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RESEARCH ARTICLE

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Arbuscular Mycorrhizal Fungal Community Shifts Driven by Heavy Metals in Cocoa Agroecosystems at Different Crop Age

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ABSTRACT

Cocoa (*Theobroma cacao* L.) is an economically important tropical crop, but its production is increasingly constrained by strict regulations related to heavy metal accumulation in soils. Arbuscular mycorrhizal fungi (AMF) are a key biological component of cocoa agroecosystems due to their role in soil processes and plant nutrition. However, information on AMF diversity and their response to heavy metals remains limited. In this study, AMF communities were evaluated in cocoa plantations of different ages established under contrasting heavy metal conditions. A total of 53 AMF taxa were identified, indicating high fungal richness across all plantations. No significant differences were found in α-diversity indices or community composition among plantation ages. In contrast, multivariate analyses showed that variation in AMF community structure was primarily associated with soil heavy metal gradients, particularly cadmium (Cd) and zinc (Zn). Species-level models revealed contrasting responses among taxa, with both positive and negative associations along Cd and Zn gradients, suggesting differential tolerance strategies. Overall, these results indicate that edaphic factors, especially heavy metal concentrations, play a more important role than plantation age in shaping AMF community composition. These findings provide a basis for identifying AMF taxa adapted to metal-enriched soils and for developing management strategies that support sustainable cocoa production.

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Glomeromycota; community assembly; agroforestry; agroecosystem sustainability

1. Introduction

Cocoa (*Theobroma cacao* L.), belonging to the Malvaceae family, is a plant native to the tropical areas of America whose production is mainly developed in agroforestry systems [1]. Currently, Africa leads its production worldwide, followed by Latin America and Asia. The main product marketed from this species is chocolate, with an annual per capita consumption that ranges between 4 and 8 kg in Europe and reaches 4 kg in the United States [2,3]. This sustained demand has consolidated a solid economic sector and expansion, particularly in the field of export.

However, since 2019, the European Union, through Regulation No. 488/2014, has established maximum permitted limits of cadmium (Cd) in products derived from cocoa, restricting levels to a maximum of 0.8 ppm, along with others heavy metals [4]. This regulation has generated a threat considerable socioeconomic impact for Latin American producers, whose soils have naturally high levels of Cd and Pb, which can be absorbed and translocated to cocoa seeds [5]. Cadmium is considered one of the most toxic heavy metals present in the environment, without any identified to date a physiological or biological function in plants [6]. In addition, it represents a direct threat to safety food and human health [7], as its accumulation in the body can cause kidney stones, kidney failure, and dysfunctions in the endocrine, reproductive and respiratory systems.

In Peru, cocoa plantations cover approximately 107,000 hectares, constituting a key resource for its positioning in the international market. During the period January–December 2022, the San Martín region led production with 64.9 thousand tons, followed by Junín (31.9 thousand tons) and Ucayali (22.7 thousand tons), representing 38%, 18%, and 13% of national production, respectively. Likewise, in the first semester of 2023, around 46.7 thousand

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tons of cocoa and derivatives were exported, reaching a value of US$ 162.8 million, which represented a year-on-year growth of 20.7% in value and 12.3% in volume [8]. These figures increased for the year 2024 with exports of 159 thousand tons in cocoa and derivatives with a value of US$ 1,249 million. These figures reflect the strategic importance of cocoa cultivation in the national economy, as well as its fundamental role in the subsistence of thousands of small producers [9].

Despite the sustained growth of the sector, the presence of heavy metals such as cadmium in agricultural soils constitutes a potential threat to the quality of the grain, competitiveness in the international market and, ultimately, the sustainability of the productive system [10]. Recent studies report the presence of Cd in leaves and seeds of various cocoa genotypes, with especially high levels in regions such as Tumbes, Piura, and Amazonas, exceeding permissible limits [11]. This has generated a reduction in Peruvian cocoa exports to Europe, and it has documented that producers in the Piura region have suffered income losses of around 31% [12]. This situation threatens the cocoa value chain and the socioeconomic well-being of the families who depend on this crop. Likewise, the presence of other heavy metals such as Pb, As, and Cr raises concerns regarding food safety risks in cocoa plantations [10].

Faced with this scenario, the development of innovative and sustainable strategies that allow mitigating or avoiding the accumulation of Cd in soils and grains of cocoa. A promising ecological alternative is the use of mycorrhizal fungi arbuscular (AMF) natives. This symbiosis, which occurs in approximately 72% of plant species capable of forming fungus–plant associations, plays a key role in ecosystem functioning [13]. AMF can reduce the bioaccumulation of heavy metals in plants, representing a valuable biotechnological tool for the management and remediation of contaminated agroecosystems (Merlos et al. 2016) [14]. A study by [15] showed that inoculation with *Glomus versiforme* and *Rhizophagus intraradices* in plants of *Lonicera japonica* grown in Cd-contaminated soils significantly improved their growth due to a greater absorption of phosphorus, while reducing Cd concentrations in roots and shoots. Thus, other heavy metals such as Cd, Cr, Ni, and Pb are also mediated by AMF in important crops such as corn (*Zea mays* L.) and soybean (*Glycine max* L.), either through phytoextraction or phytostabilization mechanisms [16,17]. These beneficial effects are attributed to the adaptive mechanisms of AMF, such as metal immobilization, transport, and compartmentalization, which in turn reduce its translocation to vegetative and reproductive organs [17–19].

Therefore, characterizing native AMF communities in soils containing heavy metals such as Cd, Pb, Hg, and As is important to better understand how these fungi respond to metal-enriched conditions in cocoa agroecosystems. Native AMF communities may be adapted to these environments and play a relevant role in soil–plant interactions under metal stress, which is important for sustainable soil management in tropical cocoa systems [21]. In this context, this study aimed to (i) assess the influence of crop age on AMF community composition and (ii) evaluate the effect of soil heavy metal concentrations on the composition of these communities.

2. Methods

2.1. Site description and sampling methods

The study was carried out between October 2023 and December 2024, at the end of the rainy season, in 35 plots distributed across seven provinces of the San Martín region, Peru: Mariscal Cáceres, Huallaga, Lamas, Picota, Tocache, El Dorado, and Bellavista (Table S1 of Supplementary Material). The selection of sampling sites considered geographic and edaphic variability, as well as the predominant agricultural use in the area (Figure 1).

The general climatic conditions of the Peruvian Amazon are characterized by average annual temperatures ranging from 23 to 33 °C, and annual precipitation averaging 1380 mm [22]. The sampled plots correspond to cacao-producing farms managed under diversified agroforestry systems, where *T. cacao* L. is cultivated in association with complementary species such as banana (*Musa* spp.), coffee (*Coffea* spp.), local fruit trees, and timber species typical of the region.

In each plot, rhizospheric soil samples were collected from *T. cacao* plants. Five plants per plot were selected, and from each plant, an individual soil sample was obtained at a depth of 0–30 cm. Sampling was conducted at three equidistant points around the trunk in order to obtain a representative characterization of the rhizosphere. Each sample consisted of approximately 1.5 kg of soil, which was placed in labeled zip-lock bags and transported at ambient temperature until processing.

A total of 175 soil samples were collected following a systematic sampling design [23]. The sampling scheme was structured as a hierarchical design, in which individual soil samples were nested within plots, allowing for the representation of within-plot

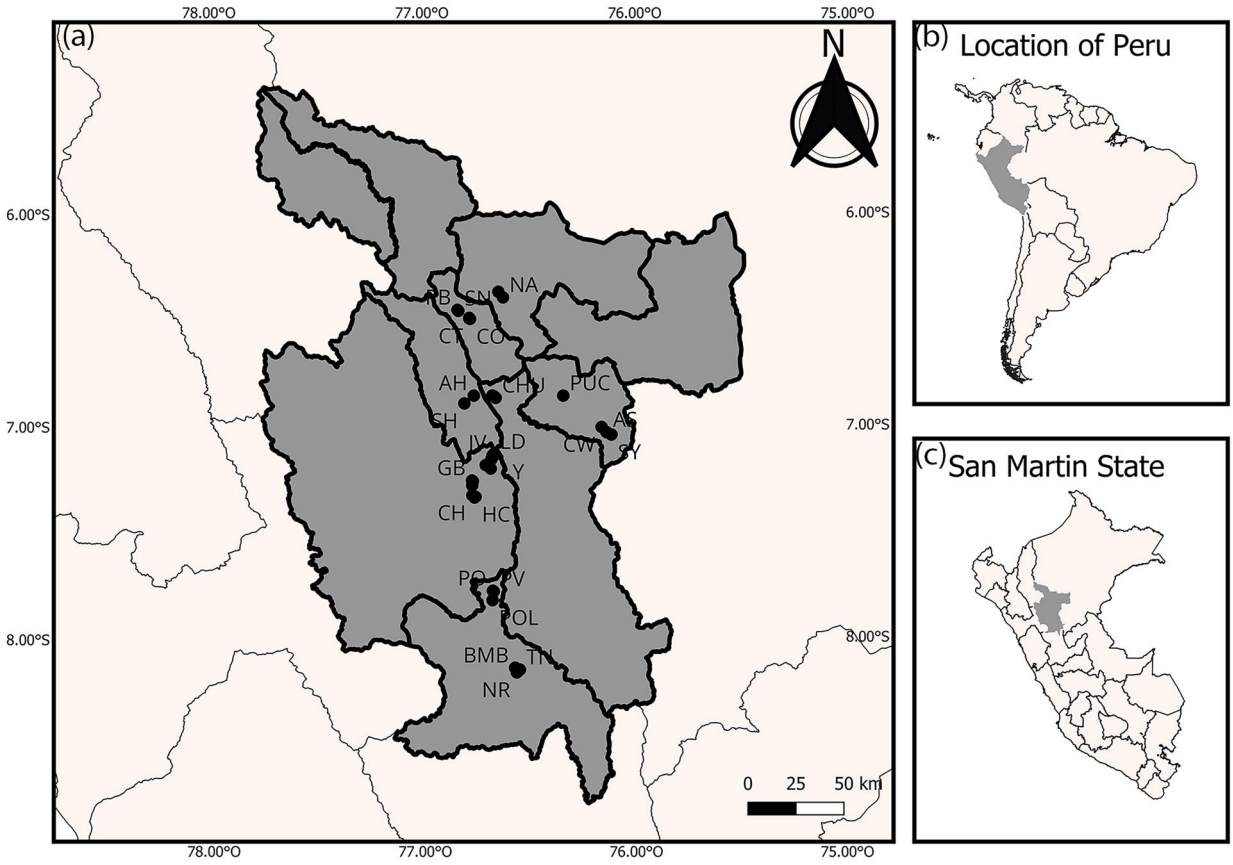

Figure 1. Geographic location of the study area and soil sampling sites in *Theobroma cacao* plantations in San Martín, Peru. (a) shows the distribution of sampling sites within the study area, (b) indicates the location of Peru in South America, and (c) shows the location of the San Martín region within Peru. Black dots represent soil sampling points.

variability while preserving the plot as the primary unit of comparison.

2.2. Physical, chemical, and heavy metal analysis of the soil

A 500 g aliquot of soil was obtained from each independent sample corresponding to each sampled plant. These aliquots were destined for analysis physicochemical and heavy metal analysis at the Laboratorio de Suelos, Aguas y Foliares (LABSAF) of the Estación Experimental Agraria – El Porvenir. The samples were dried at room temperature, disaggregated, homogenized and sieved using a 2 mm mesh. Soil pH was determined according to US EPA method 9045D [24]. The parameters matter organic (%), total nitrogen (estimated as 5% of organic matter), phosphorus available (ppm), exchangeable potassium (ppm), exchangeable bases (%), and the soil texture (%) were determined according to the provisions of the Standard Mexican Official [25]. On the other hand, the heavy metals nickel (Ni), copper (Cu), arsenic (As), cadmium (Cd), lead (Pb), zinc (Zn), and mercury (Hg) were quantified by atomic absorption spectroscopy (AAS). For the preparation and digestion of the samples, the protocols established in US EPA Method 3051 A and US EPA Method 6020B (SW-846) methods were used [26,27]

2.3. Extraction, identification, and quantification of AMF spores

The extraction of AMF spores was carried out using the wet sieving and decantation technique, according to the classic protocol described by [28], complemented by a stage of centrifugation in a 60% sucrose gradient, to facilitate the separation of the spores [29]. The spores obtained were mounted on slides using polyvinyl alcohol-lactic-glycerol (PVLG) [30] and a mixture of PVLG with Melzer's reagent (1:1) for later observation and microscopic identification.

Morphological identification was carried out using taxonomic keys provided by the International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi [31], following the classification proposed by [32], with the latest updates [33–36]. Taxa were delineated based on spore morphology and conservatively assigned at the species level only when diagnostic features were consistent with published descriptions. For taxonomic determination, morphological characteristics such as spore color, size, shape, and wall structure were considered. The observation and documentation of these structures were

performed using a Leitz Laborlux S optical microscope equipped with a Leica DFC 295 digital camera, and images were captured and analyzed with the Leica Application Suite software. To quantify spore abundance per sample, spores and sporocarps were counted as individual units.

2.4. Ecological and statistical analysis

The structure and diversity of AMF communities were evaluated by calculating species richness (S), Shannon–Wiener index (H), Simpson's dominance (D), Pielou's evenness (J), frequency of occurrence (FO), and relative abundance. These parameters were estimated for each sample using the formulas described by [37]. The frequency of occurrence was calculated as FO = Ji/k, where Ji represents the number of samples in which taxon i was detected and k is the total number of samples analyzed. According to [38], species were classified as dominant (FO > 50%), mostly common (30% < FO ≤ 50%), common (10% < FO ≤ 30%), or rare (FO ≤ 10%). Relative abundance was estimated as the proportion of individuals of each taxon relative to the total number of individuals recorded in all samples [39].

To account for the hierarchical sampling design, in which individual samples were nested within plots, different analytical approaches were applied depending on the type of response variable. For alpha diversity metrics (S, H, D, and J) and spore density, linear mixed-effects models were used, considering crop age as a fixed effect and plot as a random intercept. Prior to modeling, Shannon diversity values were transformed to exp(H). Differences among crop-age classes were evaluated using Tukey-adjusted pairwise comparisons ($p < 0.05$).

For soil physicochemical properties and heavy metal concentrations, subsamples within each plot were aggregated to the plot level ($n = 35$) to ensure independence among observations. Differences among crop-age classes were then evaluated using the Kruskal–Wallis test, with Bonferroni adjustment for multiple comparisons. Results were expressed as medians with interquartile ranges. The statistical analyses were performed using the Kruskal function from the *agricolae* [40].

Changes in AMF community composition among crop-age classes were assessed using permutational multivariate analysis of variance (PERMANOVA), based on Bray–Curtis distances, implemented in the adonis2 function from the *vegan* package [41,42], with 999 permutations. To avoid pseudoreplication, analyses were conducted using plot-level aggregated data. The homogeneity of multivariate dispersion (PERMDISP) was evaluated using the betadisper function. Pairwise comparisons between crop-age classes were performed using the pairwise.adonis function with Bonferroni correction from the *pairwiseAdonis* package. In addition, non-metric multidimensional scaling (NMDS) was used to visualize differences in community composition among crop ages, also based on the Bray–Curtis distance [43], with the aim of graphically visualizing the dissimilarities in the composition of the fungal communities between crop ages.

To explore the relationship between heavy metals and AMF community structure, a canonical correspondence analysis (CCA) was performed using plot-level data. All measured metals were initially included in the model, and their individual contributions were evaluated using permutation tests. This analysis allowed the identification of the main environmental gradients associated with changes in AMF community composition.

Based on these gradients, taxon-specific responses of AMF to heavy metal concentrations were evaluated using generalized linear models with a negative binomial distribution (GLM-NB), suitable for overdispersed count data. Only heavy metals identified as significant predictors in the CCA were included in these models. In each model, the abundance of each taxon was expressed as a function of the corresponding metal concentration, which was previously standardized (z-score transformation). The regression coefficient (β) and its 95% confidence interval were estimated, where $\beta < 0$ indicates a decrease in abundance with increasing metal concentration, and $\beta > 0$ indicates an increase. Only taxa with sufficient occurrence and variability were retained in the analysis, as rare taxa with sparse data did not allow reliable parameter estimation. To account for multiple testing across taxa, p-values were adjusted using the Benjamini–Hochberg FDR [44].

Finally, an indicator species analysis (ISA) was conducted to identify AMF taxa significantly associated with specific crop-age classes. This analysis was based on the IndVal index, which integrates the frequency of occurrence and the relative abundance of each taxon [45]. The calculation of IndVal and the evaluation of its significance were performed using the multipatt function of the *indicspecies* package, applying a Monte Carlo permutation test with 999 permutations. Taxa with $p < 0.05$ and IndVal ≥ 25% were considered significant indicator taxa.All statistical analyses were performed in R version 4.4.1 [46].

To visualize the exclusivity of taxa or taxa shared among crop-age classes, Venn diagrams were generated using the online tool "Calculate and draw custom Venn diagrams" available at https://bioinformatics.psb.ugent.be/webtools/Venn/.

3. Results

3.1. Occurrence of AMF taxa

A total of 53 taxa were identified, including seven morphotypes retained at the genus level (*Ambispora* sp. 1, *Ambispora* sp. 2, *Acaulospora* sp. 1, *Acaulospora* sp. 2, *Diversispora* sp. 1, *Dominikia* sp. 1 and *Sclerocystis* sp. 1) due to insufficient diagnostic morphological features for species-level assignment. The provisional morphotypes *Acaulospora* sp. 1 and *Acaulospora* sp. 2 differed mainly in spore size, shape, and Melzer's reactivity, whereas *Ambispora* sp. 1 and *Ambispora* sp. 2 were distinguished by differences in spore size and wall ornamentation. These taxa represent the three classes and six taxonomic orders currently recognized for the phylum Glomeromycota.

Among the most representative families, Glomeraceae stands out, including the genera *Glomus* (5 taxa), *Rhizoglomus,* and *Sclerocystis* (3 taxa each), followed by *Dominikia* and *Funneliformis* (2 taxa each), and *Funeliglomus*, *Microkamienskia*, *Nanoglomus*, *Parvocarpum*, and *Sclerocarpum*, each represented by a single species. Another family with high species richness was Acaulosporaceae, with 16 taxa belonging to the genus *Acaulospora*. Representative morphotypes of these taxa are shown in Figure 2.

Regarding crop age, 31 AMF taxa were recorded in plots aged 8–14 years, 29 taxa in plots aged 15–20 years, and 31 taxa in plots older than 20 years. Of the total taxa identified, 14 taxa were common to the three age categories. Conversely, 10 taxa were exclusive to the 8–14-year plots, 8 taxa to the 15–20-year plots, and 11 taxa to those older than 20 years (Table 1, Figure 3).

3.2. Richness, diversity, and indicator species of AMF

The analysis showed that crop age did not produce significant differences in the diversity variables evaluated: species richness ($F = 0.291$, $p = 0.750$), Shannon index ($F = 0.608$, $p = 0.551$), Simpson dominance ($F = 0.646$, $p = 0.531$), and Pielou's evenness ($F = 1.308$, $p = 0.285$) (Figure 4).

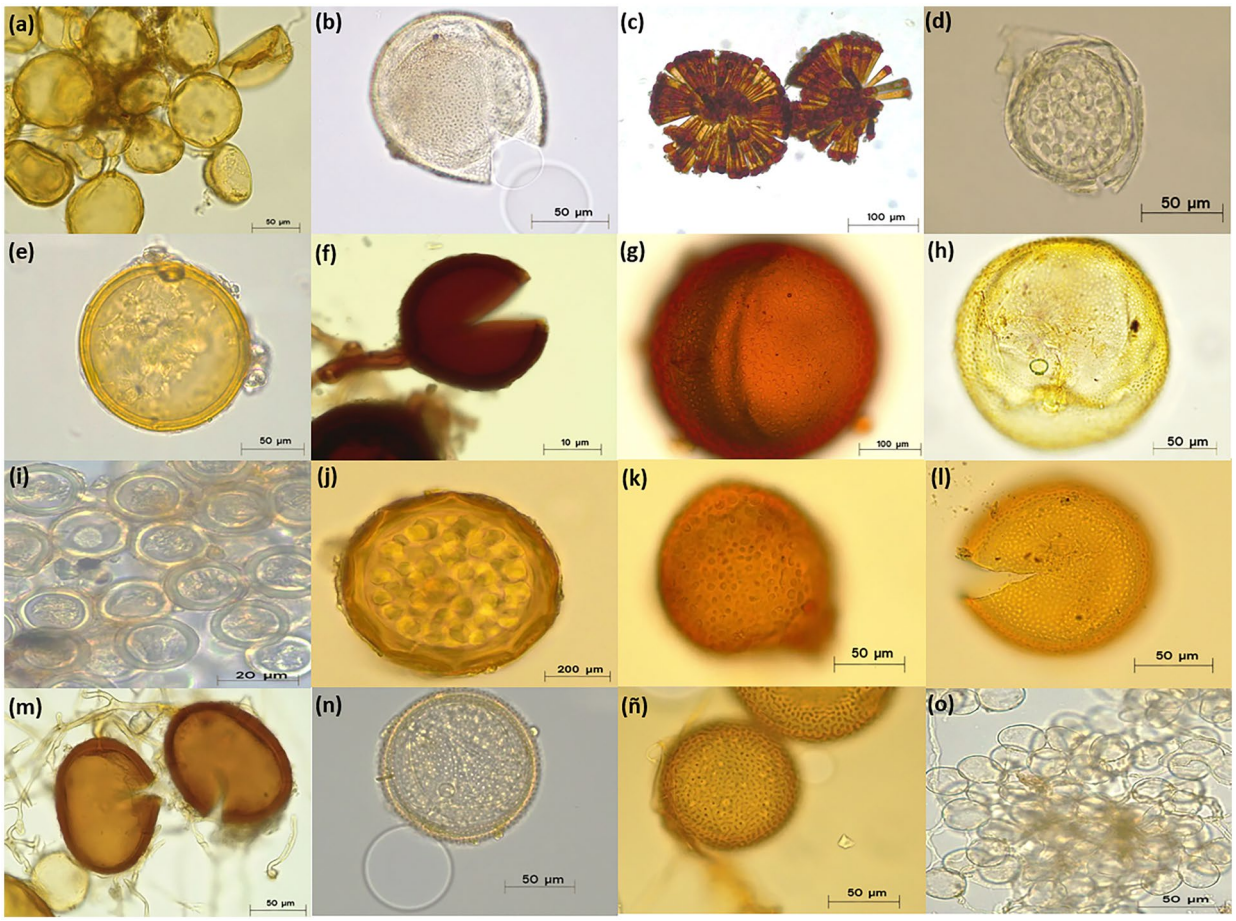

Figure 2. Representative morphotypes of arbuscular mycorrhizal fungi (AMF) spores used for morphological identification. (a–o) Correspond to the taxa recorded in this study: (a) *Rhizoglomus intraradices*; (b) *Acaulospora scrobiculata*; (c) *Sclerocystis clavispora*; (d) *Ambispora granatensis*; (e) *Acaulospora spinulifera*; (f) *Rhizoglomus fasciculatum*; (g) *Acaulospora foveata*; (h) *Acaulospora cavernata*; (i) *Sclerocarpum amazonicum*; (j) *Ambispora* sp. 1; (k) *Acaulospora reducta*; (l) *Acaulospora alpina*; (m) *Glomus trufemii*; (n) *Acaulospora paulineae*; (ñ) *Acaulospora rehmii*; (o) *Dominikia* sp. 1. Spores were mounted in PVLG and PVLG + Melzer's reagent to observe diagnostic structures. Scale bars are shown in each image.

Table 1. Relative abundance (%) and frequency of occurrence of AMF species at different crop ages of *Theobroma cacao*.

AMF species	8–14	15–20	>20	FO	Zhang
Archaeosporomycetes					
Archaesosporales					
Ambisporaceae					
Ambispora					
Ambispora gerdemannii	0	0	0.26	0.6	R
Ambispora granatensis	0	0	0.73	1.7	R
Ambispora sp. 1	0	0	0.6	2.9	R
Ambispora sp. 2	0	0	0.08	0.6	R
Glomeromycetes					
Diversisporales					
Acaulosporaceae					
Acaulospora					
Acaulospora alpina	2.31	2.78	2.23	9.1	R
Acaulospora aspera	22.03	4.02	9	37.1	MC
Acaulospora cavernata	2.02	1.57	1.58	7.4	R
Acaulospora excavata	0.32	0	0.71	1.7	R
Acaulospora foveata	0	0.2	0	0.6	R
Acaulospora herrerae	0.7	0	0	1.7	R
Acaulospora mellea	4.33	1.8	8.19	20.6	C
Acaulospora morrowiae	0	2.16	1.37	4	R
Acaulospora paulineae	0	3.18	0	1.7	R
Acaulospora reducta	0	0	1.77	2.9	R
Acaulospora rehmii	0	0	3.41	5.7	R
Acaulospora scrobiculata	15.67	10.52	13.85	40	MC
Acaulospora sp. 1	1.58	0	1.35	4	R
Acaulospora sp. 2	0.87	0	0	1.1	R
Acaulospora spinosissima	2.18	8.89	0	7.4	R
Acaulospora spinulifera	0.82	0	0	0.6	R
Diversisporaceae					
Corymbiglomus					
Corymbiglomus corymbiforme	0	0	0.38	0.6	R
Diversispora					
Diversispora spurca	0	0.58	0	0.6	R
Diversispora sp. 1	0.61	0	0	2.3	R
Otospora					
Otospora bareai	0	0	0.33	1.1	R
Gigasporales					
Gigasporaceae					
Gigaspora					
Gigaspora margarita	0.24	0	0	1.1	R
Racocetraceae					
Cetraspora					
Cetraspora gilmorei	0.44	0	0	0.6	R
Cetraspora pellucida	0	0.16	2.06	6.3	R
Racocetra					
Racocetra fulgida	0.44	0	0	1.7	R
Racocetra weresubiae	0	0	0.18	0.6	R
Entrophosporales					
Entrophosporaceae					
Entrophospora					
Entrophospora claroidea	2.92	7.24	2.94	16	C
Entrophospora etunicata	1.27	4.52	1.48	6.3	R
Glomerales					
Glomeraceae					
Dominikia					
Dominikia aurea	0	0.64	4.81	6.9	R
Dominikia sp. 1	1.53	0	0	0.6	R
Funneliformis					
Funneliformis geosporum	25.17	26.95	27.22	73.7	D
Funneliformis mosseae	0.09	1.83	0.77	4	R
Funneliglomus					
Funneliglomus sanmartinensis	3.82	2.25	8.16	18.3	C
Glomus					
Glomus ambisporum	0	0.66	0	1.7	R
Glomus glomerulatum	0.31	0	0.29	1.1	R
Glomus heterosporum	0.81	0.16	0.6	4	R
Glomus microcarpum	1.08	2.51	0.86	6.3	R
Glomus trufemii	2.55	1.4	0	4.6	R
Microkamienskia					
Microkamienskia peruviana	0	2.14	0	0.6	R
Nanoglomus					
Nanoglomus plukenetiae	0	0	0.11	0.6	R
Parvocarpum					
Parvocarpum badium	0.25	2.66	0	2.9	R
Rhizoglomus					
Rhizoglomus aggregatum	0	0	1.1	0.6	R
Rhizoglomus fasciculatum	1.22	0	0	1.7	R
Rhizoglomus intraradices	0.64	0.22	0	1.7	R
Sclerocarpum					

(*Continued*)

Table 1. Continued.

AMF species	8–14	15–20	>20	FO	Zhang
Sclerocarpum amazonicum	0	2.11	0	0.6	R
Sclerocystis					
Sclerocystis clavispora	0.21	0	0	0.6	R
Sclerocystis sinuosa	2.82	7.22	3.31	30.3	MC
Sclerocystis sp. 1	0	0.01	0	0.6	R
Paraglomeromycetes					
Paraglomerales					
Paraglomus					
Paraglomus laccatum	0.74	0.89	0.28	3.4	R
Paraglomus occultum	0	0.73	0	1.1	R

FO: Frequency of occurrence, and classification according to [102]: dominant (>50%: D), mostly common (30–50,0%: MC), common (10–30%: C), and rare (≤10%: R).

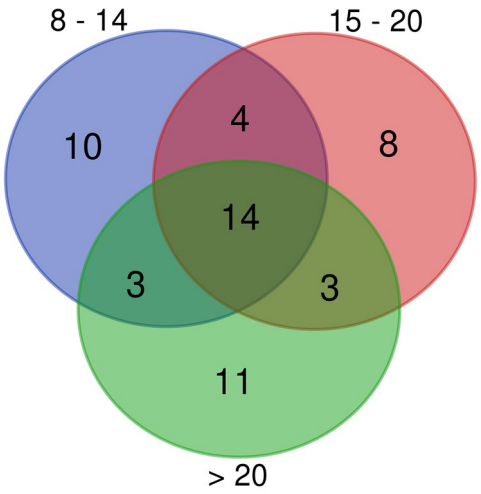

Figure 3. Venn diagram showing the number of shared and unique AMF taxa among *Theobroma cacao* plantations of different crop-age classes (8–14, 15–20, and >20 years). Numbers indicate the taxa exclusive to each age class and those shared between two or all crop-age classes.

The indicator species analysis (ISA) identified only two AMF taxa significantly associated with crop-age classes (Table 2). *Ac. spinosissima* was associated with plantations aged 15–20 years (IndVal = 0.57; $p = 0.048$), whereas *Ac. aspera* showed a strong association with both younger (8–14 years) and older (>20 years) plantations (IndVal = 0.73; $p = 0.037$).

IndVal expresses the strength of association between each species and a crop-age class, values closer to 1 indicate a stronger and more specific relationship. *p*-values indicate the statistical significance of this association.

3.3. Physical and chemical analysis of soil

Physicochemical analyses showed that OM was higher in younger soils (8–14 years) and decreased progressively with crop age, reaching the lowest values in soils older than 20 years. This trend was accompanied by an increase in sand content and a reduction in silt, together with lower pH and reduced K and Mg contents in older soils. Soils with intermediate age (15–20 years) showed chemical conditions similar to younger soils, except for slightly lower exchangeable Na. These patterns indicate gradual changes in soil texture and a progressive decline in soil fertility with increasing crop age (Table S2 of Supplementary Material).

Soil heavy metal analyses indicated that concentrations of all evaluated elements (Ni, Cu, As, Pb, Zn, Hg, and Cd) did not differ significantly among crop-age classes (Table 3), indicating that differences in heavy metal concentrations among plantations of different ages were not detected under the conditions evaluated.

3.4. Composition of AMF communities

According to the PERMANOVA results, no significant differences in AMF community composition were detected among crop-age classes ($F = 1.157$, $R^2 = 0.067$, $p = 0.26$) (Figure 5). Pairwise comparisons also showed no significant differences between groups after Bonferroni correction. The homogeneity of multivariate dispersion test (PERMDISP) indicated no significant differences among groups ($F = 0.61$, $p = 0.57$), confirming that the lack of significance in PERMANOVA was not influenced by differences in group dispersion.

Canonical correspondence analysis (CCA) indicated that heavy metals significantly influenced the composition of AMF communities ($F = 1.41$, $p = 0.048$), explaining 26.7% of the total variation (Figure 6). Among the evaluated variables, Cd ($F = 2.02$, $p = 0.001$) and Zn ($F = 2.62$, $p = 0.001$) were identified as the main factors associated with changes in community composition, whereas Ni, Cu, As, Pb, and Hg did not show significant effects.

The analysis of heavy metal effect coefficients revealed clear taxa-specific responses within the AMF community. For Zn, several taxa showed significant associations, with both positive responses such as *Glomus microcarpum*, *Acaulospora morrowiae*, *Dominikia aurea* and negative responses for *Acaulospora alpina*, *Acaulospora scrobiculata*, *Acaulospora* sp. 1, indicating contrasting ecological strategies along the Zn gradient.

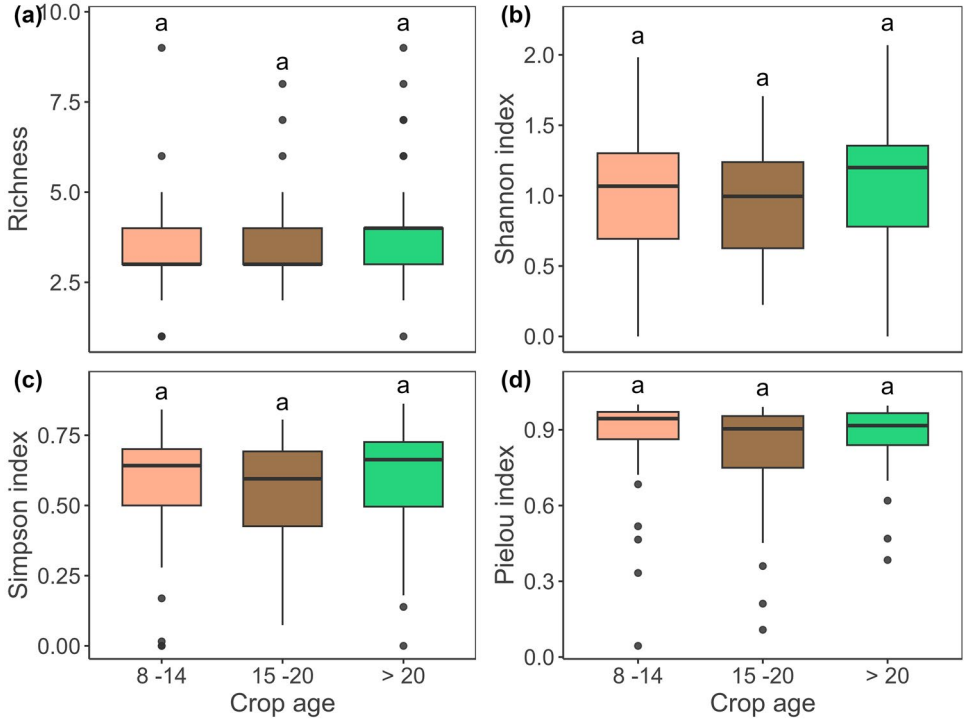

Figure 4. Alpha diversity indices of arbuscular mycorrhizal fungi in *Theobroma cacao* plantations across different crop-age classes (8–14, 15–20, and >20 years). (a) Species richness, (b) Shannon index, (c) Simpson index, and (d) Pielou evenness index. Different letters above the boxplots indicate significant differences among crop-age classes ($p \leq 0.05$); the same letters indicate no significant differences.

Table 2. Arbuscular mycorrhizal fungal species significantly associated with specific crop-age classes of *Theobroma cacao* plantations based on indicator value (IndVal) analysis.

Species	Crop age	IndVal	p-value
Acaulospora spinosissima	15–20	0.57	0.048
Acaulospora aspera	8–14/>20	0.73	0.037

In contrast, Cd exhibited stronger and more polarized effects, with multiple taxa showing significant positive responses as *Glomus microcarpum*, *Funneliformis mosseae*, *Entrophospora etunicata*, while others showed marked negative associations like *Acaulospora rehmii*, *Cetraspora pellucida*, *Acaulospora mellea*, *D. aurea*. These results highlight Cd as a key driver of species-level variability within the AMF community (Figure 7).

4. Discussion

Our study focused on assessing whether the composition of arbuscular mycorrhizal fungi (AMF) communities associated with *T. cacao* varied according to crop age as a first objective. This study revealed that, despite differences in crop age (8–14 and >20 years), no significant differences were found in species richness or overall diversity indices. Likewise, no significant differences in community composition were detected among crop-age classes, suggesting that AMF communities remain relatively stable across plantation ages. These results indicate that crop age alone does not act as a strong structuring factor and highlight the potential influence of rhizospheric and edaphic conditions on AMF community dynamics. Studies such as those by [47] and [48] emphasize the importance of soil variables, including pH, texture, nutrient availability, and associated plant species, in shaping AMF communities.

The observed stability in α-diversity may be explained by the high functional resilience of Glomeraceae, which maintain colonization rates above 50% across a wide range of soil conditions [49,50]. Moreover, mycorrhizal regeneration in tropical ecosystems does not follow a linear trend but is strongly conditioned by edaphic properties and the presence of highly mycotrophic plant species [48,51]. Consistent with this, PERMANOVA analyses did not detect significant differences in AMF community composition among crop-age classes, reinforcing the idea that crop age alone is not a primary driver of community structure. Instead, it is likely that underlying soil physicochemical properties could play a more important role in determining AMF community patterns [52,53,54]. Additionally, phylogenetically related plants may host distinct AMF communities depending on their specific symbiotic compatibility [55].

The results of the present study reveal a remarkably high richness of AMF, with a total of 53 taxa

Table 3. Kruskal–Wallis test results (with Bonferroni-adjusted multiple comparisons) for heavy metal concentrations in soil across crop age.

Crop age	As	Cd	Cu	Hg	Ni	Pb	Zn
				mg.kg^{-1}			
8–14	4.52 (3.69–5.83)a	0.4 (0.3–0.51)a	23.86 (18.63–31.09)a	0.15 (0.1–0.34)a	19.3 (13.79–25.68)a	16.65 (10.29–24.55)a	68.19 (51.9–131.81) a
15–20	5.85 (5.18–6.29)a	0.55 (0.45–0.74)a	24.54 (19.78–25.85)a	0.13 (0.11–0.14)a	17.1 (15.47–18.46)a	20.93 (17.09–23.86)a	81.99 (74.33–100.05) a
>20	6.68 (4.44–10.61)a	0.28 (0.13–0.59)a	21.92 (14.34–26.28)a	0.22 (0.11–0.25)a	15.92 (7.05–24.35)a	18.23 (8.39–20.69)a	91.75 (51.18–102.94) a

Values are presented as median (IQR). Different letters indicate significant differences among crop-age classes based on Kruskal–Wallis followed by Bonferroni post hoc test ($p < 0.05$). As: Arsenic; Cd: Cadmium; Cu: Copper; Hg: Mercury; Pb: Lead; Ni: Nickel; Zn: Zinc.

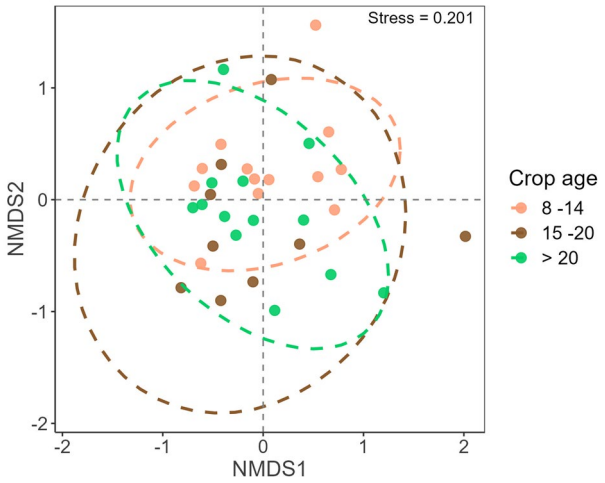

Figure 5. Non-metric multidimensional scaling (NMDS) ordination of arbuscular mycorrhizal fungal community composition in *Theobroma cacao* plantations across different crop-age classes (8–14, 15–20, and >20 years). Points represent individual soil samples, colors indicate crop-age classes, and dashed ellipses represent the 95% confidence intervals for each group.

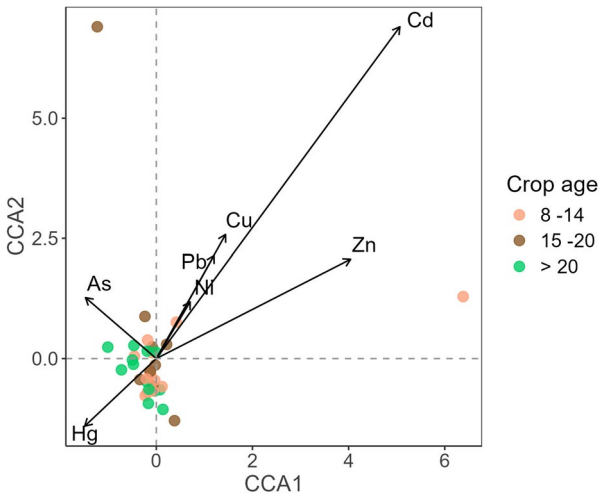

Figure 6. Canonical correspondence analysis (CCA) showing the relationship between arbuscular mycorrhizal fungal community composition and soil heavy metal concentrations in *Theobroma cacao* plantations across crop-age classes (8–14, 15–20, and >20 years). Points represent plots and arrows represent heavy metal variables (Ni, Cu, As, Cd, Pb, Zn, and Hg). The direction and length of the arrows indicate the strength and direction of their association with community composition.

belonging to the phylum Glomeromycota, distributed across three classes and six orders. This diversity aligns with previous studies indicating that cocoa is a highly mycotrophic crop, well adapted to acidic and low-fertility soils through its strong dependence on mycorrhizal symbiosis [56]. In particular, the families Glomeraceae and Acaulosporaceae were the most representative, with a high frequency of genera such as *Glomus*, *Rhizoglomus*, *Sclerocystis*, and *Acaulospora* (16 taxa). This dominance pattern is consistent with findings from cocoa agroforestry systems [57,58], and with studies conducted in Peru [59,60] and in the Brazilian Caatinga [61,62], where these genera show strong tolerance to adverse edaphic conditions, high sporulation, and wide dispersal capacity. According to [63,64], Acaulosporaceae and Glomeraceae are the dominant families due to their ecological plasticity and wide adaptability. This pattern is further supported by recent reports of species from these families in the Amazon region of Peru, such as *Acaulospora aspera*, *Ac. flava*, *Ac. flavopapillosa*, *N. plukenetiae*, and *Rhizoglomus cacao* [65–69]. The high phenotypic plasticity of genera such as *Glomus*, *Acaulospora*, and *Rhizoglomus* has also been reported in semi-arid and tropical regions of Brazil [61,70–73].

A notable representation of the orders Diversisporales, Gigasporales, and Archaeosporales was also observed. Diversisporales, dominated by *Acaulospora*, are recognized for their high sporulation capacity in dry and acidic soils [51,71]. The presence of Gigasporales was mainly recorded in tropical environments, consistent with [54], who observed that this group thrives in tropical forests with low phosphorus availability and nutrient-poor environments [61,74], while Archaeosporales, although less studied, have exhibited remarkable adaptability to degraded and acidic ecosystems, as previously reported in Amazonian soils [74,75].

Remarkably, this study reports 13 new records of Glomeromycota species for Peru, all originating from the Amazon region: *Ambispora granatensis, Acaulospora alpina, A. paulineae, A. spinulifera, Corymbiglomus corymbiforme, Otospora bareai, Cetraspora gilmorei, Racocetra fulgida, R. weresubiae, Glomus glomerulatum, G. trufemii, Parvocarpum badium, and Sclerocarpum amazonicum*. These findings increase the known richness of Glomeromycota in Peru to 106 species, contributing to the national inventory of arbuscular mycorrhizal fungi [76].

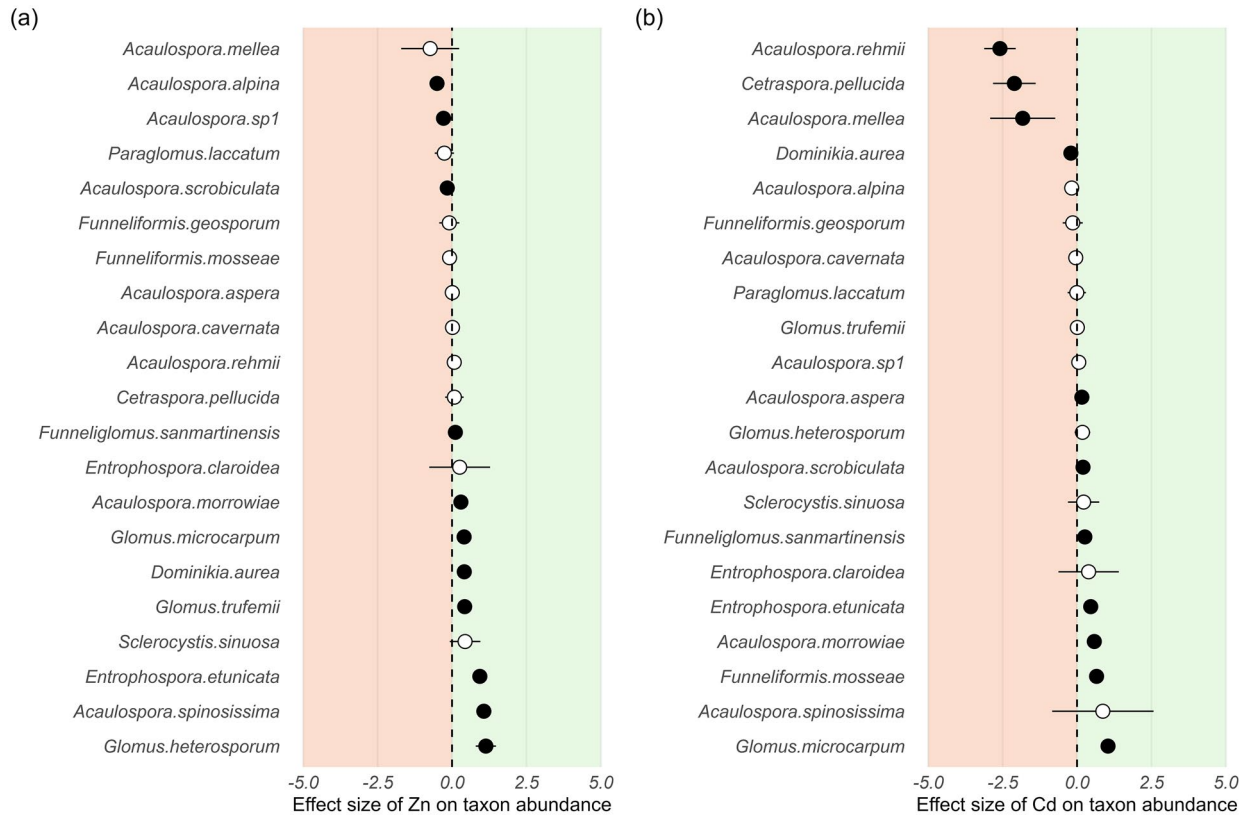

Figure 7. Taxa-specific responses of AMF taxa to heavy metal gradients based on negative binomial models. Panels show the effects of (a) Zn and (b) Cd. Points represent β coefficients and horizontal bars indicate 95% confidence intervals. Positive and negative values denote increases or decreases in abundance along the metal gradient, respectively. Filled symbols indicate significant responses after FDR correction, whereas open symbols denote non-significant effects.

Regarding the second objective, this study aimed to determine whether heavy metals shape the composition of AMF communities. Among the evaluated elements, cadmium (Cd) and zinc (Zn) were identified as the main variables associated with changes in AMF community composition. The association of Cd with AMF community variation is consistent with previous studies, which have identified this element as one of the heavy metals most frequently linked to reductions in AMF diversity and shifts in community structure [77,78]. Likewise, Zn has been reported as an important factor influencing AMF community composition in contaminated soils and has been identified, together with Pb, as a significant environmental variable shaping AMF assemblages [79]. In the present study, Cd and Zn were the metals most strongly associated with changes in AMF community composition, whereas Pb showed no significant influence. Similar findings have been reported by [78], who also found that Pb was not a major driver of AMF community structure. These contrasting results among studies suggest that the effects of heavy metals on AMF communities are context-dependent and may be influenced by local environmental conditions, soil properties, geographic location, host plant communities, and the adaptive capacity of resident AMF assemblages. According to [80,81], these metals interfere with key enzymatic pathways, thereby reducing sporulation and fungal colonization. Moreover, acidic soils exacerbate these toxic effects [82], which may explain the lower abundance of some taxa observed in certain plots.

The dominance of *Glomus* and *Acaulospora* in contaminated soils suggests a high tolerance to pollutants and reinforces their potential use as bioindicators of anthropogenic disturbance [83,84]. Several studies have reported a high presence of species belonging to the family Glomeraceae in soils contaminated with heavy metals, showing in many cases a positive relationship between their abundance and increasing metal concentrations [85,86]. However, species responses within this family are not uniform. In the present study, species-level responses showed contrasting patterns along Cd and Zn gradients, with both positive and negative associations across taxa.

For example, *Rhizoglomus intraradices* have shown a decrease in abundance as Zn concentrations increase, a pattern also reported by [87]. Similarly, *Rh. fasciculatum* and *Rh. clarum* are negatively affected by metal presence, particularly Zn, suggesting a sensitivity of the genus *Rhizoglomus* to this element [85]. In contrast, responses to Cd and Zn observed in this study indicate that tolerance is species-specific rather than uniform across genera.

Anothers species of the order Glomerales, such as *Funneliformis mosseae*, *F. geosporum*, and *Funneliglomus sanmartinensis*, maintained relatively stable abundances across the evaluated metal gradients. Nevertheless, previous studies have shown that species of *Funneliformis* may experience reduced germination and sporulation under high Zn concentrations [88], while still showing high tolerance to As [89]. A similar response may occur in *Funneliglomus*, given its close phylogenetic relationship with *Funneliformis* [90].

The family Acaulosporaceae showed high species richness in this study. Several species, such as *Acaulospora paulinae*, *Ac. mellea*, *Ac. cavernata*, *Ac. foveata*, and *Ac. rehmii*, showed reduced abundance in the presence of metals. In addition, *Ac. reducta*, *Ac. foveata*, *Ac. rehmii*, and *Ac. mellea* were negatively affected by Cd, which agrees with previous reports indicating that Cd generally reduces sporulation in Acaulosporaceae [91,92]. In contrast, species such as *Ac. morrowiae* and *Ac. spinosissima* appeared to benefit from the presence of Cd and Zn. These species could be of interest for biofertilizer development, as these metals act as essential micronutrients for plants. *Ac. scrobiculata* and *Ac. aspera* showed significant but low-magnitude responses to Cd concentrations, with β values close to zero, suggesting a relatively neutral response across the gradient, supporting its known high ecological adaptability and wide distribution across ecosystems [93].

Species belonging to the order Gigasporales have previously been described as tolerant to metals such as Al [94,95]. In the present study, these species did not show clear positive or negative changes in abundance, suggesting a general tolerance to the evaluated metals. This tolerance may be related to biochemical and molecular mechanisms involved in metal phytostabilization [96,97]. An exception was *Gigaspora margarita*, whose abundance decreased as Cu and Zn concentrations increased, indicating sensitivity to these metals.

Archaeosporales and Paraglomerales, considered ancient lineages with limited information regarding their response to heavy metals, showed variable patterns. Although Archaeosporaceae has been reported to be abundant in metal-contaminated soils [98], its presence in this study was lower compared to other families. *Ambispora granatensis* showed clear sensitivity to Cd, As, Cu, and Zn, with reduced abundance under higher metal concentrations. Regarding Paraglomerales, previous studies have reported high incidence in soils with elevated Zn and Pb levels [99]; however, the species detected in this study (*Paraglomus occultum* and *P. laccatum*) did not show significant variation across metal concentrations. These results suggest that further research is needed to better understand the ecological responses of these orders to heavy metal stress.

On the other hand, this research underscores the importance of integrating molecular tools, since several taxa were identified only at the genus level, such as *Ambispora* sp., *Acaulospora* sp., *Diversispora* sp., *Dominikia* sp., and *Sclerocystis* sp. [100.] reported discrepancies between morphological and molecular approaches, suggesting that the actual diversity observed in this study may be underestimated. The detection of uncommon genera such as *Dominikia* and *Parvocarpum* indicates that the studied ecosystem provides agroecological conditions favorable to the occurrence of rare or previously undocumented taxa. In agreement with [101], it can be inferred that mycorrhizal richness is influenced more by the microbiome and biochemistry of the host plant than by abiotic factors alone.

Finally, our findings support those of [102], who emphasized that cultural practices influence AMF community structure. This study confirms that heavy metal gradients, particularly Cd and Zn, appear to play a more relevant role than crop age in shaping AMF community structure. Overall, this research broadens the understanding of AMF diversity and functional ecology in Amazonian agroforestry systems, demonstrating that soil fertility, heavy metal presence, and physicochemical properties are key determinants of their distribution, abundance, and community composition.

5. Conclusions

The present study provides evidence of a high diversity of arbuscular mycorrhizal fungi (AMF) associated with cocoa cultivation in Amazonian ecosystems of Peru. The influence of geochemical factors, particularly cadmium (Cd) and zinc (Zn), on the structure of mycorrhizal communities was significant, highlighting their role as key environmental filtering agents. In contrast, crop age did not significantly influence community composition, indicating that AMF communities remain relatively stable across plantation ages. Likewise, the dominance of certain genera in impacted soils suggests strong functional resilience and their potential utility as bioindicators of heavy metal contamination.

These findings highlight the importance of integrating edaphic variables, particularly heavy metal gradients, into assessments of fungal biodiversity. Furthermore, they emphasize the need to incorporate molecular techniques to complement morphological identification and reveal the hidden diversity of AMF in Peruvian ecosystems. The differential tolerance observed among taxa suggests their potential application in phytoremediation

strategies aimed at mitigating heavy metal contamination in agroecosystems.

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Authors contributions

Cecil Eduardo del Aguila-Torres: Conceptualization, Methodology, Investigation, Writing—Original Draft Preparation **Maria Nelly Pezo-Torres**: Conceptualization, Methodology, Investigation, Writing—Original Draft Preparation, Writing—Review and Editing, Visualization **Sergio Sebastian Vega-Herrera**: Software, Validation, Formal Analysis, Data Curation, Writing—Original Draft Preparation, Writing—Review and Editing **Carlos Verde-Gribau**: Methodology, Investigation **Enrique Meseth**: Resources, Supervision, Project administration, Supervision, Project Administration, Funding Acquisition **Mike Anderson Corazon-Guivin**: Conceptualization, Investigation, Resources, Writing—Review and Editing, Supervision, Funding Acquisition **Jorge Luis Paz-Urrelo:** Conceptualization, Methodology, Investigation, Resources, Writing—Review and Editing, Supervision, Project Administration.

Conflicts of interest

The authors declare no conflicts of interest.

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