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Environmental drivers of AMF Diversity and Community Composition of Robusta across Coffee Systems in Tshopo, DRC

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Abstract

Robusta coffee, grown by 25 million farmers across more than 50 countries, plays an important role in smallholder farmers' livelihoods and the economies of many low-income countries. Despite robusta coffee's growing economic importance, currently accounting for 43% of global coffee production, its association with arbuscular mycorrhizal fungus (AMF) communities and how agricultural practices affect this association remains poorly understood. To address this, we characterised the AMF community composition of robusta coffee in part of its region of origin, the Democratic Republic of Congo. AMF diversity and community composition were compared between coffee monoculture, agroforestry systems and wild robusta in its native rainforest habitat. Using Illumina sequencing on 304 root samples, we identified 307 AMF operational taxonomic units (OTUs), dominated by the genera *Glomus* and *Acaulospora*. OTU richness did not vary across the three studied systems, yet large differences in community composition were found. Many unique OTUs were only observed in the coffee in the rainforest. In general, lower available soil phosphorus (P) and lower soil bulk density increased AMF diversity, yet higher available soil P and pH increased AMF diversity in the wild forest coffee. Shifts in AMF community composition across coffee systems were driven by canopy closure, soil pH, available soil P and soil bulk density. Our study is the first to characterise mycorrhizal communities in wild robusta coffee in its region of origin and shows that even low-input agricultural practices result in major AMF community shifts as compared to a natural baseline.

1. Introduction

Over the past two decades, the global demand for coffee has shown an upward trajectory, reaching 10.1 million tons during the 2022–2023 period, with projections anticipating a substantial increase in production by 5.8% (ICO, 2023). Robusta coffee (*Coffea canephora Pierre ex A. Froehner*) currently accounts for 43% of the global coffee production, compared to 56% for arabica coffee (*Coffea arabica L.*) (ICO, 2023). Among the most traded tropical agricultural commodities, Robusta coffee is grown by 25 million farmers across more than 50 countries. It plays a significant role in smallholder farmers' livelihoods and the economies of many low-income countries (FAO, 2023).

Historically, robusta coffee was a major export commodity for the DRC, second only to copper (ICO et al., 2000). In the mid-1980s, robusta production peaked at around 110 000 tons, contributing to 75% of the country's agricultural export revenues and about 15% of its GDP (ICO, 2023; ICO et al., 2000). However, production levels declined sharply from the late 1980s to the early 2000s (ICO, 2023). This decline in production was linked to a global coffee price collapse, economic mismanagement, political instability, violence, insecurity, and the resurgence of Coffee Wilt Disease (Bamenga et al., 2024). Currently, low farm gate prices are a major challenge according to coffee farmers in Tshopo province, DRC (Broeckhoven et al., 2025b).

Besides the agronomic potential, it should be noted that the DRC is a key region of origin region for robusta and plays a crucial role in preserving coffee's genetic diversity. Moreover, unique sensory profiles

were recently identified in cultivated and wild robusta in the DRC (Bollen et al., 2024). Even more so, genetic diversity is important for traits such as disease resistance (Lashermes et al., 2010; Silva et al., 2006), tolerance to climate change tolerance (Davis et al., 2012) and drought tolerance (Davis et al., 2021a), which are essential for the future of global coffee production.

Coffee and coffee farmers are already encountering significant challenges due to climate change, with a further decrease in the suitability of globally cultivated areas expected (Bunn et al., 2015). Robusta coffee is generally considered to be more heat-tolerant than arabica, making it more resilient to climate change (Jayakumar et al., 2017; Läderach et al., 2017). However, some studies suggest that robusta may be more temperature-sensitive than previously thought (Kath et al., 2020). Nonetheless, the global share of robusta production compared to arabica has been increasing over time (ICO, 2023), a trend expected to continue due to climate change (Davis et al., 2021). This shift is particularly relevant for lowland tropical regions like most of the Democratic Republic of the Congo (DRC), where robusta is produced. Robusta and arabica represent 65% and 35% of the DRC's coffee production, respectively (ICO, 2021).

Arbuscular mycorrhizal fungi (AMF) are important symbionts for sustainable crop production, particularly within low-input agricultural systems in the tropics (Herder et al., 2010; Verbruggen et al., 2010). AMF represent the most prevalent and important symbiotic association between plants and fungi, fostering relationships with approximately 70 % of plant species, including most crops (Brundrett and Tedersoo, 2018; Smith and Read, 2008; Strack et al., 2003). B colonising plant roots and establishing extensive hyphal networks, AMF provide various benefits, such as enhanced nutrient uptake of less mobile nutrients (Leigh et al., 2009; Smith and Read, 2008). Besides nutrient uptake, AMF are known to bolster resistance to soil-borne pathogens (Veresoglou and Rillig, 2012), contribute to improved drought tolerance (Augé, 2001; Raviv, 2010), soil health and soil structure (Wilson et al., 2009) and reduction of nutrient leaching (Van Der Heijden, 2010).

While the mycorrhizal literature often presents an overly optimistic view of the benefits of AMF on crop yield, the actual yield effects under field conditions are frequently challenged in agronomic literature (Ryan and Graham, 2018). While cereals are known to have limited AMF responsiveness (Ryan and Graham, 2018), for other crops like cassava (Ceballos et al., 2013; Thanni et al., 2023) and potato (Hijri, 2016), AMF have convincingly been shown to increase yield. Besides, the relevance of AMF goes beyond their potential direct yield effect. AMF are relevant because of their potential role in managing agroecosystems more sustainably, such as soil structure and phosphorus use efficiency (Rillig et al., 2019).

AMF in coffee plants have been first observed byJanse (1897) in Java. The occurrence and importance of AM symbiosis in coffee has been particularly noted in highly weathered and low-fertility soils, common in many tropical regions where coffee is cultivated (Andrade et al., 2009; Siqueira et al., 1998). Regarding the role of AMF in nutrient uptake and availability, phosphorus is undoubtedly the most important, reflected in the many reports focused on the improved P status of AM plants. This situation is no different for coffee (Andrade et al., 2009). The uptake of nutrients other than P, such as N, Ca, Mg, Fe,

Mn, Zn and Cu, is also improved by AMF, with enhanced growth as a result (Clark and Zeto, 2000; Vaast et al., 1996; Vaast and Zasoski, 1992). The high dependency on mycorrhizal associations, especially during the seedling stage(Howeler et al., 1987; Siqueira et al., 1998; Vaast & Zasoski, 1992), can be explained by the fact that young perennial plants have a high P requirement. The high P requirement may diminish with plant age (Siqueira et al., 1998). Besides the important role of AMF in coffee due to nutritional benefits, AMF have been found to improve coffee tolerance to certain pests and diseases, especially nematodes (Vaast et al., 1998).

Significant knowledge gaps remain regarding the association between AMF and coffee, despite the benefits of arbuscular mycorrhizal fungi (AMF) in boosting coffee plant fitness, especially in highly weathered soils under low-input agriculture (Andrade et al., 2009; Siqueira et al., 1998). A recent review of coffee microbiota (Duong et al., 2020), identified 36 studies dealing with AMF and coffee. Of these studies, 75 % were conducted in Central and South America, and only 14 % in frica and 11 % in Asia. Furthermore, the few African studis focused on arabia coffee in Ethiopia. Additionally, only 1 out of the 36 studies dealt with robusta coffee(Bertolini et al., 2018), whereas the other 35 dealt with arabica(Duong et al., 2020). Overall, there is a critical need for AMF studies on robusta, especially in its region of origin, the Congo Basin. Furthermore, only three studies on coffee AMF so far have utilised DNA metabarcoding, all focusing on arabica (De Beenhouwer et al., 2015a; De Beenhouwer et al., 2015b; Fulthorpe et al., 2020). Our study is the first to use metabarcoding analysis to assess the diversity of AMF in robusta coffee. Finally, a deeper understanding of the mechanisms driving coffee AMF diversity, such as the type of coffee production system and environmental variables, may provide basic information for further targeted inoculation studies to enhance coffee production (Berendsen et al., 2012; Duong et al., 2020).

Therefore, our study aimed to (i) identify the AMF taxa present in robusta coffee in (a part of) its region of origin, the Tshopo province, DRC; (ii) quantify differences in AMF diversity and community composition of robusta coffee between production systems, i.e. coffee monocultures and coffee agroforestry, in comparison to wild robusta in its native forest habitat; and (iii) assess the relative importance of soil characteristics and canopy cover as drivers of AMF diversity and community composition in robusta coffee across a management intensity gradient. Specifically, we compared (i) coffee monocultures, (ii) coffee agroforestry systems characterised by commonly cultivated and native undomesticated tree species intercropped with robusta, and (iii) forest coffee systems characterised by wild robusta growing in the natural forest understory.

2. Methodology

2.1. Study area

The research was conducted in Tshopo Province, in the North-East of the Democratic Republic of Congo (DRC). The study area (0.5–1.4°N, 24–25°E; 380 masl – 538 masl) encompassed the territories of Isangi, Banalia, and Kisangani (Fig. 1). The region’s natural vegetation consists of moist semi-deciduous

rainforest and monodominant *Gilbertiodendron dewevrei (De Wild.)* evergreen rainforest (Gilson et al., 1956). According to the Köppen-Geiger classification, the area has a tropical rainforest climate (Af) (Peel et al., 2007), with an average yearly precipitation of 1762 ± 295 mm in the Yangambi MAB reserve. The climate is characterised by two rainy seasons (August-November and April-May) and two dry seasons (December-February/March and June-July/August). Throughout the year, average temperatures remain high, with a minimum of 24.2 ± 0.4°C in July and a maximum of 25.5 ± 0.6°C in March. The dominant WRB soil type in the study area is a Ferralsol, formed from aeolian sediments, consisting mostly of quartz sand, kaolinite clay and hydrated iron oxides (Gilson et al., 1956; Van Ranst et al., 2010). However, due to seasonal inundations, Gleysols can also be found next to rivers and their tributaries (Gilson et al., 1956; Van Ranst et al., 2010).

The coffee system typology was based on canopy closure and shade tree species (Fig. 2). Coffee monocultures (MC) are characterised by robusta cultivars being the only woody species present and having an almost absent tree canopy (canopy closure < 10%). Coffee agroforestry systems (AF) are characterised by robusta cultivars being the most abundant woody species present, intercropped with commonly cultivated and native undomesticated fruit tree species and forest tree species and having a medium tree canopy closure (45.0 ± 3.2%). MC and AF are the most important coffee production systems, which will be compared, with forest coffee (FC) being the benchmark. Forest coffee systems (FC) are characterised by forest tree species being the most abundant woody species, with wild robusta in the understory, and having a high tree canopy closure (> 85%). This forest coffee is located in the Yangambi Man and Biosphere Reserve and the Ngazi Forest Reserve. Additionally, it should be noted that coffee farmers in the MC and AF systems do not use any pesticides, insecticides or mineral fertilisers. However, organic amendments are sometimes applied in low quantities. Our previous study demonstrated that coffee yields in agroforestry systems are not different from monocultures, if excessive shading (> 50%) is avoided (Broeckhoven et al., 2025a). Also, it showed the irreplaceable value of forest coffee systems in terms of biodiversity and carbon sequestration. Large differences in woody biodiversity were found between the coffee systems in the Tshopo, DRC (Broeckhoven et al., 2025a). Additionally, pollinator diversity is higher in forest coffee than in coffee monocultures or coffee agroforestry systems, with no differences between the latter two coffee systems (Depecker et al., 2025).

2.2. Data Collection

The three coffee typologies were used as the basis for the stratified random sampling design. Specifically, 27 MC, 27 AF and 25 FC 25 m x 25 m plots were established (N = 77), with a minimum of 1 km distance between plots within the same typology. Fieldwork was carried out during the main coffee harvesting season, from October to December 2021. Four mature coffee plants meeting specific criteria (Height > 1.5 m; DBH > 5 cm) were sampled in each plot, resulting in 304 root samples. These samples were collected from the upper soil layer (0 - 30 cm) surrounding the coffee plants in the four cardinal directions (Mummey and Rillig, 2008). Emphasis was placed on gathering fine young roots, known to exhibit significant mycorrhizal colonisation (Smith and Read, 2008). After sampling, roots were gently

cleansed with water, placed in paper bags, and desiccated using silica gel beads before subsequent processing.

Composite soil samples based on eight different sampling points per plot were taken at 0–5 cm depth. These composite soil samples were mixed, sundried, and then oven-dried (50°C for 72 h), after which they were ground with a ball mill and sieved with a mesh size of 2 mm. Bulk density was measured from undisturbed soil samples using Kopecky rings. Soil pH was quantified using the pH $CaCl_2$ method by mixing 10g of dried, sieved soil with a 0.01 M $CaCl_2$ solution (S/W ratio 1:5) according to ISO norm 10390. Organic Carbon and Nitrogen were determined using a Carlo Erba Elemental analyser based on dry combustion (Ravindranath and Ostwald, 2008). Olsen P was determined colourimetrically using a Lambda 25 spectrophotometer (Perkin Elmer, Waltham) after shaking 5 g dry soil for 30 min with 100ml of 0.5 M sodium bicarbonate at pH 8.5, in line with ISO norm 11263. Colour development of the extracts was done using the molybdenum blue method (Robertson et al., 1999). Canopy closure (% shade) was measured using a model A convex spherical densitometer based on four data points per plot (Englund et al., 2011; Lemmon, 1956). Canopy closure is commonly included in studies on coffee systems as a proxy for management intensity, which influences environmental variables that can potentially affect AMF diversity (De Beenhouwer et al., 2015a; Fulthorpe et al., 2020).

2.3. Root DNA Extraction, PCR Amplification and Sequencing

Further processing of the dried coffee roots started with removing PCR inhibitors and extracting total genomic DNA from 50 mg of dried roots per coffee plant. This was done using the Soil DNA Isolation Plus Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions. Subsequently, part of the small subunit (SSU) of the ribosomal RNA (rRNA) gene was amplified. The 18S SSU rDNA region, a Glomeromycota (AMF) specific marker sequence, was amplified using the AMF-specific AMV4.5NF-AMDGR primer pair (Sato et al., 2005; Van Geel et al., 2014). This primer pair was selected because it consistently results in many sequences with high AMF specificity (Van Geel et al., 2014). The primer pair was adapted for use in a dual-index sequencing strategy on Illumina MiSeq, which enabled combining multiple samples in one library (Kozich et al., 2013). The PCR mixture comprised 1 µL of extracted DNA, 0.2 µL of each 10 µM primer, 12.5 µL ALLin Hot Start Mastermix (HighQu), and 11.1 µL of PCR grade water making up a total volume of 25 µL. The PCR, performed on a Biometra TAdvanced thermal cycler (Westburg, Netherlands), started with an initial denaturation step at 95°C for 1 min, followed by 40 cycles of 15s denaturing for 95°C, 53°C for 15s for annealing and 2 min elongation at 72°C. The final extension was for 11s at 72°C. After purification of the PCR products using Agencourt AMPure XP (Beckman Coulter Life Sciences, Indianapolis, IN, USA), the concentration was measured on a Qubit fluorimeter with the Qubit dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). Samples were equimolarly pooled, and the fragment size of the pooled library was checked with gel electrophoresis. Finally, DNA fragments of the right size (ca. 350 bp) were cut from the gel and purified using the QIAquick Gel Extraction Kit (QIAgen). Sequencing was done on the Illumina MiSeq sequencing platform with the v2 500 cycle reagent kit (Illumina, San Diego, CA, USA).

2.4. Bioinformatics: Merging, filtering and taxonomy assignment

After Root DNA extraction, PCR amplification and sequencing, the demultiplexed sequencing data were processed using USEARCH following the recommended pipeline (Edgar, 2013). The pipeline included the following steps: (1) merging forward and reverse reads using the *'fastq_mergepairs'* to create consensus sequences (max 10 mismatches and min 80 %id); (2) enuring correct orientation of the sequences using the *'orient'* command against the MaarjAM database (Öpik et al., 2010); (3) check the size distribution and quality classes using the *'fastq_eestats2'* command, (4) filtering the short and low-quality sequences with the *'fastq_filter'* command, allowing a maximum expected error (MaxEE) of 1.0 for the individual sequences with truncation length set to 200 bp; (5) filtering out the singletons (unique sequences in the dataset) with the *'fastx_uniques'* command, as this has been shown to improve the accuracy of diversity estimates (Kunin et al., 2010; Tedersoo et al., 2010; Waud et al., 2014) by minimising the risk of having sequences that resulted from sequencing errors; (6) clustering the sequences into operational taxonomic units (OTUs) at sequence similarity threshold of 97% (Knight et al., 2018; Nilsson et al., 2019) and removing chimeric OTUs with the 'cluster_otus' command (Alberdi et al., 2018; Edgar and Flyvbjerg, 2015); (7) finally, using the *'otutab'* command to map each sequence to an OTU and a sample, resulting in an OTU table.

For assigning taxonomy, the consensus sequence of each OTU was BLASTed against the MaarjAM database with the following criteria for a hit: sequence similarity at least 97%, alignment at least 95% of the shorter sequence, BLAST E-value < 1e − 50 (Öpik et al., 2010). Subsequently, the remaining unidentified OTUs were manually cross-referenced against the NCBI GenBank database using the same criteria as those for MaarjAM blasting. OTUs identified as belonging to a phylum other than *Mucoromycotina* and sub-phylum other than *Glomeromycotina* were removed, as these are considered not to belong to the AMF. The remaining unidentified OTUs were also excluded. Thus, after eliminating erroneous OTUs, 313 out of the initial 540 OTUs remained.

To eliminate potential erroneous sequences generated during PCR and sequencing, three additional filtering steps were applied: (i) OTUs representing fewer than 0.01% of the sequences in a sample were discarded from that sample (Alberdi et al., 2018), and (ii) OTUs with an absolute count fewer than two across samples were removed from the dataset. This further reduced the number of OTUs from 313 to 307. Additionally, (iii) rarefaction curves of OTUs per sample were computed to validate sequencing quality. Samples that did not reliably represent the sequenced data (library size < 50) were excluded using the *rarecurve* command in the *vegan* package. As a result, 20 of the 304 samples were removed, resulting in 284 retained samples covering 307 OTUs.

2.5. Statistical Analysis

Statistical analyses were done in R, version 4.4.0 (R Core Team, 2020). Soil variables and canopy closure under different coffee systems were tested for significant differences. When comparing the coffee

systems, the Kruskal-Wallis and pairwise Wilcoxon rank sum tests with Benjamini-Hochberg correction for multiple testing were used when the data was not normally distributed. Tukey's HSD was used to compare the coffee systems in the case of normally distributed data. The *phyloseq* R package was utilised to import, store, analyse, and graphically display the sequencing data already clustered into OTUs (McMurdie and Holmes, 2013)

Before further analysis, Total Sum Normalization (TSS) without log transformation was applied to the OTU abundances. This transformed the data into proportions by dividing the reads for each OTU in a sample by the total number of reads in that sample. In the context of community-level microbiome data, proportions (and rarefied data) outperform other normalisation methods if the objective is to produce accurate Bray-Curtis dissimilarities and perform ordinations and PERMANOVAs (McKnight et al., 2019). TSS normalisation ensures the standardisation of reading depths. Normalisation is crucial for subsequent alpha- and beta-diversity analyses (McKnight et al., 2019).

Although log transformations are commonly used in many normalisation procedures, McKnight et al. (2019) showed that they can often distort community comparisons. Log transformations suppress large differences in common OTUs and amplify slight differences in rare OTUs. While rare members of an ecological community can perform important functions, dominant members tend to drive the bulk of community functionality (Fuhrman, 2009; Pedrós-Alió, 2006). Therefore, reducing the importance of dominant OTUs and amplifying the importance of rare OTUs would possibly give a misleading picture of the differences among communities (McKnight et al., 2019).

After TSS normalisation, the observed (S_{obs}) and expected number of AMF species (S_{exp}) and Hill numbers (N_1, N_2, E_1 and E_2) were calculated for each plot, following the procedure outlined by Gotelli and Chao (2013) and Hill (1973). Moreover, the expected species richness (S_{exp}) was calculated using the Chao1-bc formula as several samples had no doubletons (Głowacki, 2011).

The alpha diversity indices were first modelled against the three coffee systems using generalised linear mixed models (GLMM) from the *lme4* R-package and included 'plot' as a random factor (Bates et al., 2015). The estimated marginal means were calculated using the *emmeans* function in the *emmeans* package. Testing for pairwise differences in alpha diversity between coffee systems was done in a similar manner as for soil variables.

Nonmetric multidimensional scaling (NMDS) was then used to compare AMF community composition across the three coffee systems. The Bray-Curtis dissimilarity measure (Ricotta and Podani, 2017) and the *metaMDS* function in the *vegan* package were used for the ordination (Oksanen et al., 2018). Moreover, the Bray-Curtis dissimilarity metric is the most widely used method for comparing microbial communities (McKnight et al., 2019). Additionally, a permutational multivariate analysis of variance with 9999 permutations was performed using the *adonis* function in the *vegan* package to test for differences in community composition between the coffee systems. Pairwise comparisons with Benjamini-Hochberg correction among the coffee systems were made using the *pairwise.adonis* function in the

vegan package based on 9999 permutations (Arbizu, 2020). The indicative mycorrhizal species for each coffee system were identified using the indicator value (Dufrene & Legendre, 1997) calculated based on 9999 permutations using the *multipatt* function in the *indicspecies* package (De Cáceres & Legendre, 2009).

Before modelling the effects of soil variables, canopy closure, and coffee systems on AMF diversity, a dispersion test was conducted. This led to rejecting a Poisson distribution and favouring a Negative Binomial model, which allowed for overdispersion. Soil organic carbon content (C %) was removed due to collinearity with total soil nitrogen content (N %) (VIF > 10). After removing C %, all VIFs were less than 2, indicating no multi-collinearity issues. All soil variables were standardised prior to creating the Generalized Linear Model (GLM). Backward stepwise model selection was used based on the Akaike Information Criterion (AIC). Model selection was performed using the *dredge* function from the *MuMIn* package in R (Bartoń, 2023). This approach iteratively removed the least significant variables, retaining the model with the lowest AIC value to determine the most influential environmental variables driving differences in AMF diversity.

Finally, a Constrained Correspondence Analysis (CCA) was performed on the Bray-Curtis distance matrix, the soil variables and canopy closure. CCA enabled identifying drivers behind shifts in AMF community compositions. CCA was implemented using the *capscale* function from the *vegan* package (Oksanen et al., 2018). Forward stepwise selection was applied using the *ordiR2step* function from the *vegan* to identify the best model and the significant environmental variables.

3. Results

3.1. Variation in environmental variables across coffee systems

Canopy closure was significantly higher in the FC system (90.9 ± 0.79%) than in the AF system (45.0 ± 3.2%) ($p < 0.0001$), which in turn was much higher than in the MC system (1.1 ± 0.5%) ($p < 0.0001$). Significant differences (all $p < 0.0001$) in physical and chemical soil properties were observed between the three coffee systems (Table S1). Soil available P levels were significantly higher in AF than in MC and FC systems. pH ($CaCl_2$) was higher in AF than in MC, which, in turn, was higher in MC than in FC systems. Soil bulk density was higher in MC and AF systems than in FC systems. In contrast, no significant differences were found in the CN ratio, total nitrogen (N %) and soil organic carbon concentration (C %) (Table S1).

3.2. Mycorrhizal communities in *Coffea robusta* roots

After filtering OTUs and samples, the Illumina MiSeq analysis resulted in 307 OTUs across 284 coffee root samples. Five AMF families were identified: *Glomeraceae* (72%, 221 OTUs), *Acaulosporaceae* (18%, 56 OTUs), *Gigasporaceae* (2%, 6 OTUs), *Archaeosporaceae* (2%, 7 OTUs), and *Paraglomeraceae* (1%, 4 OTUs) (Fig. 3). These five AMF families were all present in the forest coffee (FC) system, with the

monoculture (MC) and agroforestry (AF) systems lacking members from the *Paraglomeraceae* family. *Glomeraceae* was the most dominant family in the MC and AF systems, followed by *Acaulosporaceae*, while the reverse was true for the FC system (Fig. 3).

Eight AMF genera were identified, ordered by dominance: *Glomus*, *Acaulospora, Gigaspora, Scutellospora, Archeospora, Rhizophagus, Paraglomus*, and *Dentiscutata. Glomus* was the most dominant genus in the MC and AF systems, followed by *Acaulospora*, while the inverse was found for the FC system (Fig. S1). The *Paraglomus* genus was only found in the FC system.

3.3. Variation in AMF diversity and communities across coffee systems

No significant differences were found in terms of AMF alpha diversity indices (S_{obs}, S_{exp}, N_1, N_2, E_1 and E_2) between the three types of coffee systems (Fig. S2; Table S2). In contrast, the AMF communities were found to differ significantly between all coffee systems along both NMDS axes ($F(3,281) = 10.95$; $p < 0.0001$) (Fig. S3). Moreover, the greatest differences in AMF community composition were found between FC and MC ($p = 0.00015$; $R^2 = 0.071$) and between FC and AF ($p = 0.00015$; $R^2 = 0.084$). Nonetheless, highly significant differences were also found between AF and MC ($p = 0.012$; $R^2 = 0.10$).

Out of the 307 observed OTUs, 86 were identified as indicator species. Among these, 49 indicator AMF species were identified for FC, 19 for AF and 18 for MC. Over two-thirds of all indicator species belong to the *Glomus* genera and about one-fifth to *Acaulospora* (Fig. S4). Almost all indicator species belonged to *Glomus* for MC and AF, while indicator species from 7 genera were found for FC. Nonetheless, more *Glomus* indicator species were found for FC than both other systems.

Additionally, more than one-third of all OTUs were unique (106 OTUs), defined as being found in only one of the three coffee systems (Table S3). Of the 106 unique OTUs, most were found in the FC system (48%, 51 OTUs), followed by AF (30%, 32 OTUs) and MC (22%, 23 OTUs). Two-thirds of unique OTUs belonged to *Glomus*, followed by 15% to *Acaulospora*. Furthermore, most of the unique *Glomus* OTUs were found in FC, followed by AF, with MC having the least amount of unique *Glomus* (Fig. S5). Most unique Acaulospora OTUs were found in FC, followed by MC, with AF having the least. All unique *Paraglomus* were found in FC. Furthermore, 88% of the uniquely AF OTUs belong to the genus *Glomus*, with *Acaulospora* representing only 3% of uniquely AF OTUs (Fig. S5). For FC, 65% of unique OTUs belonged to *Glomus*, while 22% belonged to the *Acaulospora* and 8% to the *Paraglomus*. In contrast, only 43% of unique OTUs found in MC belong to the *Glomus* genus, with *Acaulospora* representing 17% of unique MC OTUs.

3.4. Effects of environmental variables on AMF diversity

The Generalized Linear Model (GLM) using a Negative Binomial distribution found that OTU richness (Chao1) decreased with higher soil bulk density ($z = -2.99$, $p = 0.003$), higher available soil phosphorus content ($z = -2.22$, $p = 0.03$). OTU richness was larger in forest coffee systems than in monocultures ($z =$

2.48, $p = 0.01$). In addition, significant interaction effects were found between the FC system and soil available P as well as between FC and soil pH (Table 2; Fig. S6 and S7). Both interaction effects for FC positively affect OTU richness. Soil pH did not significantly affect the observed OTU richness ($z = -0.12$, $p = 0.11$). The GLM model for observed OTU richness (Hill N_0) gave similar results (Table S4) as the model for OTU richness (Chao1). The only difference is that no interaction effect was found between available soil P and FC in the Hill N_1 model. The GLM model for Hills N_1 only found a significant effect by soil bulk density (Table S4). No significant variables were found when modelling the other Hill numbers (N_2, E_1 and E_2) using GLM (results not shown).

Table 2
Effect of soil physio-chemical properties and canopy closure on OTU richness (Chao1), analysed using a Generalised Linear Model with a Negative Binomial distribution (n = 258).

	Reduced GLM - OTU richness (Chao1)			
	Estimate	± se	*z-value*	*p-value*
(Intercept)	2.89	0.08	34.8	**< 0.0001*** **
Soil Bulk Density	− 0.15	0.05	− 2.99	**0.0028** **
Olsen-P [mg/kg]	− 0.22	0.01	− 2.22	**0.027***
Forest Coffee (FC)	1.41	0.57	2.48	**0.013***
Agroforestry (AF)	− 0.09	0.16	− 0.54	0.59
Olsen-P x FC	0.74	0.28	2.63	**0.0085** **
Olsen-P x AF	0.04	0.13	0.29	0.77
pH	− 0.12	0.08	− 1.60	0.11
pH x FC	1.59	0.65	2.44	**0.015***
pH x AF	0.30	0.17	1.78	0.075

3.5. Effects of environmental variables on AMF community composition

The results of the CCA indicated that canopy closure ($F = 12.9$, $p < 0.0001$), pH ($F = 4.73$, $p < 0.0001$), soil available P ($F = 2.00$, $p = 0.01$), and soil bulk density ($F = 1.62$, $p = 0.04$) were all significant predictors of the variation in the Bray-Curtis distance matrix (Table 3). Canopy closure explained the largest proportion of variation in the Bray-Curtis distances (Sum of Squares = 5.46), followed by pH (Sum of Squares = 2.00), soil available P (Sum of Squares = 0.85), and soil bulk density (Sum of Squares = 0.69). These results indicate that canopy closure and soil pH are the most influential environmental variables affecting the AMF community structure. However, it should be noted that the environmental variables could only explain a minor portion of the total variability in AMF communities ($R^2_{adj} = 0.07$), similar to

Fulthorpe et al. (2020). Furthermore, canopy closure and pH had the largest effect on the differentiation between FC versus MC and AF (Fig. 4; Table 3). Soil available P was the primary factor explaining the shift in community composition between AF and MC.

Table 3
Determinants of AMF community composition variations analysed using Canonical Correspondence Analysis (CCA) based on the AMF Bray-Curtis dissimilarity matrix and environmental variables from 258 coffee root samples in Tshopo Province, DRC.

Environmental Drivers of AMF Community Composition						
	Df	Sum of Squares	CAP1	CAP2	F-value	p-value
Canopy closure [%]	1	5.46	0.88	0.37	12.9	**< 0.0001**
pH ($CaCl_2$)	1	2.00	− 0.87	0.27	4.73	**< 0.0001**
Olsen-P [mg/kg]	1	0.85	− 0.40	0.82	2.00	**0.0106**
Soil Bulk Density [kg/m^3]	1	0.69	− 0.41	0.11	1.62	**0.0450**
Residual	*254*	*107.56*				

4. Discussion

We addressed the significant knowledge gap regarding the diversity and community composition of AMF in robusta coffee. Specifically, we focused on its region of origin, including the Democratic Republic of Congo (DRC), where such data were entirely lacking. Additionally, we identified the environmental factors influencing the diversity and community composition of Robusta AMF, which had not been previously determined. Our study revealed that while the overall mycorrhizal diversity remained consistent across different coffee cultivation systems, significant variation in community composition was evident. These differences were associated with a gradient of agricultural intensification and were reflected in various soil variables and the degree of canopy closure.

4.1. Mycorrhizal Communities: Similar but Different across Coffee Systems

The most frequently encountered AMF genera in our study were *Glomus* and *Acaulospora* (Fig. 3), which is in accordance with another study of AMF in robusta coffee in Mexico (Bertolini et al., 2018), and in arabica coffee, both in Ethiopia, arabica's native range (De Beenhouwer et al., 2015a, 2015b; Muleta et al., 2007, 2008), and its introduced ranges (Arias et al., 2012; Fulthorpe et al., 2020; Júnior et al., 2019). A study on mycorrhizal associations of plantain bananas in the Tshopo, DRC, found *Glomus* being the most abundant genus, followed by *Gigaspora*, *Acaulospora*, *Scutellospora* and *Entrophospora* (Kasaka et al., 2022). Several genera, such as *Enthrophospora*, *Diversispora* and *Ambispora*, have been previously identified in arabica (Duong et al., 2020; Andrade et al., 2009; Arias et al., 2012; De Beenhouwer et al.,

2015a, 2015b; Muleta et al., 2007, 2008), but were not found in our study on robusta nor others (Bertolini et al., 2018).

We found no significant differences in AMF diversity across the three coffee systems (Fig. S1; Table S2). In contrast, mycorrhizal richness was reported to be greater in arabica roots in Ethiopian natural forests than in production coffee systems, including semi-forests, home gardens and plantations (De Beenhouwer et al., 2015b). Noteworthy, mineral fertilisers and pesticides are not used in the coffee systems studied in our research. These agro-inputs are known to change the soil characteristics and plant-microorganism interactions (Tilman et al., 2002; Verbruggen et al., 2010). The lack of using fertilisers and pesticides could partially explain the lack of differences in AMF diversity across systems, in contrast to De Beenhouwer et al. (2015b) who reported the use of mineral fertilisers and pesticides in Ethiopian plantation coffee systems. Similarly, Júnior et al. (2019) found differences between arabica coffee agroforestry and monocultures in Brazil, which they explained by the increased management intensity in monocultures, especially relating to the use of fertilisers.

Subsequent analysis of the AMF communities identified significant differences across all three coffee systems (Fig. S2). The largest differences were found between forest coffee and the two coffee production systems, in line with De Beenhouwer et al. (2015b). In addition, highly significant differences were also found between monocultures and agroforestry systems (Fig. S2). Similarly, AMF community composition in arabica plantations differed greatly from more natural coffee production systems, again likely caused by the use of chemical fertilisers and pesticides, but also by severe canopy thinning and undergrowth slashing (De Beenhouwer et al., 2015a; Júnior et al., 2019).

The three coffee systems were quite similar in terms of the presence/absence of AMF families and genera. However, they exhibited important differences in terms of dominance across the coffee systems (Fig. 3). Moreover, we found *Acaulospora* to be the most dominant genus (55%) and *Glomus* the second most dominant (40%) in forest coffee, while *Glomus* was the most dominant (70–80%) and *Acaulospora* the second most dominant (20–30%) in the monoculture and agroforestry system (Fig. 3 and S1). In contrast, De Beenhouwer et al. (2015a) found the relative abundance of the *Glomus* and *Acaulospora* genera to be similar across arabica production systems, including wild arabica coffee and plantations. The variation in the dominance of AMF genera across coffee systems in our study is understandable when considering the functional roles of these genera. *Glomus* species are known to be more host-specific and thrive in disturbed and nutrient-poor environments. They play an important role in plant growth, nutrient uptake, particularly phosphorus uptake, and stress tolerance (Smith and Read, 2008). In contrast, *Acaulospora* species are known to be less host-specific and inhabit more diverse environments, including forests. They contribute to nutrient exchange, including phosphorus uptake, though to a lesser extent than *Glomus* species, and micronutrient acquisition (Smith & Read, 2008). Muleta et al. (2007) found that shade tree species influence AMF genera's dominance in Ethiopia's arabica coffee rhizosphere, with *Acaulospora* occurring more frequently under leguminous trees than under non-leguminous shade trees. This effect could be explained by legumes being more dependent on AM fungi than non-legumes (Muleta et al., 2007). Additionally, *Acaulospora* generally prefer more acidic

soil conditions (Álvarez-Sánchez et al., 2011), and forest coffee had the lowest soil pH of all coffee systems in our study (Table S1). Overall, this could explain our finding that *Acaulospora* were more dominant in forest coffee than in monocultures and agroforestry systems, while the inverse was found for *Glomus*.

It is important to note that many unique OTUs, defined as being present in only one of the three coffee systems, were observed (Table S3). More than a third of all OTUs in this study were unique to one coffee system. In addition, many more unique OTUs were found in forest coffee compared to the two other coffee systems, with monocultures having the smallest amount of unique OTUs (Table S3; Fig. S5). Similarly, most indicator species were found for forest coffee, with forest coffee having indicator species in almost all genera identified. In contrast, almost all indicator species for agroforestry and monocultures belonged to *Glomus* only (Fig. S4). Thus, wild robusta coffee in natural forest systems is colonised by a greater diversity of unique mycorrhizae than cultivated robusta in other management systems, highlighting its important value from a biodiversity and conservation perspective. These findings align with De Beenhouwer et al. (2015a), who found forest coffee to contain many more unique OTUs than other coffee systems. Notably, 88 % of unique OTUs in the agroorestry systems were *Glomus* species. Nonetheless, in absolute terms, forest coffee still had slightly more unique OTUs belonging to the *Glomus* genus than agroforestry (Table S3).

4.2. Environmental variables as drivers behind differences in AMF diversity and community composition

Soil available P and soil bulk density negatively influenced AMF diversity overall, while available soil P and pH positively influenced AMF diversity in forest coffee. Also, AMF community composition in the coffee roots shifted significantly in response to, in order of importance, canopy closure, soil pH, available soil P and bulk density (Table 3). In contrast to other studies (De Beenhouwer et al., 2015a; Fulthorpe et al., 2020), our study did not identify an effect of soil inorganic N on AMF community composition. This is likely because none of the farmers in our study use mineral N-fertilizers.

Among the environmental variables, canopy closure was found to have the greatest impact on AMF community composition. Similarly, significant effects of canopy closure were found in studies on arabica in Central America (Fulthorpe et al., 2020), while no effect was found for studies on arabica in Ethiopia (De Beenhouwer et al., 2015a). Canopy thinning results in changes in the soil microclimate, which is known to affect soil microbial communities (Castro et al., 2010).

Available soil P affected both AMF diversity and community composition. The identification of available soil P as a driver of AMF community composition was found in our study and other studies on robusta and arabica (Bertolini et al., 2018; De Beenhouwer et al., 2015a; Fulthorpe et al., 2020; Posada et al., 2018). Furthermore, it has been shown that many fungal groups exhibited distinct preferences for specific soil phosphorus concentrations (Tedersoo et al., 2014). Vaast et al. (1996) showed that increased P-availability significantly decreased mycorrhizal colonisation of in vitro propagated arabica plants. Different AMF species presented different tolerances to increasing P-availability. Furthermore, it

has been shown that increasing P-availability can reduce the provision of plant-assimilated carbohydrates towards the mycorrhizal symbionts (Liu et al., 2012). This leads to increased competition for carbohydrates between AMF and can consequently reduce AMF diversity on the host plant. This process explains why overall AMF diversity was shown to decrease with increasing available soil P (Table 2), in line with studies on arabica (De Beenhouwer et al., 2015a; Posada et al., 2018) and other crops (e.g., Johnson et al., 2013).

In addition to the overall negative effect of available soil P on AMF diversity, a significantly positive interaction was observed between forest coffee and available soil P (Table 2; Fig. S6). Thereby indicating that for forest coffee, AMF diversity increased with higher levels of available soil P. Treseder & Allen (2002) showed that the effects of nutrient availability may differ among AMF genera and ecosystems with varying soil nutrients, for example due to N- or P-limitation. Moreover, they found that the *Glomus* genera in particular had a distinct response to nutrient availability. This might explain the interaction effect found for soil available P in forest coffee, given that soil available P was much lower in forest coffee than in agroforestry systems (Table S1) and the *Glomus* genera was relatively much less abundant in forest coffee than in agroforestry and monocultures (Fig. S1).

Although the overall effect of soil pH on AMF diversity was not statistically significant ($p = 0.11$), there was a tendency that increased soil pH decreased AMF diversity. On the other hand, higher soil pH increased AMF diversity in forest coffee systems (Table 2; Fig. S7). This can be explained by the much lower soil pH observed in forest coffee systems (pH-$CaCl_2$ = 3.40 ± 0.02) compared to monocultures (pH-$CaCl_2$ = 4.4 ± 0.2) and agroforestry systems (pH-$CaCl_2$ = 5.0 ± 0.1) (Table S1). In the extremely acidic environments found in forest coffee systems, it is reasonable to expect that AMF diversity would increase as the soil becomes less extremely acidic. In addition, soil pH was the most important soil variable driving differences in AMF community composition. This agrees with previous studies on robusta (Bertolini et al., 2018) and arabica (De Beenhouwer et al., 2015a; Fulthorpe et al., 2020; Posada et al., 2018), as well as with broader global studies (Álvarez-Sánchez et al., 2011; Hazard et al., 2013; Jansa et al., 2014). The large impact of soil pH on fungal communities has been reported before at local and global scales (Geml et al., 2012; Lauber et al., 2009; Mikryukov et al., 2023; Rousk et al., 2010; Tedersoo et al., 2014). The role of pH is important because it affects the availability of nutrients, especially P and Ca, alters competitive interaction between fungi and other soil microorganisms and can directly affect fungal growth and survival (Rousk et al., 2010).

Soil bulk density was also found to positively affect AMF diversity and drive shifts in AMF community composition. The abundance and composition of AMF communities in plant roots are known to be influenced by rhizosphere variables such as soil moisture and aeration (Fulthorpe et al., 2020; Lauber et al., 2009; Posada et al., 2018). Lower soil bulk densities allow for more interplay between air and water. Increased soil aeration and water-holding capacity positively affect the functioning of AMF (Allen et al., 2003). Under optimal soil moisture and soil aeration conditions, AMF phylotypes can be more equal competitors for root colonisation. In the case of higher soil bulk densities, the lower soil moisture

content and lower aeration may operate as an environmental filter, giving rise to fewer AMF tolerant of the prevailing habitat condition taking over (Deepika & Kothamasi, 2015).

5. Conclusion

Our study revealed substantial mycorrhizal colonisation of *Coffea canephora* roots across all coffee systems. While AMF diversity was similar across the three coffee systems, community composition varied greatly between forest coffee, monocultures, and agroforestry systems. Moreover, the dominance of multiple AMF families and genera was found to vary greatly when comparing forest coffee with monocultures and agroforestry systems. Many OTUs unique to one type of coffee system were identified, underscoring the value of all three coffee systems regarding their AMF diversity. Nonetheless, unique OTUs were most abundant in wild robusta in the forest and least in monocultures. This underscores the irreplaceable role of Congolese wild robusta coffee in its native habitat and thus emphasises the critical need for conserving tropical rainforests in the DRC.

This study showed that soil available phosphorus and soil bulk density negatively influenced AMF diversity, irrespective of the coffee system. Additionally, available soil P and pH positively influenced AMF diversity in the forest coffee system. Canopy closure, soil pH, available soil phosphorus, and soil bulk density shaped AMF composition. Although the effect of shifts in AMF community composition on coffee yields is yet unknown, research has demonstrated that AMF can significantly enhance coffee plant productivity and resilience, such as drought tolerance and pest resistance, both *in situ* and *in planta* (Duong et al., 2020). This is particularly crucial given climate change's present and future impacts on coffee production. Future research should thus focus on the practical potential of AMF associations *in situ* under field conditions to determine whether farmers should or should not incorporate AMF into their coffee management practices.

Declarations

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Author Contribution

Conceptualization: IB, OH, RM, BV; Methodology: IB, AD, OH, RM, BV; Data Collection: IB; Software: IB, AD; Formal Analysis: IB; Data Curation: IB; Writing – Original Draft: IB; Writing – Review & Editing: IB, AD, OH, RM, BV; Visualization: IB; Supervision: OH, RM, BV; Project Administration: IB, RM, BV; Funding Acquisition: RM, BV.

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Data Availability

The data has been uploaded to Zenodo and can be accessed via:
https://doi.org/10.5281/zenodo.13310433

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Chat GPT 3.5 in order to improve readability and language. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Figures

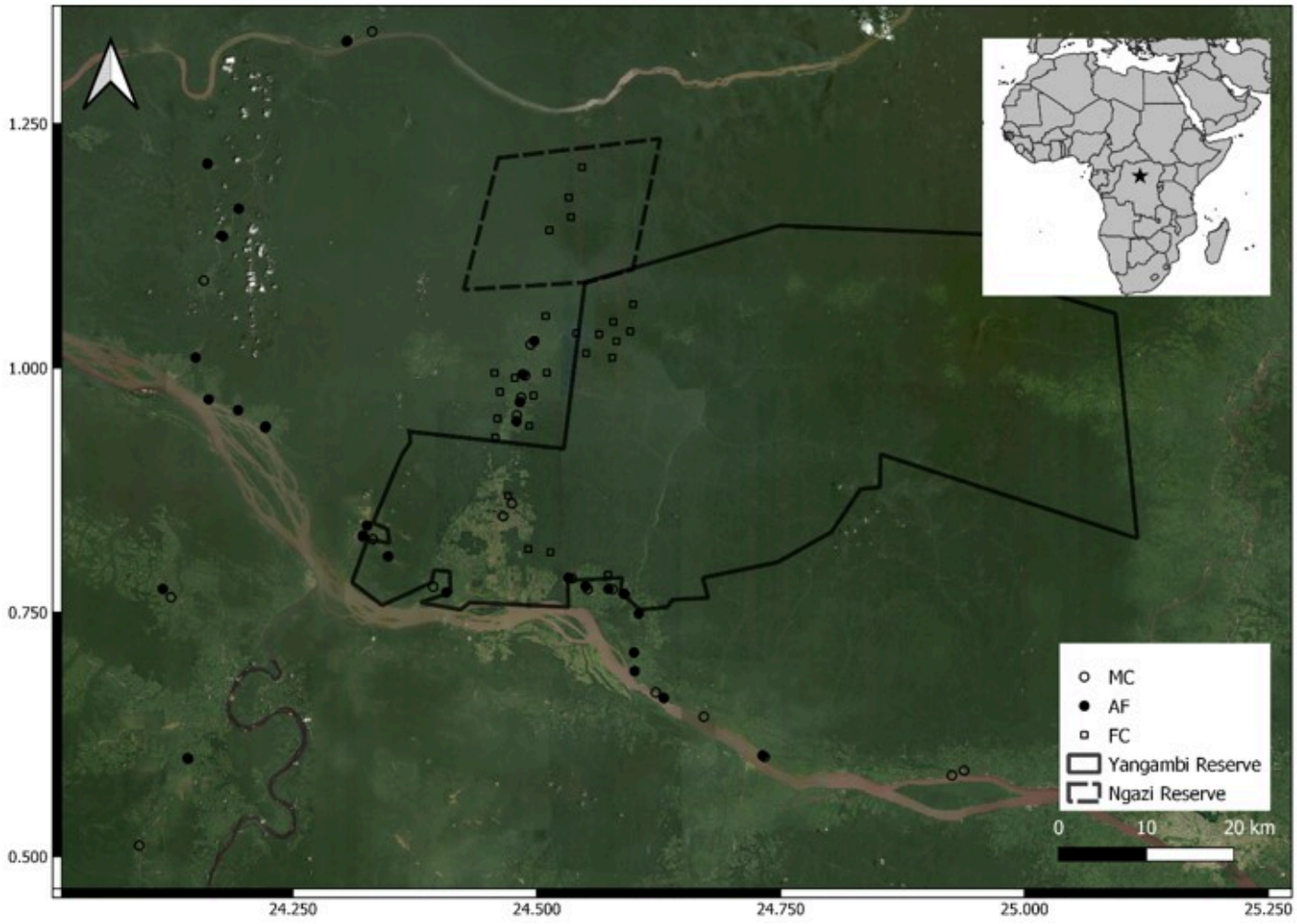


Figure 1

Map of the study area in the Tshopo province, DRC, showing the location of 77 coffee plots, encompassing the three coffee typologies: Monoculture (MC), Agroforestry (AF) and Forest Coffee (FC)



Figure 2

Representative examples of the three robusta coffee systems found in the Tshopo province, DRC: Monoculture (MC), Agroforestry (AF) and Forest Coffee (FC)

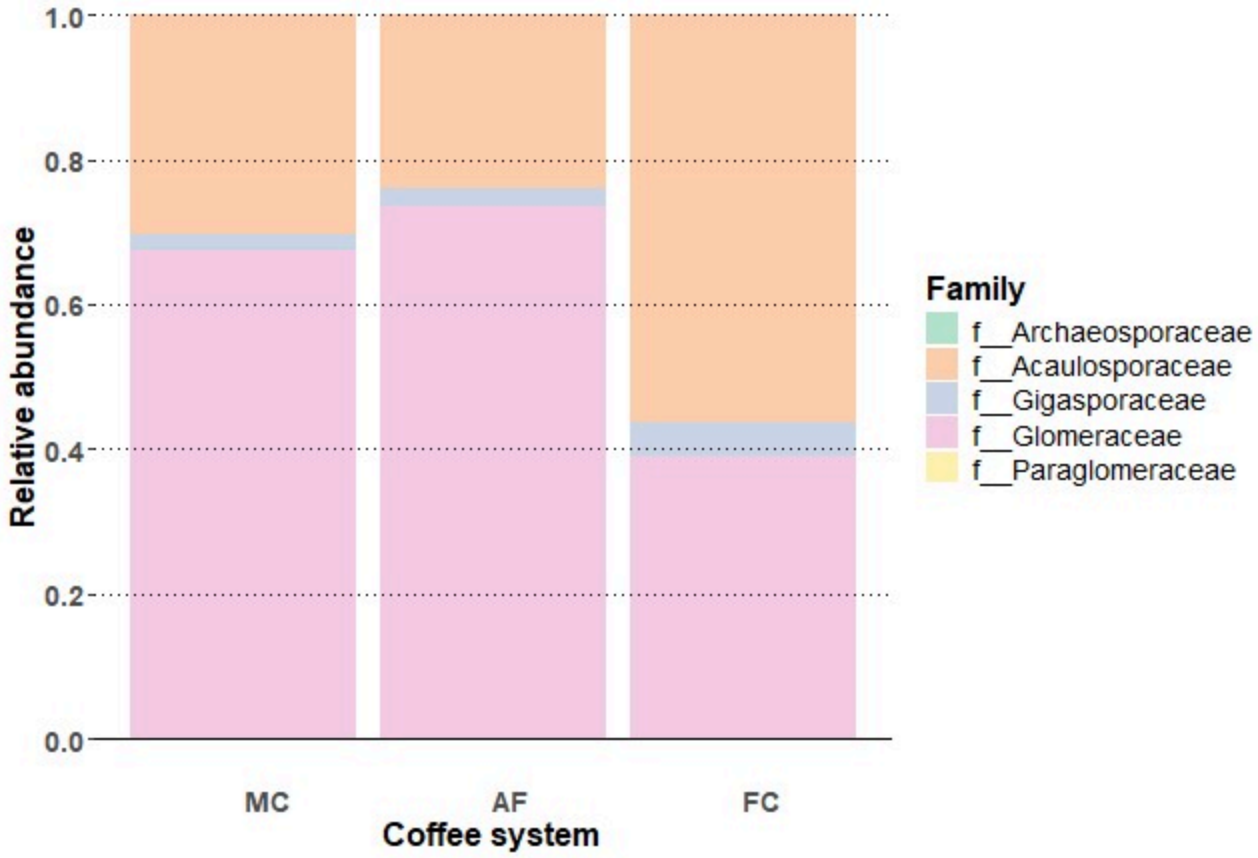


Figure 3

Relative read abundances (%) of AMF families in the roots of robusta coffee across the three coffee systems of the Tshopo province, DRC (n = 284)

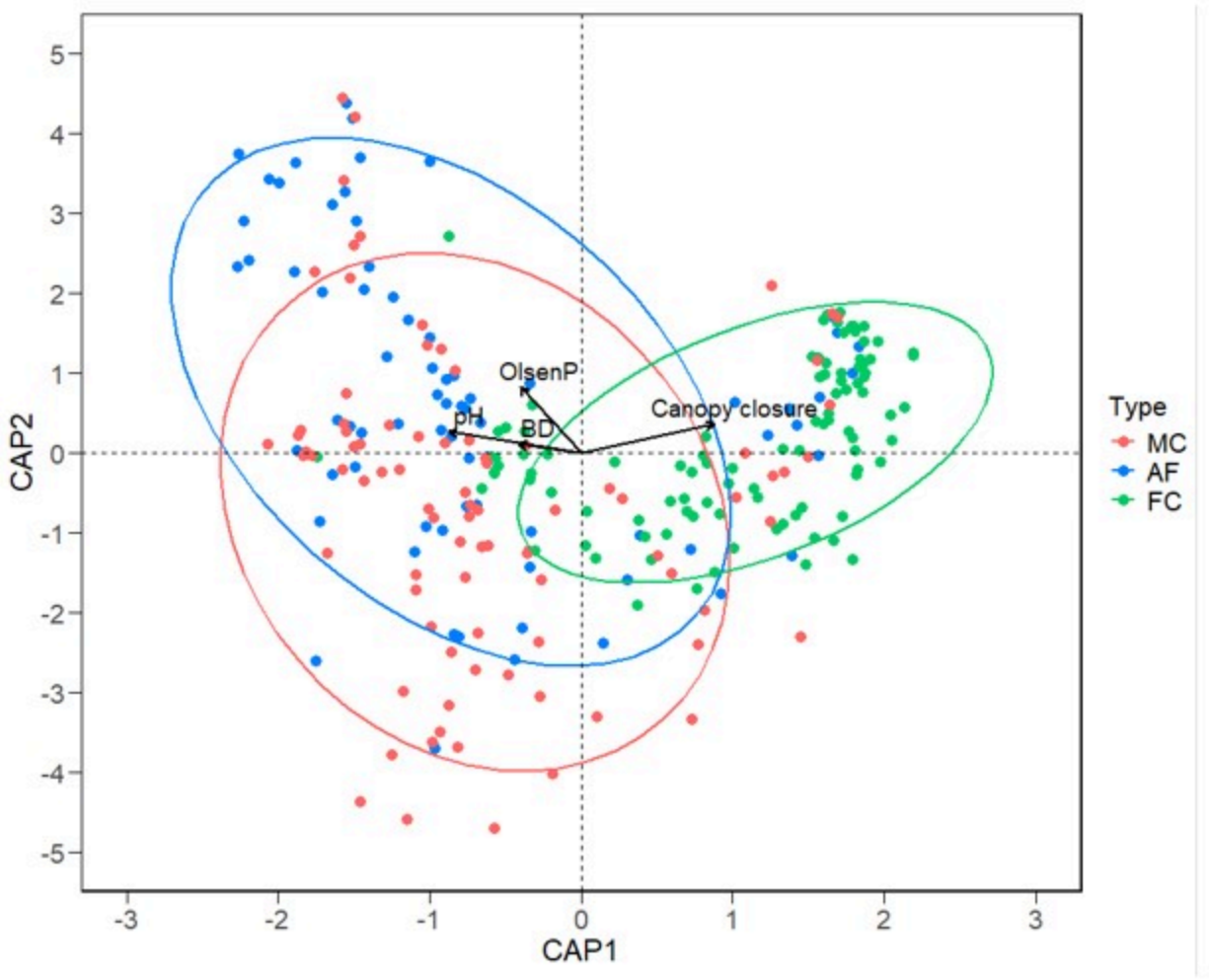


Figure 4

Ordination plot from Canonical Correspondence Analysis (CCA) showing AMF community composition in coffee roots across three coffee systems: Monoculture (MC), Agroforestry (AF), and Forest Coffee (FC). Arrows represent environmental variables significantly influencing AMF community composition (Table 3), with the angle between arrows indicating correlations (Type II scaling). Ellipses depict 90% confidence regions for each system: red (MC), blue (AF), and green (FC)

Supplementary Files

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