Article

Discovering the Diversity of Arbuscular Mycorrhizal Fungi Associated with Two Cultivation Practices of *Theobroma cacao*

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Abstract: In recent years, new data on the diversity of genera and species in the phylum Glomeromycota continue to be added and rearranged. Arbuscular mycorrhizal fungi are key to plant nutrition and agriculture. Studies report different short- and long-term cultivation practices that influence the abundance and diversity of Glomeromycota. To the best of our knowledge, there are no known studies of the fungal communities in the fine aroma cocoa cultivars. In this context, our work aims to discover the diversity of arbuscular mycorrhizae associated with two cocoa cultivation practices (conservative and semi-conservative) through the isolation of spores using microscopy and metabarcoding of the internal transcribed spacer region (ITS). Morphological analysis showed that the density of Glomeromycota spores exhibited significant differences between production systems. Although the metabarcoding analysis showed that diversity indices showed a higher increase in the roots than in the cocoa soil, independently of the cultivation practice. An abundance of 348 and 114 taxa were observed, corresponding to the conservative and semi-conservative practices, respectively. Seven genera were observed for the first time in cocoa crop agroforestry systems, including *P. scintillans*, *R. diaphanus*, *R. fasciculatus*, *R. custos*, *D. disticha*, *M. perpusilla*, and *D. bernensis*.

Keywords: arbuscular mycorrhizal fungus; diversity; fine aroma cocoa crops; ITS region; metabarcoding; morphological analysis of spores; cultivation practices

1. Introduction

Ecuador is an agricultural country by tradition, with cocoa being one of the main exportation products. [1]. Ecuador is considered the world's leading producer of fine and aromatic cocoa (*Theobroma cacao* L.), with a contribution of approximately 63%, followed by Indonesia with 10% [2]. According to the Ministry of Production, Foreign Trade, Investment, and Fisheries, the Ecuadorian cocoa sector exported USD 815.5 million in 2020, and reached USD 266.4 million between January and May 2021; the main destination countries for Ecuadorian cocoa in 2020 included the United States, Indonesia, Malaysia, and the Netherlands [3]. The average size of a cocoa crop is 3 ha, and each cocoa tree reaches a height of 4 to 8 m, except for fine aroma cocoa cultivars, which can reach up to 12 m in height. [4]. The main root of fine aroma cocoa extends up to 2 m deep, and the secondary roots develop in the upper humic layer (between the first 20 cm of depth) and extend horizontally up to 6 m around the main stem, where a wide diversity of mycorrhizal fungi cohabit [5].

Large amounts of nutrients are recycled within cocoa systems through leaf fall and pruning [6]. However, there have been losses due to the gradual depletion of soil nutrients and limits crop yields each intensive harvest. [7]. Due to the growing interest in the meeting of food demand, yield improvement, and stress resistance, governmental efforts have been made to look for alternatives [8].

Countries such as Spain, Mexico, Canada, the United States, and China, among others, have been using mycorrhizal microorganisms to develop biological products applied to commercial crops [9–11]. In this regard, the importance of arbuscular mycorrhizal fungi (AMF) has increased in the last decade, with numerous reports of beneficial effects on crops [12–15]. In recent years, new genera continue to be added to the phylum Glomeromycota, and others already classified have either been reclassified or kept their original classification following much discussion (Table A1). These fungi are associated with 90% of the plant families on earth and sustain terrestrial ecosystems through mutualistic symbiotic associations [16]. Once an interaction is established, AMF modifies the morphology of the root, intracellularly colonizing its cortex using hyphae that generate specialized structures that act as organs of nutrient exchange between the plant cell and host [17]. AMF hyphae can extend beyond depletion zones, increasing the area of mineral uptake and mineral content in the plant, exploring a larger volume of soil than could be achieved by root growth alone [18].

Some studies have reported that different short- and long-term farming systems or practices influence the abundance and diversity of AMFs. [19–21]. In recent decades, agricultural practices have been comprehensively evaluated, considering not only crop yield and soil nutrient availability, but also the composition and behavior of the microbiota, so as not to inhibit biological processes or develop dependence on synthetic fertilizers [22].

Agronomic practices in cocoa crops are generally carried out using pesticides and synthetic fertilizers to protect and increase production, respectively [22]. Despite increasing the supply of macronutrients in the soil, the nutritional demands of the crop are not sufficiently met. Therefore, much of the sustainable production of cocoa depends on the ecosystem services that the microbial community provides to the soil, such as soil aggregation, nutrient cycling, and even pathogen suppression [23]. For the usual reasons of plasticity and convergence between unrelated strains, morphological identification is tedious and requires much experience from the observer. Thus, it is not a robust indicator of the relationship between AMF types [24].

The advent of molecular techniques has revealed a much greater diversity of fungi than was known just a few years ago. Molecular identification methods are much needed for AMF studies because AMF morphology provides insufficient information to distinguish the full range of community diversity. [25]. High-throughput DNA sequencing technologies (HTS) have made information on plant-associated fungal communities available, making it possible to find microbial species among hundreds or thousands of species within a network for understanding phenomena at the scale of terrestrial ecosystems [8].

Despite its potential in basic and applied microbiology, few studies have examined AMF networks associated with cocoa crops at the metacommunity scale. Therefore, the present work explored the diversity of AMFs associated with two cocoa cultivation practices, conservative and semi-conservative, through the morphological analysis of spores using microscopy and metabarcoding of the internal transcribed spacer region (ITS). The results suggest taxa with the potential to be used in practices to improve cocoa crop production.

2. Materials and Methods

2.1. Location and Description of Cocoa Crops

Soil and root samples were collected between October and December 2020 in three cantons in Ecuador (Milagro, Vinces, and Calceta) (Figure 1 and Table A2). From each canton, five cocoa trees cultivated using conservative practices and five cultivated using semi-conservative practices were selected. Semi-conservative practice involves the low use

of synthetic fertilizers and herbicides (NPK and glufosinate-ammonium, frequency of no more than 4 times per year), supplemented by organic fertilizers and cultural practices. The sample was collected at a depth of 20 cm, in the upper humic layer of each tree. The zones were characterized into agricultural areas of cocoa, orange, and green cultivation, among other plants associated with this agroecosystem. The samples were stored in cold rooms ($-20\ ^{\circ}$C), and then subdivided into two portions: one portion for the isolation of spores and identification of AMF through microscopy, and another portion for DNA extraction and sequencing of the ITS region. The edaphic properties (macro- and microelements, pH, and organic matter parameters) were measured in each of the cocoa crops (Table A3), using the following techniques: potentiometric volumetry, colorimetry, atomic absorption, turbidimetry, and the Walkley-Black method.
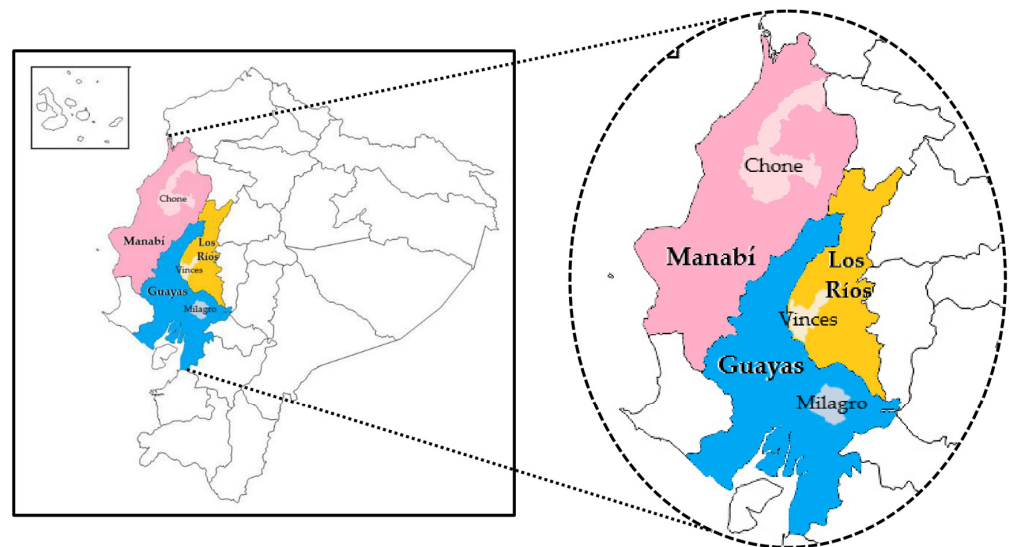

Figure 1. Geographical location of cocoa crops distributed in three provinces and cantons. The provinces (Los Ríos, Manabí, and Guayas) are represented in yellow, pink, and light blue, respectively, whereas the cantons are shown in lighter tones.

2.2. Isolation of Spores and Identification of AMF

For the isolation of the spores, wet sieving and decanting methodologies were used [26], followed by density gradient extraction [27]. The final supernatant was dissolved in water to wash the spores, and the criteria used to determine the density of AMF spores was as follows: low density corresponded to <1 spore/g soil; medium density corresponded to 1–10 spores/g soil, and high density corresponded to >10 spores/g soil [28]. Samples for morphological analysis were placed in Petri dishes and glass slides and observed under a light stereoscope (Zeiss, Jena, TH, Germany).

The identification of the predominant AMF genera was based on the morphological characteristics of the spores, i.e., their color, shape, texture, size, wall characteristics (i.e., number, thickness, color, presence of ornamentation, and Melzer reaction), layers covering the spore, the number of scars present, and the union of the suspensory hyphae to the spore [29]. These characteristics were examined using freely available morphological manuals [30–32]. Spore density from each culture system (conservative and semi-conservative practices) was compared using a nonparametric Mann-Whitney test. Homogeneity of variance and normality were evaluated using Bartlett and Shapiro-Wilk tests. The null hypothesis of both groups having an equality of spores was rejected with a $p < 0.05$. A total of 45 samples per cultivation practice (conservative and semi-conservative) were analyzed for spore count.

2.3. Staining of Colonized Roots

The colonization of AMF was observed using a binocular microscope (40×). the roots were processed according to a modified method by Phillips and Hayman [33], bleached with H_2O_2, cleaned with KOH (10% w/v, 15 min, 110 °C), and stained with trypan blue in lactic acid (0.02%, 10 min, 110 °C). Structures corresponding to colonization by arbuscular mycorrhizal fungi were examined in 1 cm long sections of roots taken at random.

2.4. Molecular Analysis: DNA Extraction and Sequencing

A total of 36 samples (18 samples from conservative practice and 18 from semi-conservative practice) from roots and soil (6 and 12 samples, respectively) were processed for genomic DNA (gDNA) extraction. Root samples were macerated in liquid nitrogen for homogenization of the fungal loads present. Subsequently, gDNA was extracted using the DNeasy mericon Food Kit (Qiagen, Carlsbad, CA, USA), following the manufacturer's instructions. In addition, gDNA from soil and spore samples was extracted using the Dneasy Power Soil Pro-Kit (Qiagen, Carlsbad, CA, USA), following the manufacturer's instructions. A 50 Ul aliquot of Gdna was dissolved in Dnase-free water. The quality and quantity of Gdna were examined on a UV-Vis NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), followed by observation on 1% agarose gel. Amplification of the ITS region was performed with two sets of primers (Table A4). The gDNA was sent to Biosequence (Quito, Ecuador) for the construction and multiplexing of the amplicon library. Paired-end sequencing was carried out with the Illumina MiSeq 300 bp platform (Illumina, San Diego, CA, EEUU). The sequences were stored at the National Center of Biotechnology Information (NCBI) Sequence Read Archive (SRA), under accession number PRJNA859242.

Sequence Analysis

Raw sequence data were demultiplexed with Casava 1.8 [34] and sequences with low quality (Phred quality score (Q) < 30) and short reads were filtered out. Amplicon sequence variants (ASV) were analyzed using Quantitative Insights into Microbial Ecology 2 (QIIME2), version 2021.4 [35]. The reads were imported for removal of redundant sequences, merging, and denoising, according to default DADA2 processing conditions. A table of ASVs was generated and the taxonomy was assigned using the public UNITE database, version 8.3. To obtain a comprehensive description of the fungal communities, alpha diversity metrics were performed (Shannon and ACE indices). The null hypothesis was evaluated through ANOVA and t-tests. Beta diversity was evaluated by measuring the Bray-Curtis dissimilarity index visualized in a principal coordinates analysis (PCoA) for a comparison of the fungal communities present in the two cultivation practices (conservative and semi-conservative), soil, and roots. The null hypothesis was evaluated using a PERMANOVA. The influence of edaphic factors on the distribution patterns of fungal communities in different cultivation practices was analyzed by means of a redundancy analysis (RDA). A Mantel test was performed to check whether there were significant correlations between soil edaphic factors and soil fungal community composition [36]. Linear discriminant effect size analysis (LEfSe) was performed to identify significantly different fungal groups in different cocoa growing practices, soil, and roots. The LDA for the different fungal taxa was adjusted to a p-value < 0.05 and a cutoff of LDA = 4 [37]. All statistical analyses were performed with microeco [38], a package implemented in R software, version 4.2.1. (RTeam, Vienna, WIE, Austria).

3. Results

3.1. Isolation of Spores and Identification of AMF

Spores belonging to AMF taxa were observed in cocoa crops under conservative and semi-conservative cultivation practices. After root staining, microstructures such as hyphae and vesicles were observed (Figure 2), with arbuscules appearing less frequently. Spore density had an average of 431 ± SEM = 131 (total of 19,402 spores), which was observed in the conservative cocoa root samples. On the other hand, an average of

321 ± SEM = 176 (total of 14,453 spores) was observed in the semi-conservative cocoa roots. Significant differences (*p* = 0.003) were observed between the spore densities present in the two cultivation practices (Figure A1).
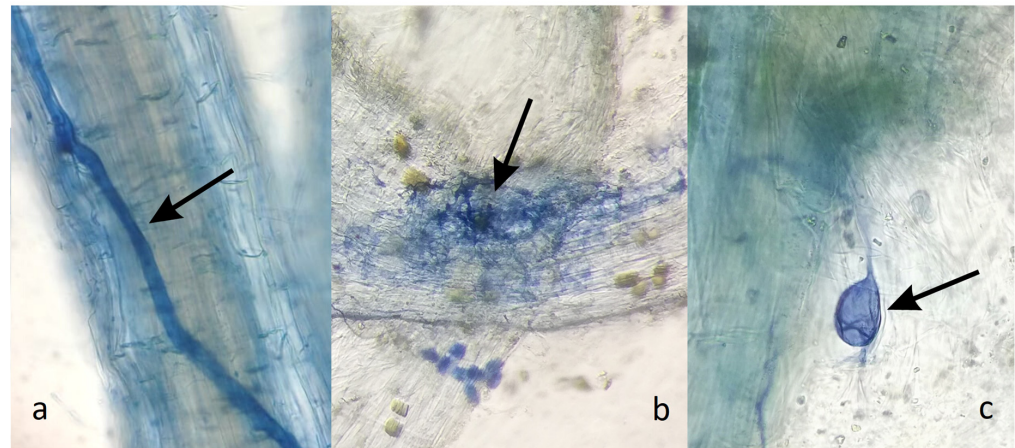

Figure 2. Characteristic cellular structures of arbuscular mycorrhizal fungi stained with trypan blue solution. (**a**) Roots colonized by hyphae (black arrow) and (**b**) roots colonized by arbuscules (black arrow) and (**c**) vesicles (black arrow). Similar structures were observed in samples from both cultivation practices.

3.2. Morphological Analysis of Spores

The morphological analysis of spores identified several genera according to shape, size, diameter range, color, number of visible walls, structure, and presence of hyphal connection. In conservative cocoa crops, the genera *Glomus*, *Acaulospora*, *Ambispora*, *Pacispora*, and *Diversispora* represented 88% of the total supply, followed by *Scutellospora*, *Racocetra*, *Entrophospora*, *Gigaspora*, *Intraspora*, *Paraglomus*, and *Archaeospora* making up the remaining 12%. In the semi-conservative cocoa crops, the genus *Glomus* represented 50% of total abundance, followed by *Acaulospora*, *Ambispora*, *Pacispora*, *Intraspora*, *Archaeospora*, *Gigaspora*, *Paraglomus*, *Entrophospora*, *Diversispora*, *Scutellospora* making up the remaining 50%. A total of 12 genera were found in cocoa crops under conservative practice and 11 in the crops under semi-conservative practice. Figures 3 and 4 show descriptions of the genera observed.

Edaphic factors were correlated with mycorrhizal colonization (*p* < 0.05) in cocoa samples under both cultivation practices. The most important influence was observed with the factors Ca, MO, NH_4, and P. Interestingly, Ca, MO, and NH_4 showed a positive correlation with colonization, whereas P had a negative correlation. On the other hand, Ca, Cu, and Fe showed the greatest influence on spore density per gram of soil, with Ca content being positively correlated and Cu and Fe being negatively correlated (Figure A2).
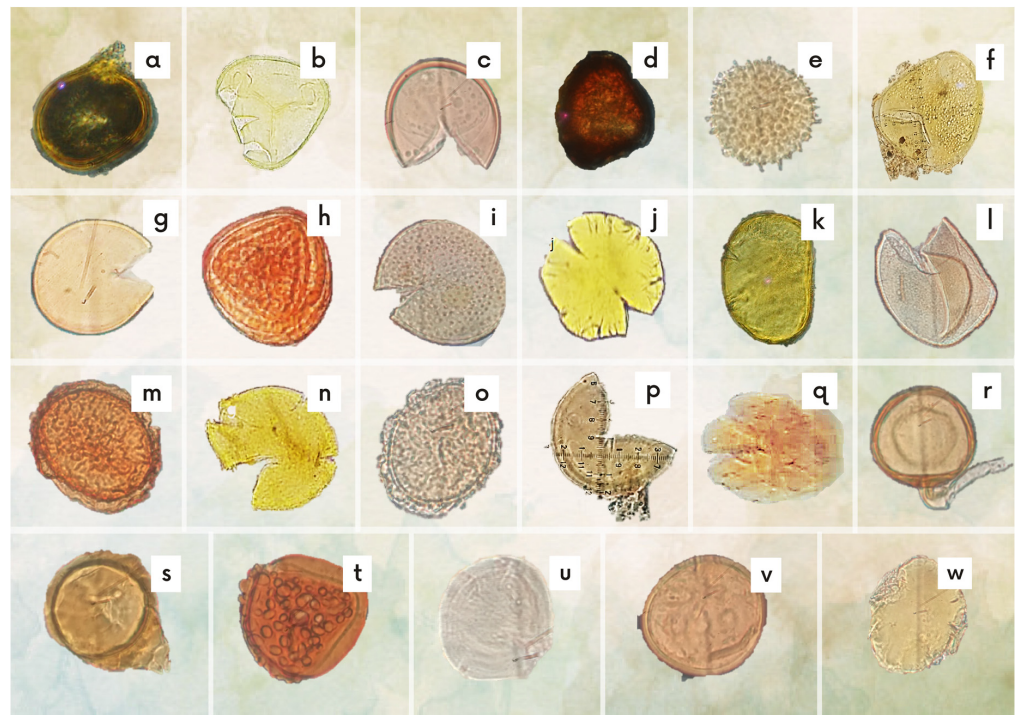
Figure 3. Genera of arbuscular mycorrhizal fungi in cocoa crops under conservative cultivation practices, identified according to spore morphological analysis: (**a–c**) *Glomus*, (**d,e**) *Scutellospora*, (**f–i**) *Acaulospora*, (**j–l**) *Pacispora*, (**m–o**) *Ambispora*, (**p**) *Racocetra*, (**q,r**) *Diversispora*, (**s**) *Entrophospora*, (**t**) *Gigaspora*, (**u**) *Intraspora*, (**v**) *Paraglomus*, and (**w**) *Archaeospora*.
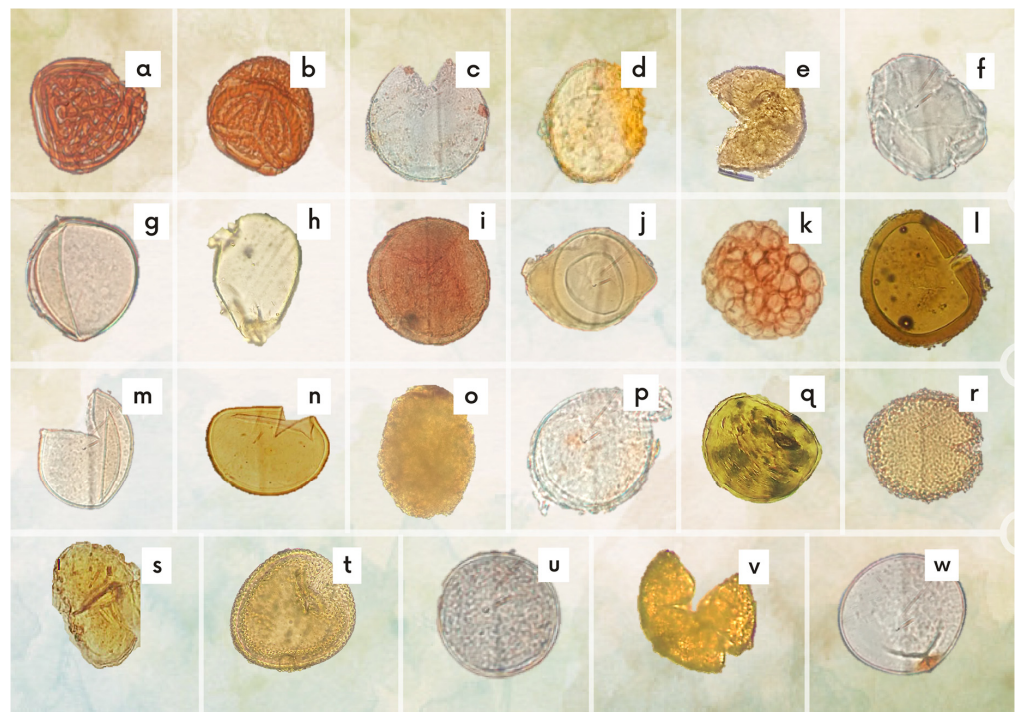

Figure 4. Genera of arbuscular mycorrhizal fungi in cocoa crops under semi-conservative cultivation practice identified after a morphological analysis of spores: (**a,b**) *Acaulospora*, (**c–e**) *Ambispora*, (**f**) *Intraspora*, (**g,h**) *Archaeospora*, (**i**) *Gigaspora*, (**j–l**) *Glomus*, (**m–o**) *Pacispora*, (**p,q**) *Paraglomus*, (**r–t**) *Entrophospora*, (**u,v**) *Diversispora*, and (**w**) *Scutellospora*.

3.3. Molecular Analysis

A total of 1019 ASVs were obtained. We identified 7 phyla, 22 classes, 47 orders, and 99 families in all cocoa soil and root samples. The alpha diversity index (Shannon) of soil and roots showed significant differences ($p = 0.001$) in fungal communities in the cocoa crop samples (Figure 5). However, no differences were observed between conservative and semi-conservative practices (Shannon, $p = 0.76$). The ACE diversity index showed no differences between cultivation systems (conservative and semi-conservative) and sampling sites (soil and roots) (Figure 5). Following beta diversity analysis, PERMANOVA results showed that the composition of fungal communities was different ($p = 0.001$) between soil and root samples (Figure 6). There were no significant differences when comparing fungal communities of the conservative versus semi-conservative practices ($p = 0.31$).
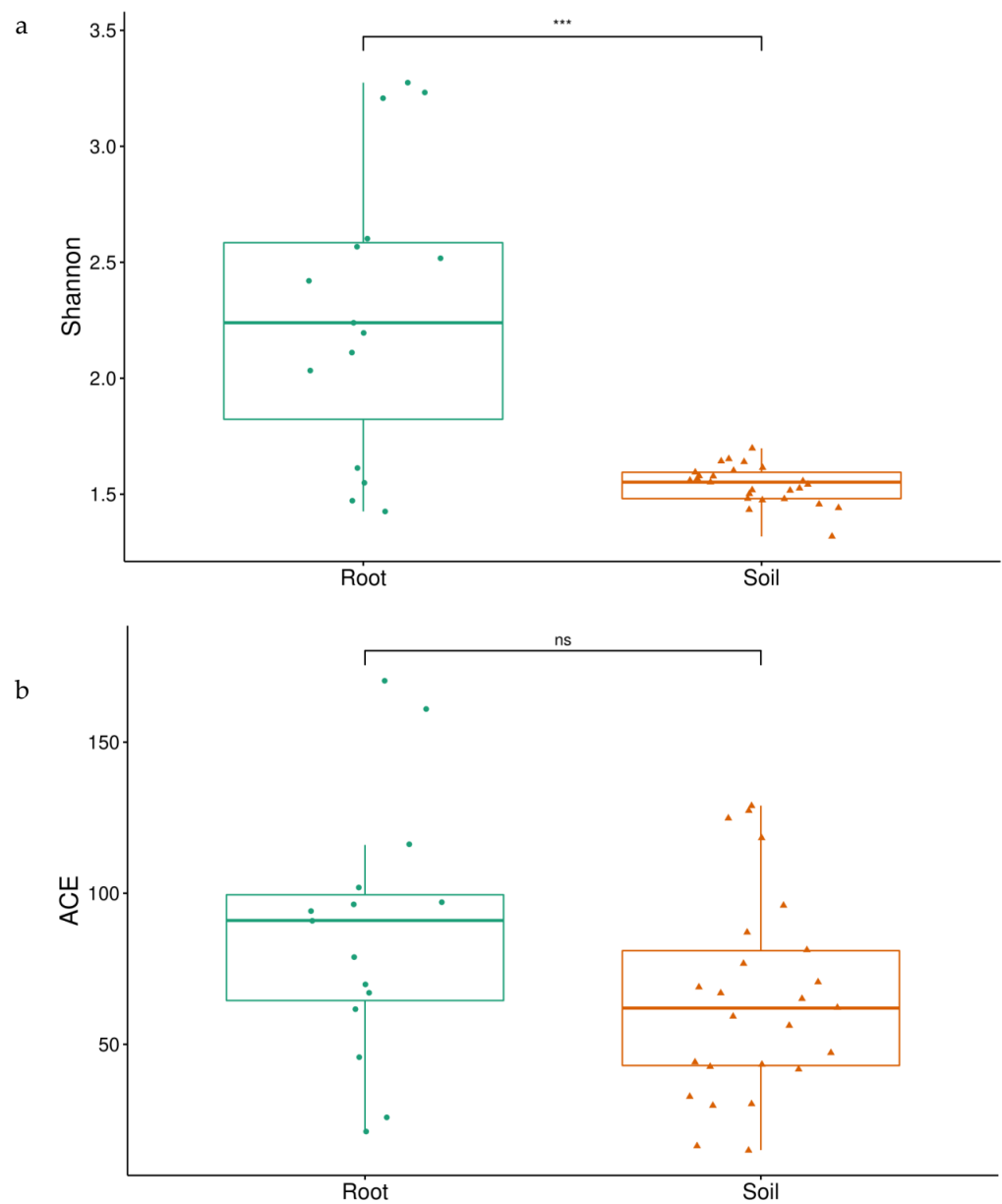

Figure 5. Alpha diversity of fungal communities present in conservative and semi-conservative cocoa crops. (**a**) Shannon index (**b**) ACE index. *** represents significant values with $p < 0.05$, ns = not significant. The circles represent fine and aroma cocoa roots while the triangles represent soil samples.
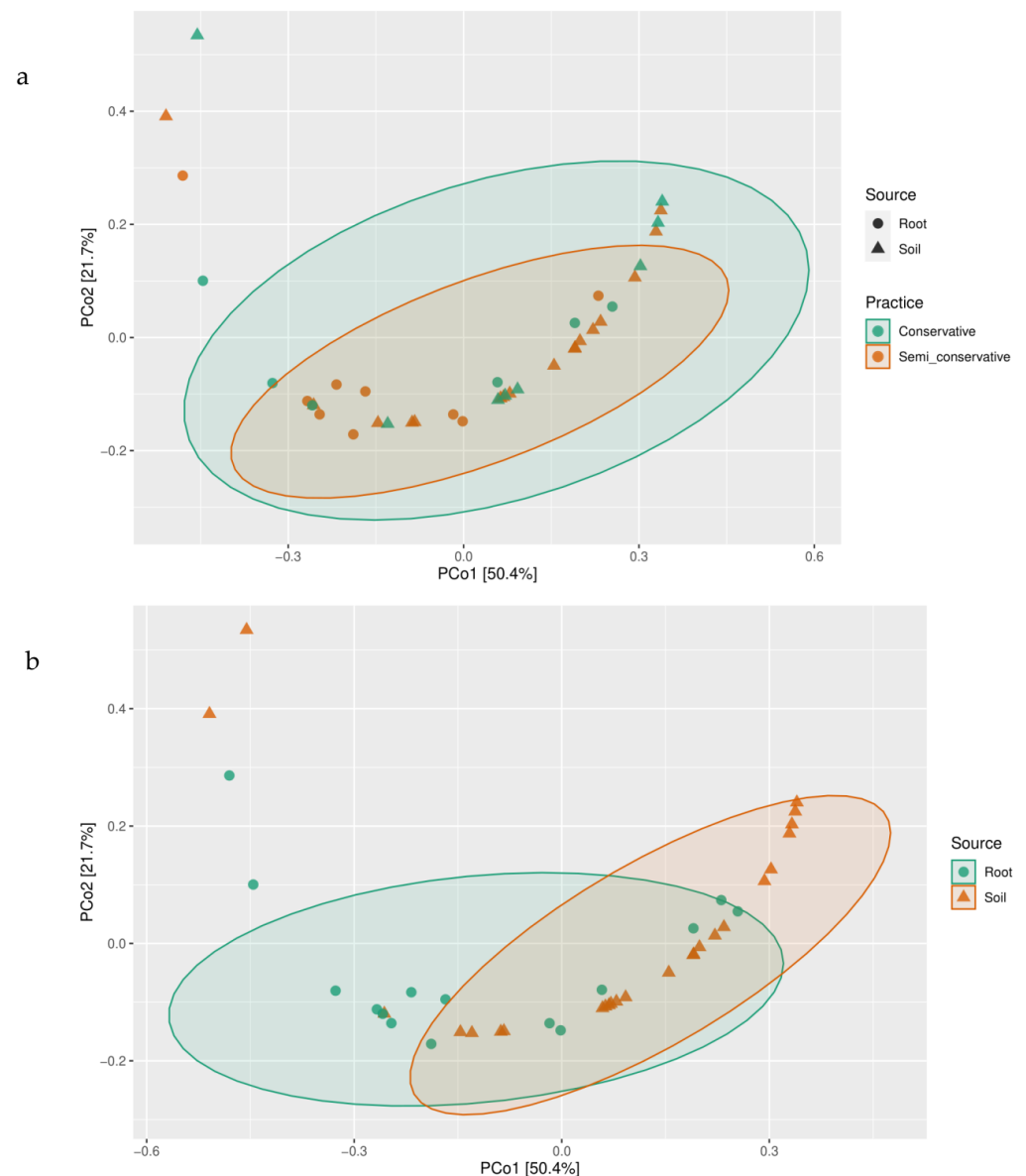
Figure 6. Principal coordinate analysis of the Bray-Curtis dissimilarity index comparing samples from (**a**) soil and roots with (**b**) cocoa cultivation practices (conservative and semi-conservative).

At the phylum level, Ascomycota dominated the cocoa soil and root samples from both cropping systems, representing 74% of relative abundance, followed by Basidiomycota (24%), and the remaining 2% composed of Glomeromycota, Mucoromycota, and Mortierellomycota.

Edaphic factors were not significantly correlated ($p > 0.05$), with fungal community composition independent of cultivation practice (Table A5). Interestingly, when the explanatory variables were used together with the fungal genera (Figure 7), the results showed that the abundance of *Neocosmospora* was explained by P, in contrast with the genus *Lasiodiplodia*. The genus *Fusarium* was explained by organic matter and NH$_4$, in contrast with the genus *Coprinopsis* (Figure 7). Lefse showed that the different phylogenetic groups at all taxonomic levels (order, family, and genus) was not significantly distinguished between the cultivation practices (conservative and semi-conservative). However, several taxa were significantly enriched when root samples were compared with soil samples. For example, the genera *Lasiodiplodia* and *Neocosmospora* were predominant in cocoa roots, whereas *Talaromyces* and *Antrodia* predominated in soils (Figure 8). According to the analysis of guilds using the

UNITE database, a total of 18 ASVs corresponding to AMF were found in the cocoa cultivation practices. All 18 ASVs are members of the phylum Glomeromycota. Table 1 shows a higher abundance of arbuscular mycorrhizae in the conservative (11 ASVs) compared with the semi-conservative practices (10 ASVs).
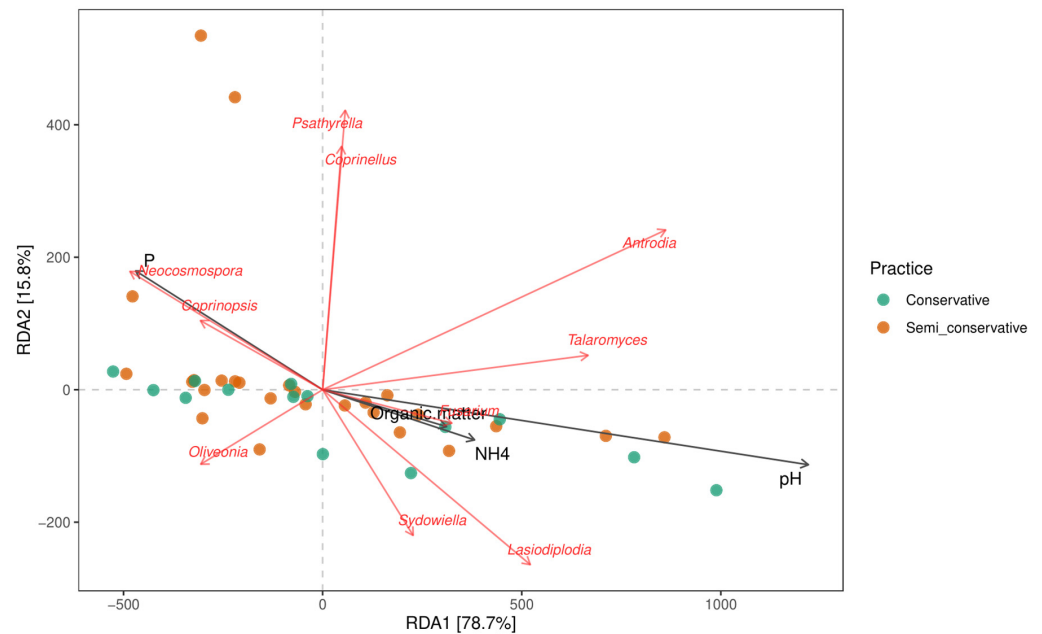

Figure 7. RDA diagram showing the relationship between edaphic properties and fungal community composition in Theobroma cacao under different cultivation practices (conservative and semi-conservative).
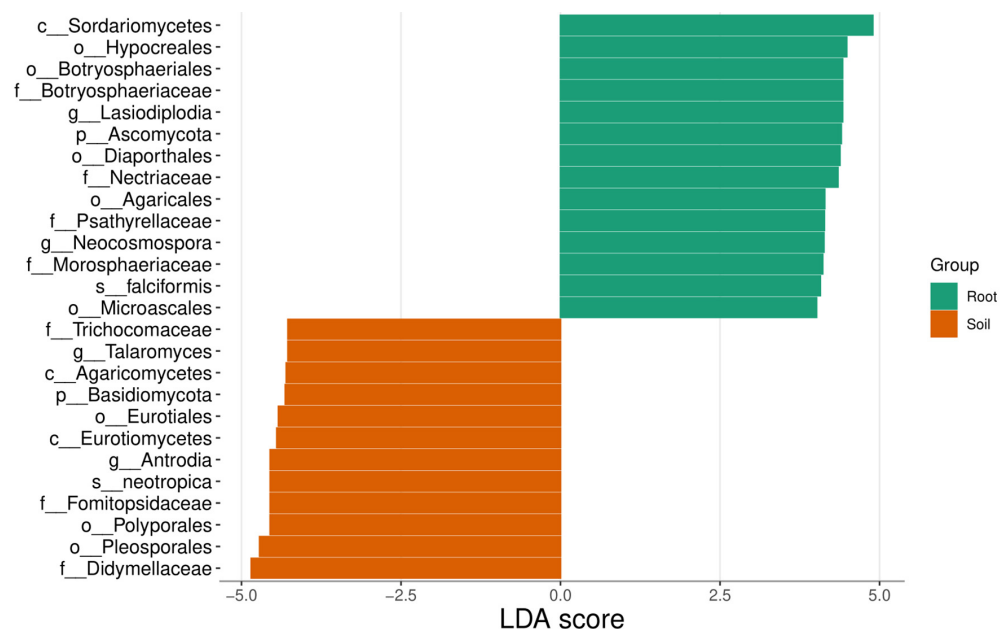

Figure 8. Lefse analysis of cocoa soil and roots. LDA shows different fungal taxa differentially significant at $p < 0.05$ (LDA cutoff = 4).

Table 1. Abundance of ASV belonging to arbuscular mycorrhizal fungi detected in conservative and semi-conservative cocoa samples. The taxa were classified by the UNITE database.

System		Classification	
Semi-Conservative	**Conservative**	**Taxonomy**	
0	3	Phylum	Glomeromycota
11	15	Class	Glomeromycetes
0	1	Order	Diversisporales
2	0	Order	Glomerales
29	125	Family	Glomeraceae
15	0	Genus	*Archaeospora* spp.
2	0	Genus	*Diversispora* spp.
3	0	Genus	*Dominikia* spp.
41	0	Genus	*Glomus* sp.
0	3	Genus	*Septoglomus* spp.
1	0	Species	*Dominikia bernensis*
8	0	Species	*Dominikia disticha*
2	2	Species	*Glomus aggregatum*
0	8	Species	*Kamienskia perpusilla*
0	10	Species	*Pacispora scintillans*
0	76	Species	*Rhizophagus custos*
0	2	Species	*Rhizophagus diaphanus*
0	103	Species	*Rhizophagus fasciculatus*
114	348	Total	

From the samples corresponding to the AMF, the phylum Glomeromycota, class Glomeromycetes, family Glomeraceae, and genera *Archaeospora*, *Diversispora*, *Dominikia*, *Glomus,* and *Septoglomus* were identified, with *Glomus* being the most prevalent, followed by *Archaeospora*. At the species level, 8 AMFs were identified, with *Rhizophagus fasciculatus* being the most abundant, followed by *Rhizophagus custos* and *Pacispora scintillans*. A strong bias of amplicons toward the genera *Rhizophagus* and *Glomus* was observed in the data set obtained (Table 1).

4. Discussion

4.1. Mycorrhizal Morphotypes in Cocoa Cropping Systems

Previous studies have reported that cultural practices in agroecosystems modify AMF community structure and diversity in crops [39]. The wide diversity of genera identified by morphology shows that fine and aroma cocoa is a mycotrophic-dependent crop and can therefore be grown in acidic soils with low fertility [40]. In the present study, classical morphological identification methods and molecular biology methods were used to discover the diversity of fungi present in fine and aroma cocoa cultivation. Although we did not perform a comparison between morphological and molecular approaches, a previous study reports that the detection of taxonomic entities is partially discordant [38]. Our findings demonstrate that AMF were present in both cocoa growing systems. The morphological characteristics of the spores found in the two cocoa-associated systems did not differ much in terms of the number of genera belonging to Glomeromycota. However, the spore density was significantly higher in the conservative cocoa crop.

The conservative practice had one exclusive genus (*Racocetra*) and another eleven genera shared with the semi-conservative practice. To the best of our knowledge, the genus *Racocetra* has not been reported on cocoa roots. Species of the genera *Acaulospora*, *Ambispora*, *Claroideoglomus*, *Diversispora*, *Funneliformis*, *Gigaspora*, *Glomus*, *Pacispora*, *Rhizophagus*, *Paraglomus*, *Archaeospora*, and *Scutellospora*, have been previously reported in agroforestry systems and the Andes of Ecuador [13,41]. These findings are consistent with a previous study suggesting that AMF community richness is not influenced by the abiotic environment, but is instead related to the host microbiome and biochemistry [42].

4.2. Arbuscular Mycorrhizal Bioindicators of Anthropogenic Intervention

Dominikia bernensis was first reported in Switzerland in 2014 [43], then in Brazil in 2018 [44], and the North Caucasus in 2021 [45]. A previous study showed that *D. bernensis* is considered an indicator species for reduced tillering, under the premise that agricultural practices might affect the species to the point of its extinction [46]. However, the present study reports the presence of *D. bernensis* in cocoa crops under a semi-conservative practice.

Dominikia disticha was reported in the South African maritime dunes in 2015 [47], in the Tunisian Saharan oasis associated with *Phoenix dactylifera* in 2020 [48], and in the North Caucasus in 2021 [45]. *D. disticha* has been isolated in ecosystems with high temperatures, high salinity, and low soil fertility. In our study, *D. disticha* was present in semi-conservative cocoa crop soils; therefore, we hypothesize that the adaptability in adverse environmental conditions of *D. disticha* could be used to produce biofertilizers. However, functional studies under controlled conditions and in the field are needed to verify its physiological adaptability [48].

Rhizophagus diaphanus has been previously reported in coastal ecosystems of Eastern China [49] and South America [50]. *R. diaphanus* has been proposed as a biofertilizer based on its mycorrhizal consortia in coffee production, its benefits [51], and its inoculation in seedlings for recovery of degraded soils [52]. The abundance of *R. diaphanus* in Murundus fields has been reduced by agricultural activity [52,53]. In our study, *R. diaphanus* was absent in the semi-conservative cocoa crops and slightly present in the conservative cocoa crops, so it could be considered one of the negative effects of agrochemical use. However, further studies are needed to determine the significant differences between these conditions.

Rhizophagus fasciculatus has been reported in South America [50] sharing environments with *R. diaphanus,* in prevalent abundance in the red sandy soils of India [54], in the vineyards of northwestern Iran [55], pastures associated with *Cenchrus clandestinus* in Colombia [56], on aerial roots of *Araucaria angustifolia* in the Atlantic rainforest of Brazil [57], and in the Lacandon rainforest of Chiapas, Mexico [58]. In addition, studies show that *R. fasciculatus* performs symbiosis and improves uptake [59], even under extreme conditions such as high salinity and water stress [60,61]. In our study, *R. fasciculatus* was absent in the semi-conservative cocoa systems, and its presence was observed in the soils and roots of the conservative cocoa crops. This fact could be explained by the fact that the use of agrochemicals affects the root colonization of *R. fasciculatus* [62].

Rhizophagus custos (Rhizoglomus) was reported in high evergreen forest ecosystems associated with the red cedar *(Cedrela odorata)* in Mexico [63]. Their presence was reported in transitional soils of an ecological preservation zone in Sinaloa, Mexico [64]. *R. custos* is attributed as a bioremediating agent for the dissipation and elimination of polycyclic aromatic hydrocarbons from the environment [65]. In our study, *R. custos* was present in both conservative and semiconservative cocoa crops.

4.3. Synergistic Contributions of AMF to Cocoa Crops

The study of the soil microbiome response to different agricultural practices has been documented. At the phylum level, Ascomycota and Basidiomycota have been reported as the dominant phyla and indicators of specific conditions when comparing agricultural treatments in vineyards versus orchards. However, our study found that Ascomycota and Basidiomycota in both cocoa cropping systems did not show variations in relative quantities [66,67].

At the genus level, *Glomus aggregatum* has been reported as a common colonizer in *T. cacao* roots [68,69], several crop species [70], and is persistent in cropping systems with agrochemical intervention [68]. A previous study showed that the combination of *G. aggregatum* and other AMF species increases the bioremediation effect against heavy metal saturation [71], promotes root growth, increases nutrient uptake, improves biomass production, and counteracts damage caused by plant-parasitic nematodes [72]. *Pacispora scintillans*, previously known as *Glomus scintillans* [73], has been reported in sugarcane crops of Iran [74], lowland wetland areas of Ethiopia, soils with crop rotation in Morocco [75,76],

and organic management systems associated with Chilean Mediterranean vineyards [77]. In our study *P. scintillans* was present in cocoa crop samples under conservative practices.

The communities of AMF were more diverse and abundant in the conservative cocoa crops, with a total of 348 taxa compared with the 114 taxa corresponding in the semi-conservative crops. Seven taxa were new records for Ecuador and the agroforestry systems of fine and aroma cacao crops: *P. scintillans*, *R. diaphanus*, *R. fasciculatus*, *R. custos*, *D. disticha*, *M. perpusilla*, and *D. bernensis*. Overall, molecular analysis captured few genera of AMFs in the roots and soil of the cocoa of both cultivation practices. The low prevalence of these taxa coincides with previous studies, in which five genera were identified in cocoa agroforestry systems in central Cameroon; *Glomus* was the most abundant, whereas *Scutellospora* and *Acaulospora* were found in smaller quantities. *Gigaspora* and *Archaospora* were found in very small quantities, so were not reported [2]. The low prevalence could be due to the difficulty in capturing the entire AMF community using the primer sets, or the lack of classification in publicly available databases.

According to our molecular analysis, the diversity (alpha and beta) of soil and roots differed significantly, independent of the cultivation practice, and AMF diversity was higher in the conservative cocoa crop. This coincides with a study where the abundance of the AMF community under organic and conventional farming conditions did not show significant variation [78]. *Neocosmospora* is an important plant endophyte and has been widely reported as a pathogen. However, it is known that some species of *Neocosmospora* have been commonly found cohabiting with orchids and citrus, and are able to synthesize antimicrobial compounds [79–82]. In our study, *Neocosmospora* was considered a biomarker in the roots of *Theobroma cacao*. *Lasiodiplodia* has been reported as a pathogen capable of causing necrotic lesions when inoculated into mango fruits and young plants of *Annona* spp. and *Spondias* spp. It has also been reported that *Lasiodiplodia* is able to produce phytohormones such as indole and jasmonic acid analogues, both important regulators of plant growth and development [83,84]. Our results show that *Lasiodiplodia* was considered a biomarker of cocoa roots.

Following differential abundance analysis, our study suggests that fungal communities have greater differences between fine aroma cocoa roots and soil than between different cultivation practices. Therefore, semi-conservative practices and the impact of low levels of agrochemical use probably cause non-significant negative effects on the quantity of some taxa. However, a small number of AMF taxa were reported absent in the semi-conservative practice, which suggests that these genera were affected.

This study contributes information on the composition and behavior of fungal communities present in the rhizosphere of fine aroma cocoa grown in Ecuador and surrounding regions, to facilitate and complement efforts in identifying best practices. The present study also made efforts to find prospective microorganisms to develop sustainable agroecosystems and reduce the negative impact of agrochemicals on productive soils in Ecuador.

Appendix A

Table A1. Updated taxonomic classification of arbuscular mycorrhizal fungi.

Phylum: Glomeromycota			
Class	**Orden**	**Family**	**Genera**
Glomeromycetes	Glomerales	Glomeraceae	*Glomus* *Funneliformis* *Dominikia* *Funneliglomus* *Kamienskia* *Septoglomus* *Microdominikia* *Microkamienskia* *Nanoglomus* *Oehlia* *Halonatospora* *Orientoglomus* *Septoglomus* *Simiglomus* *Sclerocarpum* *Silvaspora* *Epigeocarpum* *Rhizophagus* *Sclerocystis*
	Gigasporales	Scutellosporaceae *	*Bulbospora* *Scutellospora* *Orbispora*
		Gigasporaceae *	*Gigaspora*
		Dentiscutataceae *	*Dentiscutata* *Fuscutata* *Quatunica*
		Intraornatosporaceae *	*Intraornatospora* *Paradentiscutata*
		Racocetraceae *	*Racocetra* *Cetraspora*
	Diversisporales	Acaulosporaceae	*Acaulospora* *Kuklospora*
		Entrophosporaceae *	*Entrophospora* *Viscospora* *Albahypha* *Claroideoglomus* *
		Pacisporaceae	*Pacispora*
		Sacculosporaceae	*Sacculospora*
		Diversisporaceae	*Corymbiglomus* *Redeckera* *Tricispora* *Otospora* *Sieverdingia* *Desertispora* *Diversispora*
Paraglomeromycetes	Paraglomerales	Paraglomeraceae	*Paraglomus* *Innospora*
		Pervetustaceae	*Pervetustus*
		Ambisporaceae	*Ambispora*
		Polonosporaceae	*Polonospora*

Table A1. *Cont.*

Phylum: Glomeromycota			
Class	**Orden**	**Family**	**Genera**
		Geosiphonaceae	*Geosiphon*
Archaeosporomycetes	Archaeosporales	Archaeosporaceae	*Intraspora* *Palaeospora* *Archaeospora*

* The classification is still under discussion and ordering. Reference: [47,85–88].

Table A2. Location and description of cocoa crops.

Province	Canton	Farm	System	Geographic Coordinates
Los Ríos	Vinces	La Americana Edén	Conservative Semi-conservative	79°48′14.86″ West Latitude and 1°38′23.84″ Longitude South 79°49′8.02″ West latitude and 1°38′47.12″ longitude South
Manabí	Chone	San José de Olla Vieja Berto Zambrano	Conservative Semi-conservative	0°47′52.46″ Longitude South 80° 5′36.79″ West Latitude 0°47′52.04″ Longitude South 80° 8′3.70″ West Latitude
Guayas	Milagro	San José Virginia	Conservative Semi-conservative	2° 7′57.36″ Longitude South 79°29′4.35″ West Latitude 2°06′34.7″ Longitude South 79°29′47.5″ West Latitude

Table A3. Analysis of the physicochemical properties of the soil from cocoa crops under two cultivation practices.

		Texture (%)				µg/mL						
Cultivation Practice	**Farm**	**Sand**	**Silt**	**Clay**	**Organic Matter (%)**	**pH**	**NH$_4$**	**P**	**Ca**	**Zn**	**Cu**	**Fe**
Conservative	Olla Vieja	22	62	16	3.1	6.5	19	46	4938	3.8	4.1	50
Conservative	San José	16	56	28	5	6.4	30	20	4847	3.7	8.4	95
Conservative	La Americana	20	58	22	4.4	7.9	27	37	4632	9.1	6.3	109
Semi-conservative	Virginia	54	36	10	3.7	6.6	22	7	4314	3.3	8.8	117
Semi-conservative	Berto Zambrano	18	54	28	4.4	6.4	27	100	4456	5.5	6.3	42
Semi-conservative	Edén	38	42	20	2.7	6.1	16	105	2649	7.4	14.5	355

Table A4. Primers used for DNA sequencing of *T. cacao* samples.

Region	Primer	Nucleotide Sequence (5′ to 3′)	Amplicon	Reference
ITS	ITS86F ITS4	*GTGAATCATCGAATCTTTGAA* *TCCTCCGCTTATTGATATGC*	369 pb	[89]
	ITS3 ITS4OF	*GCATCGATGAAGAACGCAGC* *GTACTAGGGGAATCCTTGTT*	310 pb	[90]

ITS: Internal Transcribed Spacer.

Table A5. A Mantel test of soil edaphic factors and soil fungal community composition.

Edaphic Factors	Correlation Coefficient	*p*	*p* Adjusted
Organic matter	−0.0320031423364575	0.634	0.722
pH	0.134693332544077	0.096	0.384
NH4	−0.0262467220468201	0.586	0.722
P	−0.0227195060186735	0.722	0.722
Ca	−0.0218924855036354	0.553	0.722
Zn	0.0897857088696029	0.08	0.384
Cu	−0.0293286170633843	0.568	0.722
Fe	−0.0547996422755585	0.711	0.722

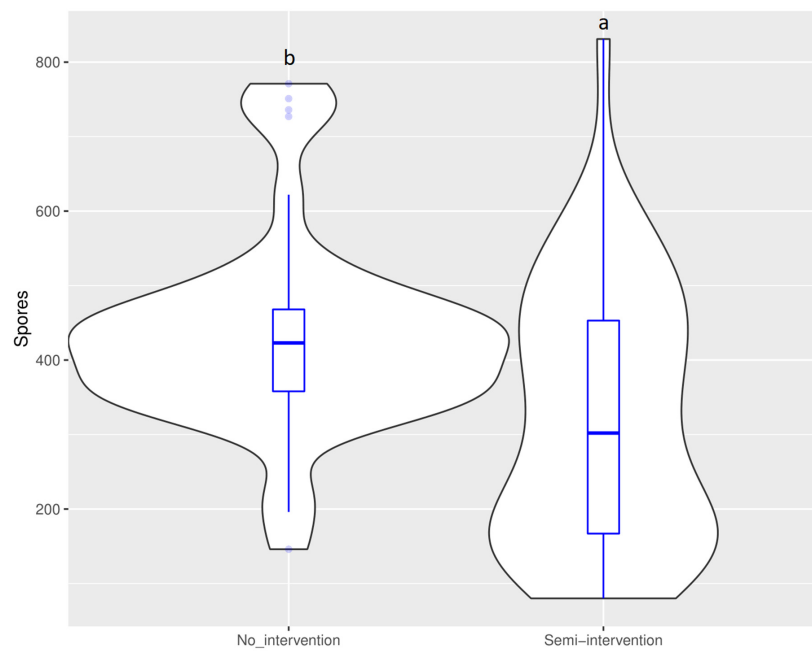**Figure A1.** Spore density present in cocoa roots from the two cropping systems (semi-intervention and no-intervention). Different letters indicate significant differences at *p* < 0.05 after the nonparametric Mann-Whitney test.
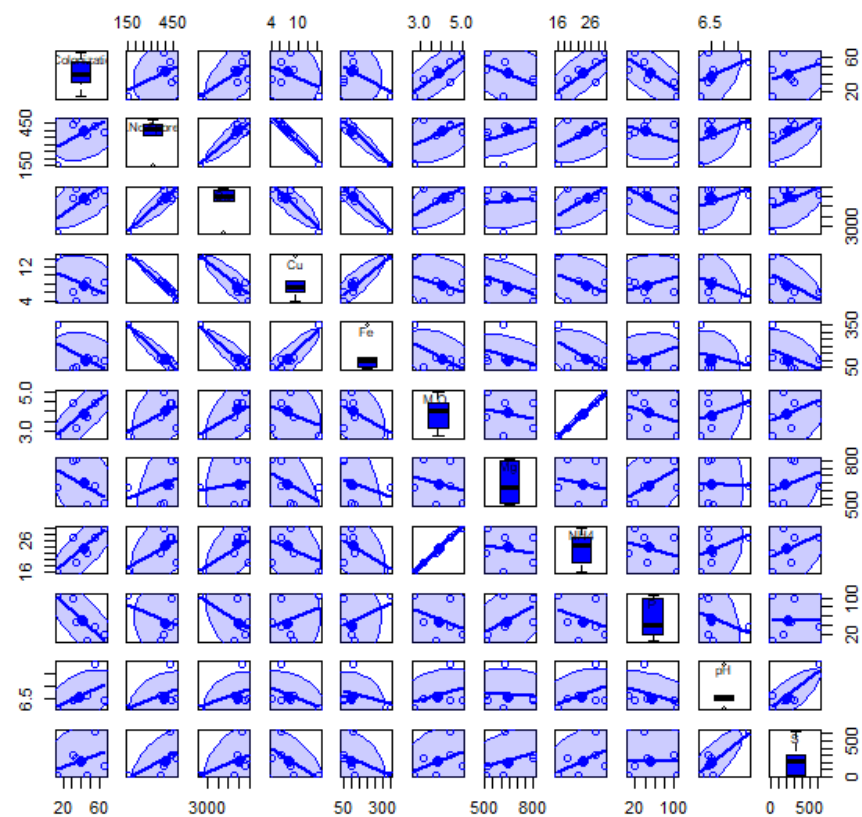

Figure A2. Scattering matrix, box and whiskers plot, concentration ellipsoid, and least squares line of physicochemical parameters versus colony and spore density.

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