong influence on the extent of belowground interspecific competition in mixed species systems (George et al., 1996). Overlap of tree root systems with the shallower roots of annual crops in agroforestry systems in upper soil layers can generate competition for water and nutrients (Bayala and Prieto, 2020). On the other hand, tree roots can contribute to soil C enrichment through root turnover and improve soil physical structure. When they are deep-rooted, trees can also reach nutrients not accessible to crops, leading to a complementary use of soil resources, known as the 'safety net' effect (Schroth, 1999). Further incorporation of these recovered nutrients into the trees' biomass, known as 'nutrient pumping', can contribute to increased soil fertility at proximity to the trees (Isaac and Borden, 2019). Trees also promote water-sharing mechanisms including redistributing water in the soil profile through the 'hydraulic lift' mechanism (Bayala and Prieto, 2020), defined as the passive movement of water through tree roots along a gradient of soil water potential (Richards and Caldwell, 1987).

In addition to the significance of rooting systems, several papers have emphasised the role of microbial communities in triggering competition/facilitation (Beule et al., 2020; Cappelli et al., 2022), with an emphasis on the role of arbuscular mycorrhizal fungi (AMF) (Battie-Laclau et al., 2020; Cardinael et al., 2020; Schroth, 1999). AMF (phylum Glomeromycota) are obligate symbionts which associate with the vast majority of terrestrial plants (Smith and Read, 2008; Watts-Williams, 2022). The symbiosis between AMF and plant species is based largely on bidirectional nutrient transfer which takes place once AMF penetrate the root cortex of host plants and form arbuscules, where nutrient exchange occurs (McGonigle et al., 1990). In the symbiosis, host plants provide photosynthate C to AMF, in exchange for nutrients which the AMF acquire in soil through extra-radical mycelium. In this way, AMF play a crucial role in plant nutrient acquisition (Barea et al., 2005; Battie-Laclau et al., 2020; Watts-Williams, 2022). Most notably, they have been shown to improve plant phosphorus (P) uptake, particularly under P-limited conditions (García and Mendoza, 2008; Sylvia et al., 2001; Yao et al., 2005; Zhang et al., 2021), as well as plant nitrogen (N) uptake and N use efficiency (Veresoglou et al., 2012). Moreover, AMF may contribute to intra- and interspecific competition (Jakobsen and Hammer, 2015; Merrild et al., 2013) and/or facilitation (Van Der Heijden and Horton, 2009) via common mycorrhizal networks (CMN), which may connect plants of different species (Begum et al., 2019), by influencing the supply rate of belowground resources to the plants (Battie-Laclau et al., 2020; Johnson, 2010). Dassen et al. (2021) studied the effect of severing ingrowth of AMF hyphal networks from the surrounding grassland vegetation on the growth of seedlings from eight grassland species and found that connection to AMF networks can have a negative impact on forb and grass seedling establishment. Still, despite advances, knowledge on the functioning of AMF in complex systems remains scarce (Dierks et al., 2024). In agroforestry systems, the effect of trees on AMF and the role of AMF in interactions between trees and crops remains poorly understood (Battie-Laclau et al., 2020; Beule et al., 2022; Cardinael et al., 2020), particularly in subsoils, though these account for the majority of agricultural soils compared to the thinner topsoil layer (Naylor et al., 2022). Bainard et al. (2011) reviewed the effect of temperate and tropical agroforestry systems on the abundance and diversity of AMF and found conflicting results, which they attributed to differences in climatic conditions, tree and crop species, or age of the trees. In a more recent review, Beule et al. (2022) found that soil microbial abundance, diversity, and functionality increased through agroforestry. However, only one study (Beule et al., 2020) has looked at the effect of agroforestry (in two temperate alley cropping systems) on soil microorganisms below 30 cm depth while also taking into account the spatial heterogeneity of agroforestry systems. They found that tree rows increased microbial abundance in both topsoil and subsoil (down to 60 cm depth). The strong positive response of the subsoil community to tree rows was explained by increased resources with proximity to trees, including root exudates and root litter, and direct symbiosis between the trees and AMF.

The present study focused on temperate silvopastoral systems, a form of agroforestry that combines trees and grazing livestock in grasslands. Grasslands make up 70 % of the global surface dedicated to agriculture (Török et al., 2021), store 20 % of global SOC stocks (FAO, 2023; Puche et al., 2019; Stockmann et al., 2013) and are therefore highly relevant to global C cycling and agricultural production (Smith, 2014). The aim of our study was to investigate the effects of apple trees, whose association with AMF is well documented (e.g. Huang et al., 2020; Przybyłko et al., 2021; Wang et al., 2022), on AMF colonisation and nutrient stocks in the roots of the understory grassland plant community (GPC). A field study on two commercial farms was carried out using a paired-site approach to compare AMF biomass, AMF colonisation, GPC root biomass, and the N:P ratio in GPC roots between silvopastoral plots and identically managed (except for the presence of trees) adjacent treeless pastures. It was hypothesised that trees would have a direct positive effect on AMF biomass and colonisation in the roots of the GPC, and that this would result in higher GPC root biomass and lower nutrient limitations.

2. Materials & methods

2.1. Study sites and experimental design

The study was carried out in Haute-Normandy, France on two organic-certified commercial dairy farms: the Domaine de Merval (Bremontier-Merval) (49°30′56" N, 1°36′22″ E) and the Ferme de Hyaumet (Dampierre-en-Bray) (49°32′55" N, 1°40′50″ E) (Fig. 1). The farms included treeless pastures with a mix of grassland species and well-established silvopastoral fields with around 15 different subspecies of apple trees (*Malus domestica*). The sites had been permanent pastures for at least 40 years before the study and apple trees were planted in rows at a density of 70–80 trees/ha in the silvopastoral plots 34 to 39 years ago in site 1, and 29 years ago in site 2. High-stem apple trees were chosen to allow for grazing by dairy cows in the understory, with a stocking density of ~1.1 LSU per hectare at both farms. Cows graze on both pasture and silvopasture plots in rotation for 6 to 8 months per year, in Spring, Summer and Autumn, with additional occasional winter grazing (Farm manager B. Cailly, personal communication, 26 October

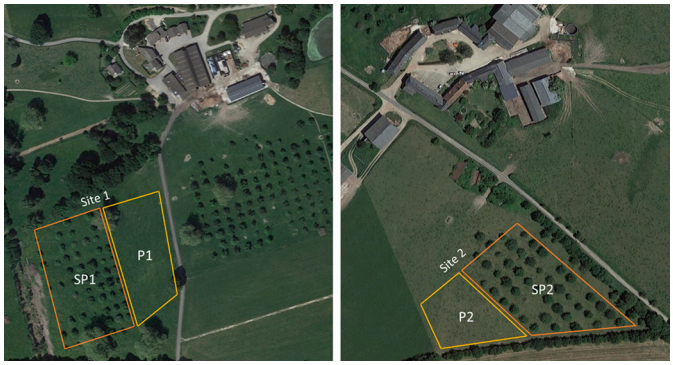

Fig. 1. Aerial view of the three study sites, with site 1 at the Domaine de Merval (left) and site 2 at the Ferme de Hyaumet (right). Each site includes one paired pasture (P)-silvopasture (SP) plots: P1-SP1 on site 1, P2-SP2 on site 2. Approximate area of plots (ha): P1 = 0.7; P2 = 0.3; SP1 = 0.7; SP2 = 0.6. Source: Google Earth, accessed 31/01/2023.

2022; L. Moinet, personal communication, 28 October 2022). Both farms produce milk and cheese from Normande cows as well as apple-derived products such as apple juice and cider. The soil textures were silt loam at Merval and silty clay loam at Hyaumet. The average yearly temperature in Haute-Normandy is approximately 11 °C with around 1000 mm rainfall per year (Meersmans et al., 2012).

The experimental design was a matched paired design used to compare silvopasture plots to adjacent treeless pasture plots (controls) at one site per farm (Fig. 2). Silvopastoral plots and controls shared identical management and environmental conditions except for the presence of trees, providing good conditions to study the effect of trees on AMF and GPC roots. Soil samples were collected following a semi-random sampling strategy. An online random generator was used to choose three trees per silvopasture plot. Rows of trees were excluded at the first two sites next to large Cottonwood trees (*Populus* sp.) on the field margins. From each of the three randomly selected trees, samples were collected systematically at three distances from the tree: (1) 1 m from the tree (2) halfway between trees rows (L2), and (3) in the middle of four trees (D/2) going towards the centre of the plot (Fig. 2) in order to capture variability on the plot. The exact same sampling pattern was followed in the three control plots using three randomly generated locations instead of trees (Fig. 2). All samples were collected at two different depths: 0–20 cm (considered the topsoil) and 20–60 cm depth (part of the subsoil). This design therefore led to a total of 18 samples per plot: (3 trees (SP) or 3 random locations (P) x 3 distances × 2 depths), yielding a total of 36 samples per site (18 × 2 (P/SP)), and 72 samples in total (36 × 2(sites)). GPS coordinates for each sampling location were recorded.

2.2. Sampling methods

Soil samples were collected from 24 October to 28 October 2022. The standard procedure involved collecting topsoil (0–20 cm) and subsoil (20–60 cm) cores at each sampling location using a 9 cm diameter auger with a serrated cutting edge. First, the auger was pressed down and twisted into the soil in 10 cm increments up to 20 cm depth to collect the topsoil sample. The auger was then placed in the borehole again and the procedure was repeated down to 60 cm depth to collect the subsoil sample. At the Ferme de Hyaumet, it was not always possible to sample down to 60 cm depth due to shallow soil, so subsoil samples went from 20 to 40–60 cm depth. Soil samples were stored in plastic bags and refrigerated at 4 °C until they were processed within the following two weeks. The soil samples collected were either used for root-based analyses or for soil-based analyses. At each sampling location, an additional undisturbed soil sample was taken in the middle of the soil layer under investigation (at 7.5–12.5 and 37.5–42.5 cm depth respectively) to assess bulk density using 100 cm^3 bulk density rings.

2.3. Root analyses

Roots were separated from the soil samples by hand-washing the samples under running water according to Böhm (1979) for analyses of biomass, AMF colonisation and N and P content. Due to low sample weight in the subsoil samples, AMF colonisation and N and P contents were measured only on topsoil samples. To isolate the roots, the whole soil samples were placed over 2 stacked sieves: a 4 mm sieve at the top where larger roots were retained, and a finer 100 µm sieve where smaller roots were retained. Roots retained in the top sieve were collected using tweezers. Large roots were separated from the root samples when they were identified as tree roots. To collect the roots retained by the bottom fine sieve, the sieve was partially submerged in water and the water sprinkler was placed in such a way that the soil-root-water mixture could circulate in the sieve. This resulted in the suspension of the fine roots, making the collection easier. For consistency, exactly five minutes were allocated to collect fine roots in each sample following this process. Approximately 35 min was taken to separate roots from soil samples.

2.4. Root biomass

The fresh weight of topsoil and subsoil root samples was recorded after 1 g of fresh roots was removed from each topsoil sample and stored

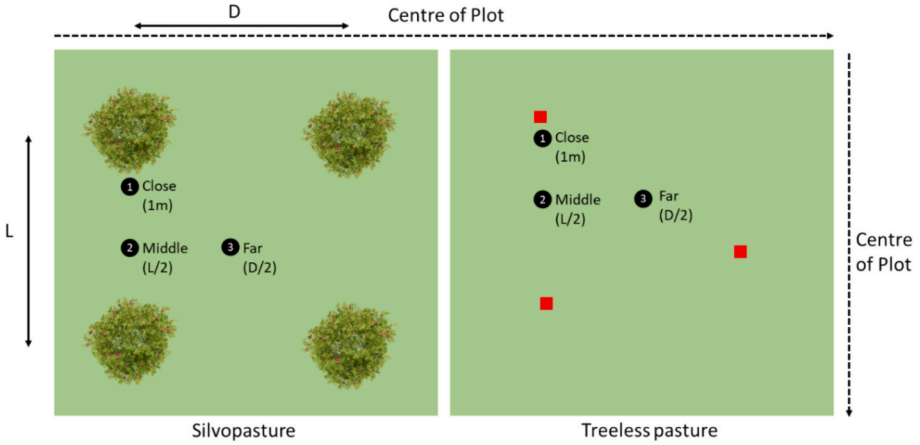

Fig. 2. Visual representation of the sampling pattern used on the silvopastoral plots (left) for each of the three randomly selected trees, and treeless pasture plots (right) for each of the three randomly selected locations in the plot. This pattern was repeated three times per silvopasture plot and three times per pasture plot.

in ethanol for AMF colonisation analysis. The root biomass was measured as the weight of roots after samples were oven-dried at 70 °C for 24 h divided by the volume of the auger used to sample the soil. For the topsoil, 1 g equivalent root dry mass was added to the sample weight after calculation of the water content of the root samples to account for the 1 g removed for AMF colonisation measurements.

2.5. N:P ratio in roots

This study was limited to analysing N and P in the roots of the GPC because these are two of the most prominent nutrients transferred from AMF to host plants (Denison and Kiers, 2011) and because GPCs are often co-limited by N and P (Schleuss et al., 2020). To measure these, dried roots were milled to 0.5 mm using the Cullati Grinder Type MFC CZ13 (Gemini B.V., Apeldoorn, The Netherlands) which combines the actions of a hammer mill and a knife mill. The milled roots were digested using a mixture of salicylic acid and sulfuric acid (H_2S0_4) (Novozamsky et al., 1983). The digestion mixture was heated, followed by the addition of hydrogen peroxide (H_2O_2). After decomposition of the excess H_2O_2 and evaporation of water, the digestion was completed with concentrated H_2S0_4 at 330 °C using selenium (Se) as a catalyst. This procedure was repeated three times in order to obtain the final digest (0.8 M H_2S0_4). In this digest, total N and total P were measured spectrophotometrically with the San++ segmented-flow system (Skalar B.V., Breda, The Netherlands). N (g N kg^{-1} soil) and P (g P kg^{-1} soil) contents and N:P ratios were then determined. N and P stocks were calculated as:

$$NorP\ stocks\ (g\ m^{-3}) = NorP\ content\ (g\ kg^{-1})\ x\ GPC\ rootbiomass\ (kg\ m^{-3}) \quad (1)$$

2.6. AMF colonisation

AMF colonisation of GPC roots was assessed from 1 g of fresh roots for each of the topsoil root samples. The roots were stained using the technique outlined by Vierheilig et al. (1998). Briefly, the roots were cleared by boiling them in 10 % KOH (potassium hydroxide) in an autoclave at 121 °C for 15 min and then rinsed several times with tap water. Cleared roots were then autoclaved further at 105 °C for 1 min in a 5 % ink - 5 % acetic acid solution. The stained roots were analysed for AMF colonisation under the microscope using the magnified intersections method presented by McGonigle et al. (1990). Approximately 5 root segments of ~1 cm length were selected and mounted on an object slide to make 5 horizontal rows of root segments. 100 microscope observation points were made per sample at 10× low power magnification (Fig. 3). At each observation point, the presence (or lack thereof) of arbuscular, vesicular, and hyphal structures was recorded. Only structures observed inside plant roots were taken into account, as these are considered to comprise the symbiotically active fungi (Öpik et al., 2006). AMF colonisation (%) was calculated using eq. 2 and arbuscular/vesicular colonisation (%) were calculated using eq. 3.

$$AMF\ col.(\%) = \frac{No.AMF\ structures\ per\ obs.point}{No.obs.points} x100 \quad (2)$$

$$Arbuscular/vesicularcol.(\%) = \frac{No.structures\ per\ obs.point}{No.obs.points} x100 \quad (3)$$

2.7. Soil analyses

For the soil-based analyses, samples were sieved using a 2 mm sieve to remove rocks, plant and animal materials, and to break down large aggregates. The samples were then prepared either for measurements of soil chemical and physical properties or for neutral lipid fatty acid (NLFA) analysis. Sieved soils were oven-dried at 40 °C for Mineral N (NO_3-N and NH_4-N), Organic C, and pH analyses, 70 °C for Olsen P, and 105 °C for soil water content. For NLFA analysis, samples were frozen at −80 °C and then freeze-dried and kept at −20 °C until analysis. Water content was calculated from the difference between fresh sieved sample weight and oven-dried (105 °C for 24 h) sample weight.

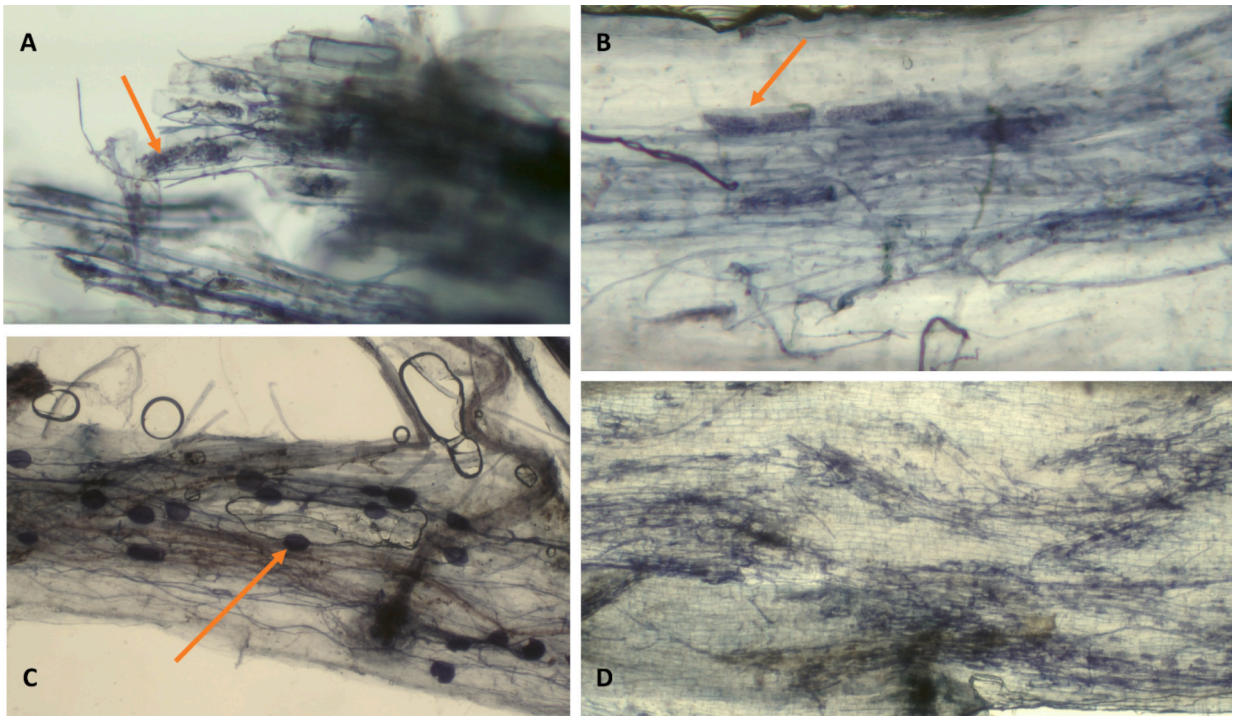

Fig. 3. Grassland plant community roots stained with ink-vinegar solution to observe mycorrhizal colonisation. The arrow in panel A and panel B point to arbuscules, where nutrient exchange between AMF and host plants occurs; the arrow in panel C points to a vesicle, the storage compounds of AMF. Panel D shows heavy root colonisation by hyphae. Microscope images were taken with 10× magnification.

2.8. AMF biomass

Analysing both AMF biomass and AMF colonisation of GPC roots is important to provide an accurate assessment of AMF communities, as several studies have found that molecular analysis of soil and root samples from the same sampling can show different results (Bainard et al., 2011). AMF live between host plants and the soil, with different implications for ecosystem processes: intra-radical mycelium affect ecosystem processes indirectly through their relations to host plants, while extra-radical mycelium can modify soil microbial community structure and composition, enhance soil aggregation, and distribute C in the soil (Barceló et al., 2020). Both intra- and extra-radical colonisation are therefore relevant to consider (Denison and Kiers, 2011). AMF biomass was assessed through NLFA analysis. NLFA analysis is widely used to measure the abundance of AMF in soils (Buyer and Sasser, 2012), as NLFAs are the main structural component of the neutral lipid molecule and an essential energy storage component of eukaryotic organisms. Briefly, the analysis started with a Bligh & Dyer lipid extraction of the freeze-dried samples (Bligh and Dyer, 1959). The total lipid extract was then divided into fractions of different polarity, with the chloroform fraction containing non-polar NLFAs. NLFA concentrations were determined by gas chromatography–mass spectrometry (GC–MS). Identification was then carried out automatically using the Sherlock™ PLFA Software Package (high-throughput PLFA method co-developed between MIDI, Inc. and the USDA-ARS), which automatically named all the PLFAs and NLFAs in the samples, categorising them by microbial origin (e.g. AMF) and performing biomass calculations and ratios (e.g. fungi:bacteria). Classification of the microbial groups was based on the publication by Buyer and Sasser (2012). The NLFA 16:1 w5c biomarker was used as a proxy for the estimation of AMF biomass (Bååth, 2003; Buyer and Sasser, 2012).

2.9. Olsen P

2.5 g of air-dry soil was weighed and put into PE-bottles. 50 ml of 0.5 M sodium hydrogen carbonate ($NaHCO_3$) solution was added to the samples. The samples were put in a shaker for approximately 30 min, after which the samples were filtered using filter paper. 4.8 ml of 0.15 M hydrochloric acid (HCl) was pipetted into a sample tube, together with 1. 2 ml of the sample filtrate. The samples were homogenised and put into an ultrasonic bath to remove any CO_2 bubbles. P-concentrations were analysed using Segmented Flow Analysis (SFA).

2.10. Mineral N and pH

Mineral N was measured in 0.01 M calcium chloride ($CaCl_2$) extracts of air-dry soil samples using SFA. The $CaCl_2$ extraction was done following the protocol by Houba et al. (2000). 3 g of air-dry soil was mixed with 30 ml of $CaCl_2$ standard solution 20 °C and shaken for 2 h. Before centrifuging the samples, pH was measured using a pre-calibrated pH-meter. After centrifugation, the supernatant was filtered into 50 ml Greiner tubes. Then, 10 ml of this supernatant was collected into a new set of tubes with an additional 25 µl of HCl 5 M for subsequent chromatography.

2.11. Soil organic carbon (SOC) stocks

To measure the soil organic carbon (SOC) content in the bulk soil, samples were first fumigated to remove any inorganic C. Approximately 5 mg of soil was weighed and put into 8 × 5 mm silver cups for fumigation. After this, 100 ml of HCl fuming (37 %) was added in a 150 ml glass cup and put into a fumigation chamber together with the samples. The samples were left in the fumigation chamber overnight. Following this, samples were transferred into new tin 8x5mm cups as the silver cups had become corroded due to fumigation. Organic C concentration of the samples was then analysed using an elemental analyser (Thermoscientific Flashsmart™). SOC stocks were calculated based on an Equivalent Soil Mass basis, based on von Haden et al. (2020), which accounted for the different depths of the subsoil samples. The R-script provided by von Haden et al. (2020) was used to calculate SOC stocks using the bulk density of the samples, the depth increment from which the sample was taken and the organic carbon concentrations measured as described above. Stocks were calculated in g C $cm^{-3,}$ which were then converted to Mg C ha^{-1}.

2.12. Statistical analyses

All statistical analyses were conducted in Rstudio version 2023.06.2 (Rstudio Team, 2023). Differences were considered statistically significant at $p < 0.05$ but marginally significant results ($p < 0.1$) were also highlighted.

Root biomass in the subsoil samples was not sufficient to allow for AMF colonisation and N and P content to be measured. Therefore, for AMF colonisation and N:P ratio in the GPC roots, statistical analyses were carried out for topsoil samples only. Topsoil and subsoil data were treated separately for the rest of the analyses.

To characterise variability of soil physico-chemical soil characteristics across depths, two-sided *t*-tests were carried out to compare Olsen P, SOC concentrations and stocks, mineral N, pH, water content, and bulk density between the topsoil and subsoil. Linear mixed-effects models (LMEs) were then carried out for each of the properties in the topsoil and subsoil separately. We tested for the effects of sites and the presence of trees and their interaction by including the interaction between factors tree and site as fixed effects in the model, whilst accounting for the nested structure of the sampling design by including the sampling tree number (or random location number for the treeless control plots) as a random effect. This was to account for the non-independence of samples taken at different distances from the same point within sites.

A simple linear regression was used to test if AMF biomass was significantly correlated with AMF colonisation in the topsoil. Two-sided *t*-tests were carried out to compare AMF biomass and GPC root biomass in the topsoil and subsoil. We then assessed the fixed effects of sites and trees and their interaction on both AMF biomass and GPC root biomass in the topsoil and subsoil separately using LMEs, using the same random structure described above. For topsoil samples, additional LMEs were performed to study the effect of site and tree treatments on AMF colonisation, particularly arbuscular colonisation and vesicular colonisation, as well as N and P in the GPC roots. When LME model assumptions were not met, the data was log-transformed.

Simple linear models were used to study the relationship between AMF biomass and GPC root biomass, and between AMF biomass and the N:P ratio in the GPC roots in the topsoil.

3. Results

3.1. Soil physical and chemical properties

There were significantly higher values for Olsen P, SOC concentrations and stocks, mineral N, water content, and bulk density in the topsoil compared to the subsoil (Two-sided t-tests: $p < 0.001$). The mean values ± standard errors ($n = 72$) for all soil variables as well as the main effects of the statistical tests are presented in Table 1. All soil variables to the exception of bulk density in the subsoil, were significantly different between the two sites for both the topsoil and subsoil. SOC concentrations and stocks, pH and mineral N were higher at site 2 compared to site 1, while Olsen P was higher at site 1. For the topsoil, trees had a statistically significant negative effect on SOC concentration and stocks and soil water content in the topsoil only at site 2. Bulk density, contrastingly, was higher in the presence of trees in the topsoil only at site 1. Mineral N, Olsen P and pH remained unaffected by the presence of trees. Trees were not found to have a significant effect on any of the soil properties in the subsoil.

Table 1
Soil physical and chemical properties measured at each of the three sites (S) in plots with and without trees (T), i.e. in pasture (P) and silvopasture (SP), at two sampling depths (D): topsoil (0–20 cm) and subsoil (20–60 cm), and results of LMEs to study the (interactive) effects of trees and sites on the properties. Letters beside each soil property show the results of Tukey's post hoc test. Capital letters show differences between P and SP of sites 1 and 2 in the topsoil. Small letters show differences between P and SP of sites 1 and 2 in the subsoil. Cells highlighted in grey show (marginally) significant *p*-values ($p < 0.05$). Mineral N [mg N-(NO_3-N and NH_4-N) kg^{-1} soil], Olsen-P [mg PO_4-P kg^{-1} soil], Organic C [%], SOC [Mg C ha^{-1}]. pH [−], Water content [water weight $g.g^{-1}$soil], Bulk density [g cm^{-3}].

Site (S)	Trees (T)	Depth (D)	Olsen P (mg kg^{-1})	Mineral N (mg kg^{-1})	SOC concentration (%)	Bulk density (g cm^{-3})	SOC stock (Mg C ha^{-1})	pH (−)	Water cont. (g g^{-1})
Site 1	P	Top	34.2 ± 0.67^{A}	13.4 ± 0.63^{A}	2.90 ± 0.06^{A}	1.26 ± 0.02^{A}	68.2 ± 1.56^{A}	5.64 ± 0.14^{A}	0.26 ± 0.00^{AB}
		Sub	29.4 ± 0.68^{a}	3.28 ± 0.50^{a}	0.77 ± 0.03^{b}	1.49 ± 0.02^{a}	50.7 ± 2.12^{ab}	6.08 ± 0.11^{a}	0.15 ± 0.00^{ab}
	SP	Top	33.2 ± 0.92^{A}	15.8 ± 1.56^{AB}	3.25 ± 0.13^{A}	1.15 ± 0.03^{B}	74.8 ± 2.86^{A}	5.82 ± 0.10^{A}	0.27 ± 0.00^{A}
		Sub	28.6 ± 0.13^{a}	4.18 ± 0.67^{a}	0.9 ± 0.07^{b}	1.47 ± 0.03^{a}	52.5 ± 4.73^{a}	6.08 ± 0.10^{a}	0.17 ± 0.01^{b}
Site 2	P	Top	29 ± 0.47^{B}	29.2 ± 9.55^{BC}	4.46 ± 0.10^{B}	1.14 ± 0.02^{B}	106 ± 2.35^{B}	7.43 ± 0.02^{B}	0.25 ± 0.00^{B}
		Sub	26 ± 0.06^{b}	14.4 ± 4.98^{b}	1.38 ± 0.07^{a}	1.57 ± 0.04^{a}	39.3 ± 2.97^{b}	7.69 ± 0.02^{b}	0.14 ± 0.01^{b}
	SP	Top	29.1 ± 0.58^{B}	22.7 ± 1.78^{C}	3.89 ± 0.14^{C}	1.19 ± 0.02^{AB}	92.7 ± 3.32^{C}	7.45 ± 0.02^{B}	0.22 ± 0.01^{C}
		Sub	26.8 ± 0.35^{b}	9.12 ± 0.80^{b}	1.47 ± 0.08^{a}	1.49 ± 0.04^{a}	43.2 ± 1.86^{ab}	7.63 ± 0.01^{b}	0.14 ± 0.01^{b}

			Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil
LMEs	Tree	coef.	−0.005	0.005	1.98	1.09	0.34	0.11	−0.11	−0.04	6.56	2.79	0.007	−0.002	0.01	−0.001
		SE	0.009	0.004	1.14	0.68	0.16	0.07	0.03	0.05	3.69	3.56	0.007	0.009	0.006	0.009
		df	9	9	9	9	8	9	8	9	8	9	9	9	8	9
		p	0.59	0.21	0.12	0.14	0.06	0.18	< 0.01	0.44	0.11	0.45	0.33	0.85	0.08	0.9
	Site	coef.	−0.06	−0.04	5.88	4.75	1.55	0.59	−0.11	0.04	37.65	−10.35	0.11	0.1	−0.01	0.1
		SE	0.009	0.004	1.44	0.68	0.16	0.07	0.03	0.05	3.69	3.56	0.007	0.009	0.006	0.009
		df	9	9	9	9	8	9	8	9	8	9	9	9	8	9
		p	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.01	0.44	< 0.001	< 0.05	< 0.001	< 0.001	0.06	< 0.001
	Tree: site	coef.	0.01	0.015	−0.77	0.42	−0.91	−0.03	0.15	−0.04	−19.77	2.1	−0.01	−0.004	−0.04	−0.03
		SE	0.02	0.007	2.32	1.42	0.23	0.16	0.04	0.11	5.22	7.52	0.01	0.02	0.008	0.04
		df	8	8	8	8	8	8	8	8	8	8	8	8	8	8
		p	0.47	0.07	0.75	0.78	< 0.05	0.83	< 0.01	0.71	< 0.05	0.79	0.39	0.84	< 0.01	0.41

3.2. Effect of trees on AMF biomass and colonisation of the GPC roots

There was significantly higher AMF biomass in the topsoil (Two-sided *t*-tests: mean ± se 8433 ± 396 pmol NLFA g^{-1} soil, $n = 35$) compared to the subsoil (3987 ± 289 pmol NLFA g^{-1} soil, $n = 34$).

Trees had a significant effect on AMF biomass in the topsoil (LME: β = −1990.48; SE = 752.79; $p < 0.05$), while sites did not (β = 990.18; SE = 752.79; $p = 0.22$). The tree-site interaction was also not significant (β = 634.6; SE = 1588.39; $p = 0.7$), suggesting a negative effect of trees on AMF biomass consistent across sites (Fig. 4a). In the subsoil, trees had a marginally significant effect on AMF biomass (β = −804.95; SE = 396.72; $p = 0.07$), while AMF biomass differed significantly between the two sites in the subsoil (β = 2448.19; SE = 396.72; $p < 0.001$) (Fig. 4b). The tree-site interaction was not significant (β = −392.09; SE =830.18; $p = 0.65$).

AMF biomass was found to be correlated significantly with AMF colonisation (Linear models: F(1,32) = 6.73; $p < 0.05$) as well as arbuscular colonisation (F(1, 32) = 6.1; $p < 0.05$). AMF biomass was not correlated with vesicular colonisation (F(1, 32) = 0.34; $p = 0.56$) (Supplementary fig. 1). In both pastures and silvopastures, there was higher vesicular colonisation than arbuscular colonisation (Table 2).

In the topsoil, there was a significant negative effect of trees on AMF colonisation (LME: β = −15.33; SE = 5.47; $p < 0.05$) and no site effect (β = −2.67; SE = 5.47; $p = 0.64$). The site-tree interaction was not significant (β = 2.85; SE = 11.56; $p = 0.81$). When taking a closer look at colonisation rates of specific AMF structures in the topsoil, trees were found to have a significant negative effect on arbuscular colonisation (β = −6.45; SE = 2.77; $p < 0.05$), while site (β = −3.66; SE = 2.77; $p = 0.21$) or the site-tree interaction (β = 2.85; SE = 11.56; $p = 0.81$) were not significant. It must be noted that the residuals of the model were not normally distributed, even after removing outliers and log-transforming the data. For vesicular colonisation, both trees (β = −3.95; SE = 1.86; $p = 0.06$) and sites (β = −10.16; SE = 1.86; $p < 0.001$) had a significant effect on vesciular colonisation, with significantly lower vesicular colonisation in site 2 compared to site 1. The tree-site interaction was not significant (β = 3.1; SE = 3.75; $p = 0.43$).

3.3. Effect of trees on GCP root biomass and N:P ratio

There was significantly higher GPC root biomass in the topsoil (Two-sided t-tests: mean ± se 4.01 ± 0.56 kg m^{-3} soil, $n = 36$) compared to the subsoil (0.25 ± 0.56 kg m^{-3} soil, $n = 36$). In the topsoil, there was a marginally significant negative effect of trees on GPC root biomass (LME: β = −0.33; SE = 0.16; $p = 0.07$) while sites had no effect (β = 0.17; SE = 0.16; $p = 0.33$). The site-tree interaction was not significant (β = 0.07; SE = 0.35; $p = 0.84$) (Fig. 4c). On the other hand, GPC root biomass was unaffected by trees in the subsoil (β = −0.15; SE = 0.13; $p = 0.29$), while sites did have a significant effect (β = 0.47; SE = 0.13; $p < 0.01$). The site-tree interaction was not significant (β = −0.08; SE = 0.28; $p = 0.79$) (Fig. 4d).

Neither trees (β = 0.03; SE = 0.02; $p = 0.16$) nor sites (β = −0.01; SE = 0.02; $p = 0.52$) had a significant effect on the N concentrations in the GPC roots. The site-tree interaction was considered, the site-tree interaction was not sigificant (β = −0.06; SE = 0.04; $p = 0.13$), despite markedly lower values in the pasture as compared to the sivlopasture at site 1 only (Fig. 5a) . Trees had no significant effect on the P concentrations in the GPC roots (β = −0.17; SE = 0.13; $p = 0.22$) but sites did (β = −0.45; SE = 0.13; $p < 0.01$), with lower P concentrations at site 2. The tree-site interaction was not significant (β = 0.36; SE = 0.26; $p = 0.21$), despite markedly higher values in the pasture as compared to the sivlopasture at site 1 only (Fig. 5b). There was a significant tree-site interactive effect on the N:P ratio in the GPC roots (β = −2.19; SE =

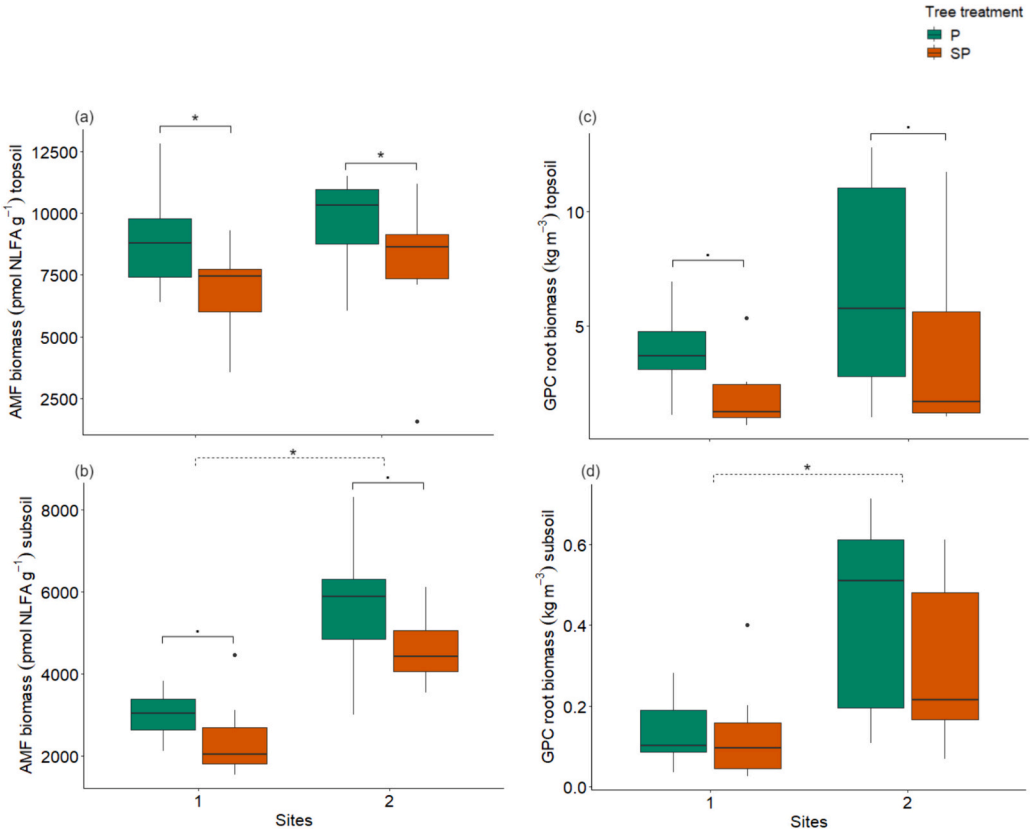

Fig. 4. Arbuscular mycorrhizal fungi (AMF) biomass (pmol NLFA g^{-1} soil) in the subsoil, 20–60 cm (a) and topsoil, 0–20 cm (a) and grassland plant community (GPC) root biomass (log-transformed) in the subsoil (c) and topsoil (d), in pasture (P) and silvopasture (SP) plots, at sites 1 and 2. Solid lines indicate differences between P and SP and dotted lines indicate differences between sites. Significance levels: '***': $p < 0.001$, '**': $p < 0.01$, '*': $p < 0.05$, '.'; $p < 0.1$.

Table 2
AMF biomass (pmol NLFA g^{-1} soil) and colonisation (%) in pasture (P) and silvopasture (SP) in the topsoil (0–20 cm) and subsoil (20–60 cm), and results of LMEs to study the (interactive) effects of trees and sites on these. Cells highlighted in grey show (marginally) significant p-values ($p < 0.05$). Values are mean ± standard error ($n = 18$ for P; $n = 17$ for SP).

Sites (S)	Trees (T)	Depth (D)	AMF biomass (pmol NLFA g^{-1} soil)	Total AMF colonisation (%)	Arbuscular colonisation (%)	Vesicular colonisation (%)
Site 1	P	Top	9038 ± 688	67.7 ± 3.71	13.9 ± 2.78	18.7 ± 1.62
		Sub	3008 ± 237		–	–
	SP	Top	6742 ± 698	50.5 ± 3.57	3.25 ± 0.8	13.1 ± 2.76
		Sub	2402 ± 307		–	–
Site 2	P	Top	9726 ± 570	63.6 ± 5.39	6.22 ± 2.03	7 ± 1.04
		Sub	5655 ± 519		–	–
	SP	Top	8038 ± 907	49.7 ± 6.32	3.78 ± 1.23	4.56 ± 1.89
		Sub	4666 ± 299		–	–

			Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil	Topsoil	Subsoil
LMEs	Tree	coef.	−1990.48	−804.95	−15.33	–	−6.45	–	−3.95	–
		SE	752.8	396.72	5.47	–	2.77	–	1.86	–
		df	9	9	9	–	9	–	9	–
		p	<0.05	0.07	<0.05	–	<0.05	–	0.06	–
	Site	coef.	990.18	2448.19	−2.67	–	−3.66	–	−10.16	–
		SE	752.8	396.72	5.47	–	2.77	–	1.86	–
		df	9	9	9	–	9	–	9	–
		p	0.22	<0.001	0.64	–	0.22	–	<0.001	–
	Tree:site	coef.	634.6	−392.09	2.88	–	8.12	–	3.1	–
		SE	1588.39	830.18	11.56	–	5.14	–	3.75	–
		df	8	8	8	–	8	–	8	–
		p	0.7	0.65	0.81	–	0.15	–	0.43	–

0.95; $p = 0.05$. Tukey's post-hoc test showed that the N:P ratio in the GPC roots differed significantly at site 1 only, with higher N concentrations and lower P concentrations leading to a significantly higher N:P ratio in silvopasture compared to pasture (Fig. 5c).

3.4. Effect of AMF biomass on GPC root biomass and nutrient concentrations

There was an overall significant positive correlation between AMF biomass and GPC root biomass (Linear models: $F(1, 67) = 84.14$; $p < 0.001$). When studying the topsoil and subsoil separately, we found a positive correlation between AMF biomass and GPC root biomass in the subsoil ($F(1, 32) = 16.16$; $p < 0.001$) but not in the topsoil ($F(1,33) = 0.92$; $p = 0.34$) (Supplementary fig. 3). There was no significant correlation between AMF biomass and either N concentrations ($F(1, 33) = 1.72$; $p = 0.2$) or P concentrations ($F(1, 33) = 0.54$; $p = 0.47$) measured in the GPC roots. There was logically also no significant correlation between AMF biomass and the N:P ratio measured in the GPC roots ($F(1, 33) = 2.09$; $p = 0.16$) (Fig. 6).

4. Discussion

In this case study, we used a paired site approach to investigate the effect of apple trees on AMF biomass and colonisation of understory GPCs in two commercially managed grazed temperate silvopastoral systems and the consequences for GPC root biomass and root nutrient status. Our results led us to reject our leading hypothesis that trees would have a direct enhancing effect on AMF biomass and colonisation which in turn would result in higher GPC root biomass and nutrient uptake. Instead, we found that trees had an overall negative effect on both AMF biomass and AMF colonisation of the GPC. Considering the significant role of AMF in agroecosystems and evidence from other studies that show that tree-based intercropping systems can support a more abundant and diverse AMF community compared to conventionally management systems (Bainard et al., 2011), our results point towards a limited role for AMF in tree-GPC interactions relative to the effect of trees themselves.

4.1. Effect of trees on AMF

Our results showed that trees had a negative effect on AMF biomass and colonisation of the GPC. The significant positive correlation between AMF biomass and AMF colonisation of the roots (Supplementary fig. 1) in our study suggests a proportionate distribution of plant C between AMF root and soil compartments (Barceló et al., 2020). Moreover, the GPC in pastures showed greater abundance of AMF hyphae and arbuscules than in silvopastures (Table 2). Since arbuscules are indicators of active and efficient nutrient exchange (Denison and Kiers, 2011), this suggests that there was lower nutrient exchange between AMF and the GPC in silvopastures compared to pastures. To our knowledge, our study is the first assessment of the effect of apple trees on AMF colonisation and biomass in silvopastoral systems, so direct comparison to other studies is not possible. However, our results are in line with a recent study of temperate alley agroforestry systems with poplar and willow trees (Giray et al., 2024), in which the authors found a 12 to 19 % decrease in AMF in tree rows of alley agroforestry systems compared to the alleyways. Apple trees, like poplars and willows, associate with AMF and so an increase in AMF biomass in the soil would have been expected at least due to tree-AMF symbiosis. Although the purpose of including specific distances from trees in our spatial design was aimed at capturing the non-random heterogeneity generated from the presence of trees rather than at providing a fine analysis of the spatial effects of trees within silvopasture, we further analysed the effect of distance from trees. This showed that AMF colonisation of the GPC increased with increasing distance from the trees (Supplementary fig. 5). AMF biomass was also lowest close to the trees, though this trend was not statistically significant (Supplementary fig. 6).

In the same study by Giray et al. (2024), the authors found that, whereas AMF decreased linearly with increasing distance from the tree rows, the mean total PLFA content (including main fungal and bacterial groups) was 30 % higher in poplar or willow tree rows compared to the alleyways. This was driven by an increase in BAM (Basidiomycota + Ascomycota + Mucoromycota) fungi, some of which are ectomycorrhizal fungi (EMF) with which poplar and willows associate. In our case, apple trees are not ectomycorrhizal, but GPC species can be and EMF provide 12 % of fungal taxa in temperate grass- and shrublands (Tedersoo et al., 2014). One plausible explanation for the decrease in

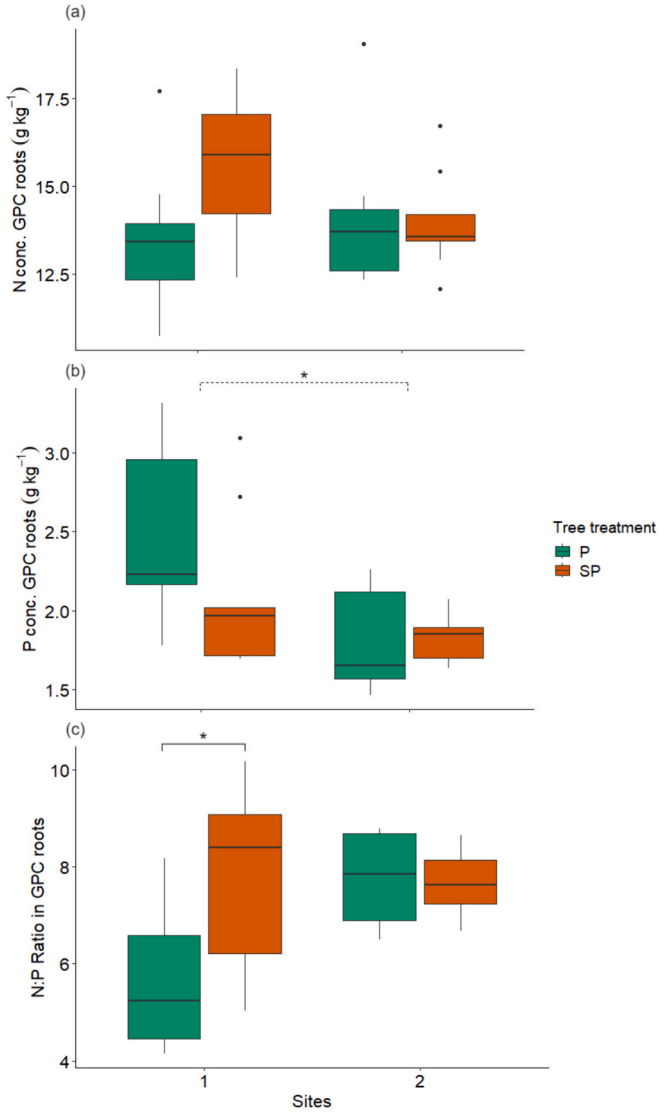

Fig. 5. Nitrogen (N) concentrations (g kg^{-1})) (a), phosphorus (P) concentrations (g kg^{-1}) (b), and N:P ratio (c) measured in the grassland plant community (GPC) roots collected in the topsoil (0–20 cm) of pasture (P) and silvopasture (SP) plots, at sites 1 and 2. Solid lines indicate differences between P and SP and dotted lines indicate differences between sites. Significance levels: '***': $p < 0.001$, '**': $p < 0.01$, '*': $p < 0.05$, '.'; $p < 0.1$.

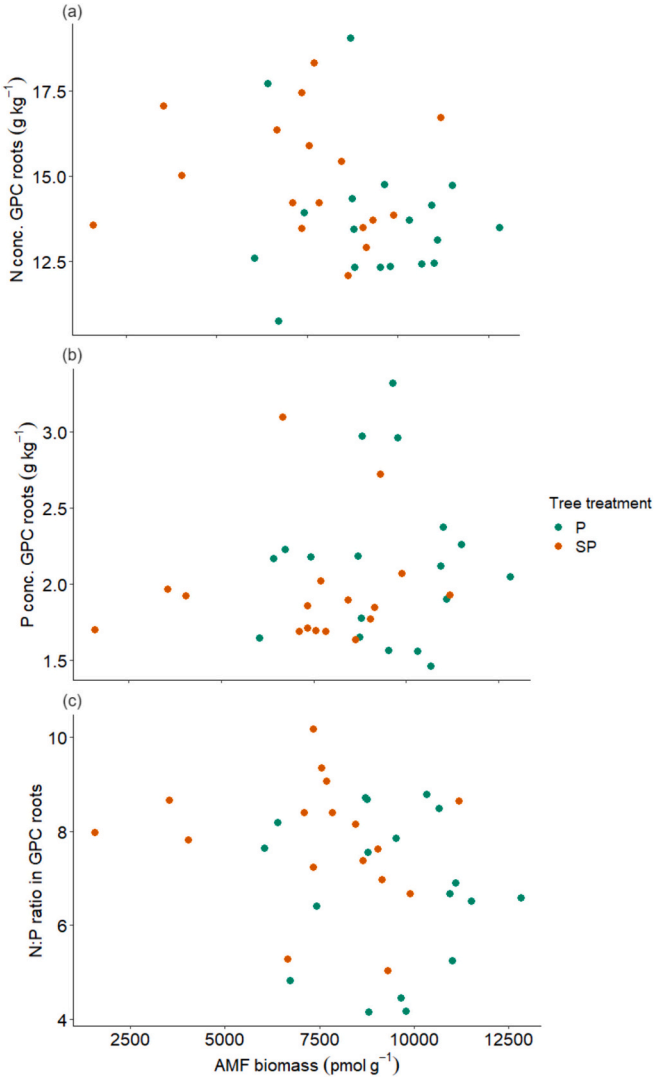

Fig. 6. Relationships between AMF biomass (pmol NLFA g^{-1} soil) and nitrogen (N) concentrations (a), phosphorus (P) concentrations (b), and N:P ratio (c) in the grassland plant community (GPC) roots collected in the topsoil (0–20 cm) of pasture (P) and silvopasture (SP) plots. Green dots represent samples from pastures (P); orange dots represent samples from silvopastures (SP). There were no significant linear relationships between AMF biomass and any of the variables. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

AMF in the presence of trees at our study sites is that apple tree litter promoted the growth of microbial competitors to AMF like EMF or saprotrophic fungi, at the expense of AMF. Indeed, apple trees provide litter inputs to the soil, which present a source of C and N for soil organisms including fungi, but these have different capacities for breaking down litter and obtaining these elements (Read and Perez-Moreno, 2003). Functional differences between fungi can therefore lead to positive feedbacks between trees and certain fungi that are better able to decompose the litter (Becklin et al., 2012). Unlike AMF, EMF or saprotrophic fungi which may have also been present in our study sites have the ability to utilise organic nutrient sources and degrade recalcitrant substrates (e.g. lignocellulose compounds) by secreting extracellular enzymes with strong oxidation potential (Heklau et al., 2021; Moll et al., 2015). Becklin et al. (2012) also found that willow leaf litter decomposition in alpine grassland sites may generate positive plant-fungal feedbacks by promoting the growth of EMF associated with willows to the detriment of AMF. A limitation of our study is that we did not assess the diversity and abundance of microbial communities beyond AMF at our sites, which would have allowed us to verify this interpretation.

Yet, the possibility that trees promoted microbial competitors to AMF in our systems is also supported by an analysis of correlations between AMF and soil chemical and physical properties (Supplementary fig. 9). In the topsoil of both pastures and silvopastures, we did not find evidence for a relationship between AMF biomass/AMF arbuscular colonisation and soil properties, including SOC concentrations and stocks and available N and P, suggesting low AMF activity. Since the topsoil is most influenced by recalcitrant plant material (Moll et al., 2016), the effect of trees on the promotion of EMF and saprotrophic fungi at the expense of AMF would have been particularly pronounced in the topsoil. This might explain the lack of AMF activity in the topsoil of silvopastures. However, this does not explain the similar results in pasture. Interestingly, in the topsoil of both silvopastures and pastures, there were significant negative correlations between AMF vesicular colonisation and SOC concentrations and stocks and significant positive correlations with soil water content. Since vesicles function as fungal storage organs (Johnson and Gehring, 2007), this would suggest that

increased soil organic matter and water led to AMF storing nutrients rather than exchanging them with the GPC.

The correlation results in the subsoil are very different from the topsoil, with strong, and sometimes contrasting, positive and negative correlations between AMF biomass and soil properties in pasture and silvopasture (Supplementary fig. 9). These significant correlations are in line with studies which show that AMF are influenced by biotic and abiotic factors (Lauber et al., 2008; Lekberg et al., 2007; Moll et al., 2015; Tedersoo et al., 2014). There was a significant negative correlation between AMF biomass and Olsen P in the soil, which we could expect considering AMF are known to increase P uptake by their host plants (García and Mendoza, 2008; Sylvia et al., 2001; Yao et al., 2005; Zhang et al., 2021), though we did not observe increased P in the GPC roots (Fig. 5).

The contrasting correlations between AMF and soil properties in the topsoil and subsoil are somewhat surprising, but in line with our hypothetical explanation that AMF competitors would have been promoted to a larger extent in the topsoil than in the subsoil. Moreover, literature shows that AMF communities can vary at different soil depths. Oehl et al. (2005) studied AMF community structure down to 70 cm in two permanent grasslands and found that, while AMF decreased with depth, there remained a large diversity of AMF species in deep soil layers (50–70 cm), concluding that AMF communities in deep soil layers are diverse and different from the topsoil. Bahrman et al. (2014) also found strong vertical variation and absence of nested patterns in mycorrhizal fungal communities in forest soil, with vertical variability in fungi communities stronger than horizontal and temporal variation. It is therefore possible that the AMF species present at our study sites preferred subsoil environments. Interestingly, Oehl et al. (2005) found evidence for the survival of sensitive AMF species in the subsoil under adverse conditions caused by intensive farming practices such as tillage in a maize field, leading to AMF finding a preferred habitat below ploughing depth. In our study, the potential positive effect of trees on competitive microbial communities at the expense of AMF might have led to AMF moving deeper into the soil profile and be more active at deeper depths, causing more correlations between AMF and soil properties in the subsoil.

Site-specific effects of trees on AMF biomass could have been expected from the contrasting results found in studies with other tree species. For example, in temperate regions, a study found lower AMF abundance in the topsoil of 25-year old black walnut (*Juglans nigra L.*)-based silvopastures than in adjacent open pastures (Poudel et al., 2022), while another study looking at two tree-based intercropping systems (one with alternating rows of hybrid poplar clones and alternating rows of black walnut (*J. nigra*) and white ash (*Fraxinus americana L.*); the other with rows of black walnut (*J. nigra*) and silver maple (*Acer saccharinum L.*)) found a positive effect of trees on AMF abundance (Lacombe et al., 2009). In tropical regions, some studies found greater AMF biomass and colonisation of crop roots at proximity to trees relative to further away from trees, while others did not, likely due to differences between tree species (Dierks et al., 2021). In our study, the clear negative effect of apple trees on AMF biomass and colonisation was consistent across the two sites, despite contrasting pH and nutrient availabilities at those sites and clear site-specific tree effects on SOC concentrations and stocks.

4.2. Effect of trees on the GPC

The presence of trees at our sites was also associated with an overall decrease in GPC root biomass. There can be a number of explanations for this, the first being above- and belowground competition by the apple trees with the GPC. Aboveground competition for light may have contributed to a reduction in GPC root biomass, which is supported by the higher shoot:root ratio observed from a quick survey performed at site 2 the previous year (Supplementary fig. 4). One study in a strip crop system with apple trees found that understory cocksfoot (*Dactylis glomerata L.*) intercepted 23–26.5 % of the photosynthetically active radiation, indicating strong competition for light by the apple trees (Wang et al., 2019). Pezzopane et al. (2019) also found that Piata-grass yields decreased closer to trees because of shading in Brazilian integrated crop-livestock-forestry systems. However, other studies have shown a positive effect of light to moderate shade on a number of grass forage species' yield and quality in agroforestry practices relative to forages in open pasture, when root competition is minimised (Pang et al., 2019). Studying the microclimate created by trees, including temperature, soil moisture, and irradiance, is recommended for future studies seeking to understand below-ground dynamics in agroforestry systems. Moreover, between 80 and 90 % of root biomass in grasslands is concentrated in the top 30 cm of soil (Cleland et al., 2019) while tree roots can explore deeper soil layers, but Cardinael et al. (2015) found that there was coexistence between tree and crop roots in both the upper and deep soil layer in an alley-cropping system. It is therefore possible that the presence of apple tree roots inhibited GPC root growth due to spatial constraints. While our study is limited by the lack of assessment of root distribution and densities to support this, we did find that trees had a negative effect on GPC root biomass in the topsoil and not in the subsoil.

The lower GPC root biomass in silvopastures compared to pastures might also be related to nutrient availability at the sites. N:P ratios measured in the roots of the GPC at both sites (ranging from ~5 to 9) indicated N-limitations in both silvopasture and pasture plots. N:P ratios are a good indication of nutrient limitations for plants (Koerselman and Meuleman, 1996; Tessier and Raynal, 2003) and while studies have found wide differences in thresholds of nutrient limitations for vegetative growth, these range from 6.7 to 16 for N-limitations according to a review by Tessier and Raynal (2003). At site 1, we did find a higher N:P ratio in the GPC roots in silvopasture compared to pasture (Fig. 5), which is in line with the positive but statistically non-significant effect of trees on soil mineral N at this site (Table 1). Moreover, analysis of the effect of distance from trees on N:P ratios in the GPC roots showed these were highest closest to the trees (Supplementary fig. 8). This might reflect a positive effect of trees on nutrient cycling and tree-mediated alleviation of N limitations and this site, but since this was only observed at one site, we cannot draw conclusions.

4.3. Links between AMF and the GPC

We had hypothesised that AMF biomass and GPC root biomass would be strongly correlated due to beneficial effects of the symbiosis for GPC nutrient acquisition, which we did not show in this study. It would be justified to assume that the lower GPC root biomass which resulted from the presence of trees led to a decrease in AMF because AMF are obligate symbionts that depend on host plants for survival and reproduction (Kiers et al., 2011). The ability to reproduce utilising C resources from host plants, repopulate the soil, live in the soil between hosts, and discover and infect new hosts, are all necessary for AMF to persist (Denison and Kiers, 2011). Decreases in plant root length densities are known to decrease the rate of spread of AMF, due to a lower number of plant roots available to colonise (Van Noordwijk et al., 1998). However, we found no correlations between AMF biomass and GPC root biomass in silvopastures, in either the topsoil or subsoil (Supplementary fig. 9), suggesting that if the lower GPC root biomass led to a reduction in AMF biomass, it was not the only mechanism involved.

In addition, we failed to identify a relation between AMF biomass and N and P concentrations in the GPC roots (Fig. 6), suggesting that the role of trees in GPC nutrient dynamics at our sites was greater than that of AMF. Nutrient availability has a strong influence on the level of symbiosis with AMF, as host plants no longer invest in the fungal partner if nutrients are accessible to them in sufficient quantities (Corcoz et al., 2022; Nouri et al., 2014). Since the GPC appeared to be N-limited in both our study sites, we would have expected nutrient exchange between AMF and the GPC. AMF are also known to increase plant P uptake

(García and Mendoza, 2008; Huang et al., 2020; Sylvia et al., 2001; Yao et al., 2005), so we had hypothesised that AMF biomass would be positively correlated with P concentrations in GPC roots. Instead, we found no significant correlation between AMF biomass and root P concentrations (Fig. 6). This might be explained by sufficient availability of P in the soil, which is also supported by the finding that there was higher vesicular colonisation compared to arbuscular colonisation of the GPC roots at all sites (Table 2). Indeed, soil nutrient availability can influence the formation of these structures, with a general positive influence of N on arbuscule formation and a positive influence of P on vesicle formation (Corcoz et al., 2022). In addition, highest vesicular colonisation was measured in the pasture of site 1 (Table 2), the site where there was a significantly lower N:P ratio in the GPC roots of the pasture compared to silvopasture (Fig. 5). The fact that vesicular colonisation was highest where the N:P ratio was lowest supports the finding that P was not a limiting factor for the GPC at this site.

It is thought that AMF can contribute to increased competition in mixed-species systems by favouring the plants with a higher carbon source in the CMN (Dierks et al., 2024). It might therefore be reasonable to consider that AMF contributed to reducing GPC root biomass by linking tree and GPC roots via a CMN to the benefit of the higher carbon source strength of adult plants in the mycorrhizal network. However, if this is the case, we would have also expected to find negative correlations between AMF biomass and nutrient concentrations in the GPC roots, which is not what we found (Fig. 6). Still, analysing the apple tree roots in addition to the GPC roots to study their colonisation and using isotopic tracing in future research to study whether trees and the GPC are linked through a CMN, and investigate the flow of nutrients between the agroforestry species, would be beneficial to gain a better understanding of the role of AMF in these systems.

It is often reported that cattle prefer shade when it is accessible to them, particularly in warm weather (e.g. Schütz et al., 2009, 2011). Because we know that the presence of cattle and their grazing activities can have a large influence on the structures and functions of grassland ecosystems (Hao and He, 2019), it might be tempting to assume that our results are due to differences in grazing between the pasture and silvopasture plots, with cows preferring shaded silvopasture plots in summer, rather than to the presence of trees. A limitation of our study is that, in our attempt to isolate the effect of trees, we did not consider cows at our sites. However, two recent reviews on the effects of silvopastoral systems on the behaviour of cattle recently concluded that, while silvopastoral systems improve the thermal environment for cattle, their effect on cows' behaviour (e.g. resting, rumination) and physiology (e.g. internal temperature) are inconclusive (Deniz et al., 2023; De-Sousa et al., 2023). The studies included in the review by Deniz et al. (2023) consistently found no difference in rumination between silvopastoral systems and treeless pastures. Other studies have found that animals graze longer in treeless pastures than in silvopastoral systems, likely as a result of higher forage quality (De Souza et al., 2010; Lopes et al., 2016). De-Sousa et al. (2023) also found that of the six studies that evaluated walking behaviour in their review, three found higher walking frequency in treeless pastures compared to silvopastures. Future studies should address the animal component by including sites with/without the presence of cattle to further understand the potential of silvopastoral systems for resilience to climate change.

5. Conclusions

The aim of this study was to contribute to improved understanding of silvopastoralism in temperate grazed grasslands, by studying the effects of silvopastoral trees on AMF biomass, colonisation of the GPC, and subsequent root nutrient concentrations of the GPC. We address an important gap in studies of microbial communities in agroforestry systems which are often limited to the topsoil while not accounting for the spatial heterogeneity of silvopastoral systems. While our results are limited by the small number of study sites, our results show clearly that trees had a direct negative effect on GPC root biomass, AMF biomass, and AMF colonisation of the GPC. These results lead us to suggest that studying the role of AMF as an intermediary in tree-GPC interactions in silvopastoral systems may not be as relevant as studying the direct effects of trees on the GPC. Although our approach capitalising on collaboration with farmers to characterise tree-crop interactions in real commercial farms does not allow to conclude on mechanisms, we explore a number of mechanisms which might explain our results. Considering the significance of AMF for agroecosystems and evidence from other studies which show that tree-based intercropping systems can support a more abundant and diverse AMF community compared to conventionally management systems, more mechanistic studies are necessary to confirm these results and understand the role that AMF play in tree-crop interactions in silvopastoral systems.

CRediT authorship contribution statement

Shevani Murray: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis. **Bram Avezaat:** Methodology, Investigation, Formal analysis. **Robin Guilmot:** Methodology, Investigation, Formal analysis. **Anne A. Hogenboom:** Methodology, Investigation, Formal analysis. **Don H. Lareau:** Methodology, Investigation, Formal analysis. **Brigitte Wear:** Methodology, Investigation, Formal analysis. **Gabriel Y.K. Moinet:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization.

Declaration of competing interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2024.105539.

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