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Arbuscular mycorrhizae in two national cocoa production systems (organic and chemical fertilization) in the province of Los Ríos, Ecuador

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***Abstract*---**The objective of this study was to evaluate the behavior of mycorrhizae in national cocoa plantations (*Theobroma cacao* L.) with different production systems, through the relationship between soil physicochemical characteristics and the ecology of arbuscular mycorrhizae as bioindicators of soil quality. Sixty soil and root samples were taken from the cantons of Quevedo, Buena Fé, Valencia, La Maná, and Mocache, to recover and count arbuscular mycorrhizae that inhabit the cocoa rhizosphere. Descriptive statistical methods were used for the general representation of the results of the observations; inferential methods were used to determine if the study factor (fertilizer application) in the influence on the ecological parameters of mycorrhizae. In terms of arbuscular mycorrhizal fungi (AMF) species richness, the PERMANOVA test showed that there were no significant statistical differences in fungal genus richness between farms with fertilization (p: 0.9936). In addition, there was a greater dominance of fungal genera in soils with fertilizers and there were 7 AMF genera found in all sampling sites (soils cultivated with national cocoa with and without fertilization): *Scutellospora, Glomus,*

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Acaulospora, Rhizophagus, Gigaspora, Ambispora y *Diversispora*, where, spore richness was higher on farms without fertilizer application located in San Carlos parish with a total of 3075 AMF spores.

***Keywords*---**mycorrhizae, bioindicators, cocoa, rhizosphere, fertilizer, phytosanitary products.

Introduction

The cultivation of *Theobroma cacao* L (cocoa) is the third most important crop in the group of agricultural commodities, after coffee and sugarcane, worldwide, and represents an important source of economic income for the countries where it is grown (Wanger *et al.,* 2018). Due to its biodiversity, Ecuador is suitable for the cultivation of Fino de Aroma cocoa, which is one of the most sought-after cocoa varieties by the main chocolatiers and chocolate manufacturers worldwide. Along with Fino de Aroma cocoa, a cloned variety known as CCN51 is also produced. However, since 2011, the government of Ecuador has initiated a national rehabilitation plan aimed at improving yields, quality, certification, and marketing of Fino de Aroma cocoa (Saravia *et al.*, 2020). As a traditional crop and source of economic income for smallholders in Ecuador, who generally do not mechanize their operations and make intensive use of fertilizers, these farmers have yields averaging 0.67 tons per hectare (Ibarra, 2019).

Improving agricultural productivity through the use of improved technologies, such as fertilizer application, is a critical pathway to increasing farm incomes, minimizing poverty, and addressing soil nutrient extraction (Ali *et al.*, 2018). The percentage extension in yield due to fertilizer application can increase by about 34 % at all levels of management and climatic conditions, but the absolute yield increase achieves higher when climatic conditions and management are favorable (Hoffmann *et al.*, 2020).

Soil applications of organic matter significantly increase yields reflecting improvements in stem length and leaf number. The right level of OM can not only improve soil fertility and cocoa growth but also reduce the severity of cocoa diseases through direct effects on soil inoculum levels and inducing plant resistance (Doungous *et al.*, 2018). The positive effect of compost on cocoa yields can potentially be attributed to physical changes that increase soil water availability, chemical improvement of nutrient availability, and biologically by promoting the activity of beneficial organisms. Dolomite application increased soil pH and Ca and Mg contents (Fungenzi *et al.*, 2021); however, organic fertilizers include the slow release of nutrients into the soil, high transportation cost, high product cost, and limited availability (Avane *et al.*, 2021).

Cocoa producers face many problems related to the acquisition of quality planting material on their farms to ensure a good annual yield of cocoa beans, and current research is based on the diversification of production systems to reverse this trend. One of the promising methods is the promotion of the use of mycelial microorganisms such as mycorrhizae that allow obtaining vigorous plants resistant to various soil pathogens (Djenatou *et al.*, 2020). The use of arbuscular mycorrhizal fungi (AMF) is considered one of the effective organic ways to increase crop productivity (Koda *et al.*, 2018), therefore understanding the composition of mycorrhizal communities in tropical areas becomes especially relevant since crop productivity in tropical soils typically of low fertility, manage to improve with the use of these fungi increasing height, stem diameter and aerial biomass (Gomes *et al.*, 2018).

Materials and Methods

The research was carried out in the cantons of Buena Fe, Quevedo, Mocache, Valencia, and La Maná, where soil sampling, extraction, and collection of AMF spores and observation of mycorrhizal colonization in national cocoa plants applying fertilizers and not applying fertilizers were applied to evaluate soil quality through the use of physicochemical parameters and biological indicators.

Soil sampling

In order to evaluate the relationship between AMF occurrence and soil physical and chemical characteristics, 5 soil samples (1 kg each) were collected in each of the 6 cocoa farms 50 meters from the initial sampling point (30 samples in farms with unfertilized cocoa and 30 in farms with fertilized cocoa). Each sample contained 5 subsamples collected evenly over 1 m2. The soil was collected with the help of a Dutch auger at a depth of 0-10 cm, the auger was cleaned with 70 % ethanol between each sample to avoid contamination of the samples. Soil samples were packed in plastic bags and stored in thermal boxes containing ice and taken to the Biotechnology Laboratory of Quevedo State Technical University. The samples were stored in a refrigerator between 4 and 10°C to evaluate the AMF community structure by morphological methods and for chemical and physical analysis (texture)

Extraction and evaluation of AMF spore density

The extraction of AMF spores was carried out by the wet sieving method followed by sucrose gradient centrifugation, proposed by Gerdemann and Nicolson (1963). It consisted of diluting 50 g of soil in 1.5 L of water, this solution was beaten in a blender for 15 seconds, and the material was allowed to settle and then sieved on 50 and 25 μm meshes. This sieving procedure was repeated 3 times for each soil sample. The material retained on the sieves was collected and transferred to falcon tubes containing a sucrose solution (60%). This solution was then centrifuged (3000 rpm for 3 minutes) and the supernatant was transferred to a 25 μm mesh sieve and rinsed with sterile distilled water to remove excess sucrose. The contents of the sieve were collected in glass containers and poured into Petri dishes with 1.25 cm grid cells between lines to facilitate counting. Subsequently, spore counting was performed with the aid of a stereo dissecting microscope.

Morphological evaluation of AMF spore diversity

Parallel to spore counting, spores were grouped into morphotypes according to their observed morphological characteristics such as size, color, and spore wall ornamentation. Spores of each morphotype were permanently mounted on polyvinyl-lactoglycerol (PVLG) and PVLG slides mixed with Melzer's reagent (1:1, v/v) and examined under a compound microscope. Spores were identified to genus level based on spore wall structure analysis, Melzer's reaction, and other taxonomic information such as characteristics and comparisons with those originally described (Blaszkowski, 2012), and online references of described species on the INVAM website of the West Virginia University, USA (INVAM, 2020). Spores with gigasporoid characteristics that differentiate two or more germ walls and Glomus spores that cannot be attributed to the species were placed in the genera *Scutellospora* and *Glomus*, respectively, following the nomenclature and species classification proposed by Redecker *et al.* (2013).

Chemical and granulometric soil analysis

About 200 cm3 of each soil sample was taken to the soil and water laboratory of the Quevedo State Technical University. The particle size and chemical parameters analyzed were texture by the pipette method, before being analyzed the soil samples were air-dried, deterronized, passed through a 2 mm diameter sieve and homogenized to determine the particle size distribution. In the laboratory, the following chemical parameters were determined: pH, organic matter, P, K, Ca, Mg, Al, S, Cu, Fe, Zn and B. Soil pH was measured with distilled water (1:1), F, K, Cu, Fe, Zn and Mn were extracted in Mehlich-1 solution. Ca, Mg and Al values were extracted in (CH3COO)2 CaH2O solution. Sulfur was extracted in SO4 solution. Boron was extracted in hot water. Organic matter was evaluated by oxidation of soil samples with a sulfochromic solution and was determined using the method proposed by Teixeira et al. (2017).

Sampling of roots of Theobroma cacao L. (cocoa)

In the 6 farms cultivated with cocoa, to evaluate the mycorrhizal colonization present in this species, 300 g of roots were collected from 5 adult cocoa individuals in each of the farms, these samples were collected in the vicinity of the place where the soil samples were collected, totaling 60 root samples (30 in the farms with fertilized cocoa and 30 in the farms with unfertilized cocoa), the root samples were washed with running water to remove soil and stored in bottles containing 50% alcohol until the analysis of the mycorrhizal colonization rate.

Thinning, coloration, and evaluation of mycorrhizal colonization in cocoa roots

The bleaching and staining process of stored cocoa roots was carried out according to the methodology proposed by Koske and Gemma (1989). It consisted of cutting the roots into pieces of up to 1 cm and placing them in glass jars with airtight lids containing a 10% KOH solution, remaining at rest overnight. However, as the cocoa roots were very dark, an additional lightening treatment was necessary by placing them in a hydrogen peroxide solution (3%) for 20 minutes. They were then immersed in a 1% HCl solution and then stained with a 0.05% trypan blue solution in lactoglycerol (1:1:1 glacial lactic acid, glycerol, and water). Finally, the roots were washed with distilled water and stored in glass jars containing 50% glycerol for evaluation of the mycorrhizal colonization rate.

The evaluation of the mycorrhizal colonization rate present in cocoa roots was carried out following the method proposed by Giovannetti and Mosse (1980). The estimation of this parameter consisted of analyzing the presence of fungal structures in the cortex region of the roots (hyphae, arbuscules and vesicles occurring inter- and intracellularly). Root segments previously stained and cut into 1-cm pieces were spread on Petri dishes containing 1-cm square lines on the bottom. The presence and absence of colonization were inspected with the aid of a dissecting stereomicroscope, using the vertical and horizontal lines on the plate, exactly at the point where the roots cross the lines, a minimum of 100 points were made and subsequently the percentage of colonization was estimated.

Statistical analysis

Data on total spore abundance were log (x + 1) transformed before analysis to meet the requirements of normality and homogeneity of variance; the Kolmogorov-Smirnov test for normality and Levene's test for homogeneity were performed. To examine whether AMF community composition differed between cocoa production types, a PERMANOVA test using the Bray-Curtis dissimilarity index with presence-absence data using 999 permutations of group association was performed using the VEGAN package of the R software platform (R Core Team, 2015).

To identify the relationship between soil physic-chemical characteristics and AMF community ecology, Multivariate methods were applied such as Redundancy Analysis (RDA) which served to correlate the physic-chemical variables that influence AMF ecology, similarity analysis (ANOSIM) to identify similarities between AMF of the soils of the farms using or not using fertilizer and finally a dimensional scaling analysis (NMDS) to identify proximities between AMF of the soils of the farms using or not using fertilizer.

Results and Discussion

Identification and enumeration of AMF genera in soils cultivated with domestic cocoa with and without fertilizer application

Seven genera of arbuscular mycorrhizae (*Scutellosora, Glomus, Acaulospora, Rhizhopagus, Gigaspora, Ambispora, and Diversispora*) were present in all sampling sites, and the highest number of spores was found on farms without application of chemical fertilizers as part of cultural management, highlighting the sampling areas of the San Carlos parish of Quevedo canton with a total average of 3075 spores/ 100 g soil (Table 2). In farms with national cocoa associated with an agroforestry system, it is possible to appreciate males in concentration fluctuating between 1712 to 2028 and spores per 100 grams of soil, which contributes to a decrease in the number of spores present (Prieto et al., 2012). Another factor that limits the diversity of mycorrhizal genera is related to climatic conditions and the type of crop, in the region of Uyacali and Madre de Dios in Peru, there is the presence of AMF only of the genus Glomus associated with wild cacao (Arévalo, 2016).

AMF genera	San Carlos		La Esperanza		El Vergel		Mocache		Buena Fe		El Moral	
	Con fert.	Sin fert.	Con fert.	Sin fert.	Con fert.	Sin fert.	Con fert.	Sin fert.	Con fert.	Sin fert.	Con fert.	Sin fert.
Scutellospora	216	409	188	415	204	403	172	454	188	383	175	404
Glomus	204	463	212	423	199	453	173	415	170	431	168	365
Acaulospora	222	443	191	399	162	431	182	395	195	411	167	472
Gigaspora	161	442	212	475	198	414	186	407	182	409	186	430
Rizhopagus	181	471	199	450	205	411	204	419	174	486	197	373
Ambispora	187	407	205	383	171	443	182	365	185	421	168	439
Diversispora	163	440	156	421	182	407	172	410	207	472	187	434
Total spores	1334	3075	1363	2966	1321	2962	1271	2865	1301	3013	1248	2917

Table 1. Number of spores of arbuscular mycorrhizal fungi found in 50 g of soil.

AMF genus richness in national cocoa under fertilizer and organic application systems

The richness of AMF fungal genera on domestic cocoa farms did not show similarity between treatments (ANOSIM p: 0.0001). The PERMANOVA test revealed that there were significant differences between treatments (p: 0.0001) for the richness of genera in farms with and without fertilizer application (Figure 1). The presence of AMF associated with cocoa is related to genera *Glomus, Ambispora, and Diversispora* in soils with low and high presence of Cadmium (Pineda *et al.*, 2020) being genera that were reported in the study, demonstrating the cosmopolitan capacity that these individuals have.

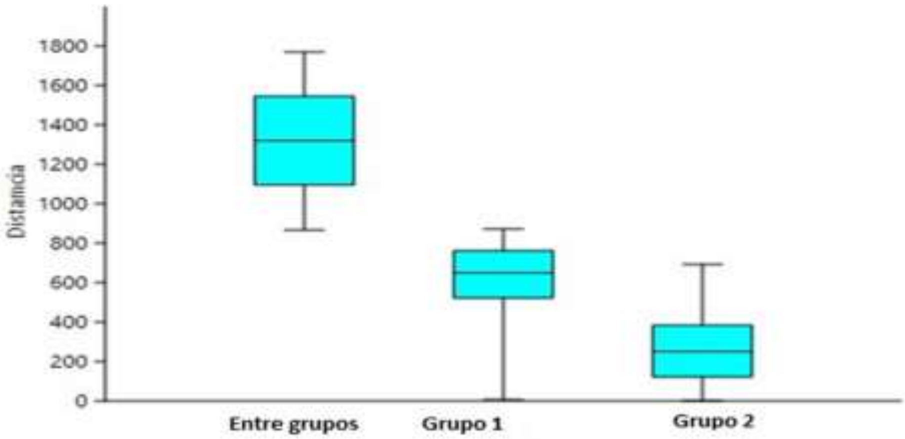

Figure 1. ANOSIM test for AMF fungal genus richness in national cocoa farms: Group 1 (with fertilizer); Group 2 (without fertilizer); Group 3 (with fertilizer); Group 4 (without fertilizer); Group 5 (with fertilizer).

The central region of the Ecuadorian humid tropics is very diverse, with great variability in soil characteristics and some microclimates. Thus, it can evidence the existence of distinct ecological regions or regions with biotic communities that differ from each other in short spaces of land, i.e., some factors affect the distribution of AMF species and, consequently, the density of spores and their colonization (Arévalo, 2016). On the other hand, spatial distribution, which integrates: climate, edaphic factors, latitude, spatial separation, management history, host plant communities, and interspecific and intraspecific interactions, all associated with the ability of dispersal, speciation, and extinction of AMF species, in addition to the importance of not disturbing the soil with fertilization, it is necessary to verify whether other factors are involved in the richness of AMF species in soils (Varma, 2008).

NMDS distance analysis in cocoa production systems with organic and chemical fertilization

The NMDS distances reveal the formation of two separate clusters (Stress = 0.16; R2 axis1= 0.92; R2 axis2= 0.03), indicating that there are significant statistical differences in the diversity of AMF present in the soils of farms cultivated with national cocoa in production systems with the presence and absence of synthetic fertilizers (Figure 2). The differences observed in the present study in the richness of AMF present in soils cultivated with cocoa with and without fertilizer were also observed in other sites with chemical soil disturbances.

Pineda *et al.* (2020) state that the results show that samples of *rhizospheric cacao* soil with high Cd concentration showed lower richness, abundance, and diversity of AMF morphospecies compared to those found in the nearby locality with low Cd concentration or little chemical fertilization. A high concentration and availability of macro- and microelements associated with a sedimentary parent material in the region impose a continuous and constant stress condition that modifies the AMF community structure (Millar and Bennett, 2016). Similar results were found in soils contaminated by anthropogenic activities with high concentrations of macro- and micronutrients as a result of uncontrolled fertilization, which presented lower AMF richness, abundance, and diversity (Hassan *et al.*, 2011).

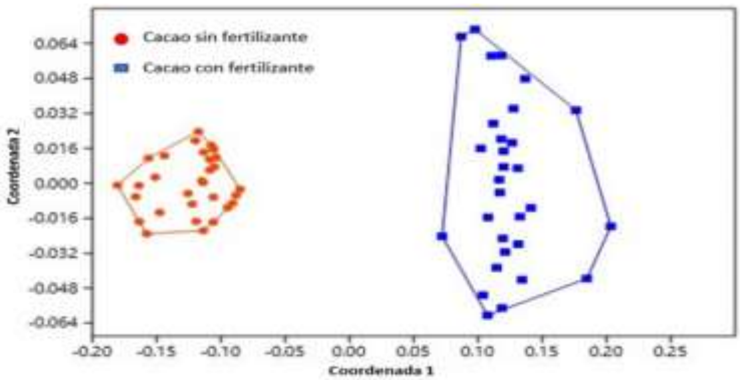

Figure 2. NMDS distance analysis of AMF from six farms grown with domestic cocoa with and without fertilizer application

Correlation of physical-chemical parameters and mycorrhizal colonization

The correlation coefficient of the model was significant at 0.94 ($p = 0.001$) for the national cocoa farms with fertilization, all correlations except for clay proved to have significant statistical differences. The correlation coefficient of the national cocoa farms without fertilization model is 0.81 and significant ($p = 0.001$), there were significant statistical differences with the following soil parameters clay, P, Ca, B and Mn. These characteristics are indispensable for mycorrhization both positively and negatively. In this case, factors such as acidity, concentrations of organic matter, phosphorus, potassium, nitrogen, aluminum, copper, zinc, and magnesium in the soil interfere with the establishment and performance of the symbiosis (Hernández-Hernández *et al.*, 2017), which is reflected in the ability of the host (plant roots) to colonize and in the spore production of AMF (Peña-Venegas *et al.*, 2007).

Table 3. Correlation of soil physicochemical parameters with mycorrhizal colonization percentage values

Soil physicochemical parameters	Farms with fertilizer	Farms without fertilizer
	R^2 adjusted	R^2 adjusted
Clay	-0.02	0.13*
Silt	0.07*	-0.03
Sand	0.31*	-0.03
pH	0.54*	-0.03
P	0.36*	0.10*
K	-0.02	-0.02
Cu	-0.03	-0.03
Ca	0.11*	-0.01*
Mg	0.07*	-0.04
Al	0.05*	-0.03
B	0.05*	-0.003*
S	-0.02	-0.04
Zn	0.51*	-0.04
Fe	0.17*	-0.03
Mn	0.53*	-0.01*
M.O.	0.28*	-0.03

* Significant statistical differences between treatments.

Wealth of AMF individuals

The comparison estimated by the Mann-Whitney test showed that the richness of individuals found between farms is different with averages of ($W = 900.0$ P-value = 2.99445E-11). There was a higher richness of individuals on the farms without fertilizer application compared to the sites where fertilizers were applied (Table 3). However, interactions between mineral fertilization and plant motorization did not show any significant or adverse effects on symbiotic processes (Rodríguez *et al.*, 2011). Crop inoculations with mycorrhizal formulations reduce the amounts of fertilizers and pesticides used and represent a sustainable technique to improve crop yield and profitability.

Table 3. Analysis of the richness of AMF individuals under different production systems

	HMA richness with fertilizers	HMA richness without fertilizers
Count	30	30
Average	261,233	593,267
Median	266,0	585,0
Standard Deviation	21,2371	37,2937
Coefficient of Variation	8,12954%	6,28617%
Standard Error	3,87734	6,80887
Minimum	206,0	526,0
Maximum	304,0	666,0
Range	98,0	140,0

Diversity of HMA individuals

The descriptive analysis and the Mann-Whitney test performed on the diversity of AMF individuals in the soils cultivated with cocoa of the national type in the six sampling sites showed that there is greater diversity in the soils without the application of fertilizers in cocoa cultivation (W = 877,0 P-value = 2,78305E-10) compared to soils treated with chemical fertilization (Table 4). The diversity and richness of AMF achieved significantly decreased with long-term P fertilization, such as NP, NPK, and OM. At the same time, the AMF community structure changed.

Table 4. Analysis of the diversity of HMA individuals

	AMF diversity with fertilizer	AMF diversity without fertilizer
Count	30	30
Average	1,91003	1,93477
Median	1,91	1,9345
Standard Deviation	0,0124166	0,00404017
Coefficient of Variation	0,650071%	0,20882%
Standard Error	0,00226695	0,000737631
Minimum	1,887	1,928
Maximum	1,934	1,942
Range	0,047	0,014

Dominance of AMF individuals in farms cultivated with national cocoa with and without fertilizer

The Mann-Whitney test defined that there is a greater dominance of AMF genera in soils where synthetic fertilizers were applied (W = 24.0 P-value = 3.13882E-10) (Table 5). Generally, a higher dominance in a disturbed environment where dominant species influence the local environment, distributing the spatial structure of the site in a certain way, and regulating the existence of natural resources (Carteron *et al.*, 2022). The abundance of the two dominant groups of arbuscular mycorrhizae is mainly associated with climate and anthropogenic influences, mainly nitrogen (N) deposition and fire suppression, along with climate change, have increased dominance over the last three decades, given that most mycorrhizal-dominated forests are supported by soils with high N availability (Jo *et al.*, 2019).

Table 5. Analysis of the dominance of AMF individuals in six farms cultivated with national type cocoa under a production system with organic and synthetic fertilization

	AMF diversity with fertilizer	HMA diversity without fertilizer
Count	30	30
Average	0,152987	0,14601
Median	0,15285	0,146
Standard Deviation	0,00366848	0,00117513
Coefficient of Variation	2,39791%	0,804829%
Standard Error	0,00066977	0,000214548
Minimum	0,1459	0,144
Maximum	0,162	0,148
Range	0,0161	0,004

Redundancy analysis for farms with fertilizer application

Redundancy analysis (RDA) made it possible to study the relationship between two equalities of Y and X variables, soil physicochemical parameters and AMF species density/enrichment. In RDA, the components extracted from X are correlated with Y variables. In this sense, the redundancy analysis (RDA) model is significant, presenting a coefficient of determination of 0.589112; for most of the descriptor parameters obtaining VIF scores less than 5 (Figure 3). Collinearity steps forward analysis revealed that the descriptor variables of the model are: Mg + S + Mn + M.O., these descriptor variables are positively correlated with AMF genera *Rhizhipagus, Glomus*, *Scutellospora,* and *Acaulospora* for cocoa farms grown with fertilizer application.

Soil microorganisms are genetically diverse, abundant, and functionally important organisms (Roesch *et al.*, 2007), therefore, understanding biogeographic patterns and what drives them is critical to maintaining ecosystems in a changing environment. Currently, 270 AMF species in the phylum Glomeromycota have already been described using spore morphological taxonomy (Redecker *et al.*, 2013). However, this is probably an underestimate of the overall richness of the AMF community (Kivlin *et al.*, 2011).

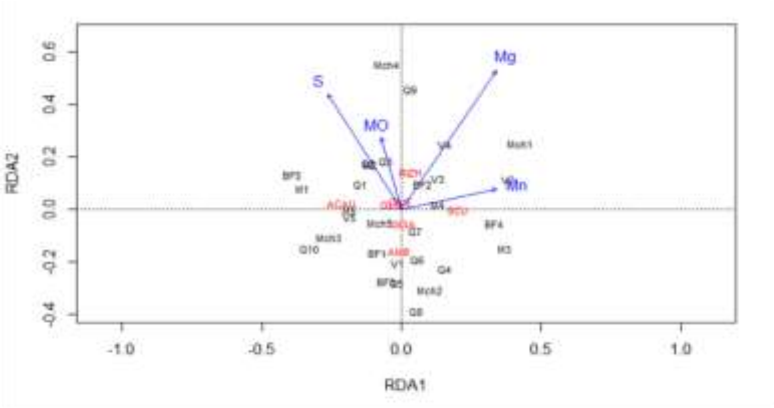

Figure 3. Redundancy Analysis (RDA) between soil physicochemical parameters with density and species richness of AMF in cocoa farms with fertilizer application.

Current knowledge of the geographic distribution of AMF species is limited. Several factors have been identified that may influence AMF distributions in soil, including abiotic (e.g., physicochemical properties of the soil), biotic (e.g., host plant), and intrinsic properties of the species (e.g., dispersal or colonization ability) (Chaudhary *et al.*, 2008). In this regard, Hazard et al. (2013) suggest that the distribution of AMF in the landscape is driven by environment rather than geographic distance. Previous studies based on the taxonomic identification of AMF using spores have shown that physicochemical parameters and various land use systems influence AMF sporulation (Lovelock et al., 2003). In addition, chemical elements such as sulfur, which is not very mobile in the soil and which in the present research was representative of the RDA in the two cocoa production conditions (organic and chemical fertilization), Toro et al. (2008) mention that AMF help in the mobility of this element, since arbuscular mycorrhizae allow an improvement in the early development of plants and sulfur mobilization plays an important role in this process (Arnawa et al., 2019).

Redundancy Analysis for farms without fertilizer application

The redundancy analysis model (RDA) for farms without fertilizer application was not significant, presenting a coefficient of determination of 0.61; where most of the descriptor parameters reached VIF scores lower than 5. The step forward collinearity analysis proved that the descriptor variables of the model are: P + K + K + Ca + S + Zn, these chemical parameters would be positively correlated with the presence of AMF spores belonging to the genera *Acaulospora*, *Gigaspora,* and *Scutellospora* (Figure 4).

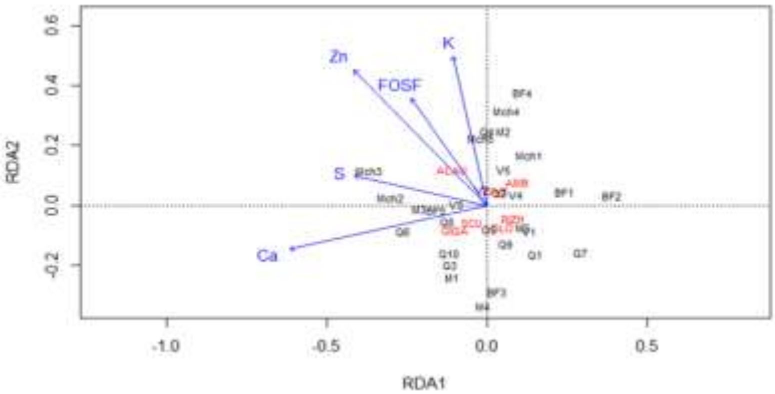

Figure 4. Redundancy Analysis (RDA) between soil physicochemical parameters with density and species richness of AMF in cocoa farms without fertilizer application

Conclusions

The richness of AMF individuals in the six farms cultivated with national cocoa with and without fertilizer application was different. There was a higher richness of individuals on the farms without fertilizer application compared to those with fertilizer application. Soil physicochemical factors such as P, K, Ca, S and Zn in farms with national cocoa without fertilizers influenced the composition of AMF genera, specifically *Acaulospora*, *Gigaspora,* and *Scutellospora*, respectively. While for cocoa soils with fertilization, the parameters Mg, S, Mn, and M.O were determinants in the presence/absence of *Rhizophagus*, *Glomus*, *Scutellospora,* and *Acaulospora*.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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