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*Rosa María Arias Mota * , Yadeneyro de la Cruz Elizondo , Laura Celina Ruelas Monjardín , Yamel del Carmen Perea-Rojas

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The propagation of Arbuscular Mycorrhizal Fungi with Trap Plants: A Proposal For Sustainable Management of Coffee Plantations

Rosa María Arias Mota [1,*], Yadeneyro de la Cruz Elizondo [2], Laura Celina Ruelas Monjardín [3] and Yamel del Carmen Perea-Rojas [4]

[1] Instituto de Ecología A. C., Carretera Antigua a Coatepec, No. 351. Col. El Haya, 91070 Xalapa, Veracruz, México; rosa.arias@inecol.mx
[2] Facultad de Biología. Universidad Veracruzana. Campus Xalapa, circuito Gonzalo Aguirre Beltrán s/n. CP 91090 Zona Universitaria Xalapa, Veracruz, México; ydelacruz@uv.mx
[3] TecNM/Instituto Tecnológico Superior de Xalapa. Reserva Territorial SN, Col. Santa Bárbara, Xalapa, Veracruz, México; laurarm@xalapa.tecnm.mx
[4] Doctorado en Micología Aplicada, Centro de Investigación en Micología Aplicada, Universidad Veracruzana Médicos No. 5, U.H. del Bosque 91010, Xalapa, Veracruz, México; yperearojas@gmail.com
*Correspondence: rosa.arias@inecol.mx

Abstract: Arbuscular mycorrhizal fungi are soil microorganisms that provide various benefits, such as increasing soil fertility and enhancing plant growth. The present study aimed to propagate arbuscular mycorrhizal fungal spores in trap plants. Soil sampling was carried out in October 2021 on five coffee farms in the central area of Veracruz, Mexico. Soil from coffee plantations and sterile sand were mixed in pots and used for the propagation of arbuscular mycorrhizal fungi, and Sorghum vulgare was used as a trap plant. The plants were kept in a greenhouse for 120 days. The abundance of spores in the sorghum trap plants increased significantly ($p<0.05$) in the soil from the coffee-growing areas between the start and end of the propagation period for all the analyzed sites. Among the 10 morphotypes detected in the coffee soils, the *Glomus* and *Acaulospora* genera were dominant, with *Glomus* sp1 and *Glomus* sp3 being the most common in the trap plants. A high percentage of mycorrhizal colonization was detected. It was concluded that S. vulgare can be used for propagating the spores of the native AMF on coffee plantations.

Keywords: bioinoculants; coffee plants; Sorghum vulgare; mycorrhizal colonization; spores

1. Introduction

Coffee is cultivated in 14 areas of Mexico, generating 1 million 25 thousand tons of cherry coffee: four areas—Chiapas, Veracruz, Puebla and Oaxaca—account for 90% of the country's coffee production. The value of coffee production in 2022 reached 6,535 million pesos. In 2021, Mexico ranked 12th in coffee production globally, while exports amounted to 428 million dollars, the main commercial destination being the United States of America. In Veracruz, coffee crops cover 139 thousand hectares, with high-quality varieties of Coffea arabica, such as typica, bourbon, mundo novo, caturra and garnica, being the most cultivated. The countries that stand out in terms of the production of Arabica coffee are Brazil, Colombia, Ethiopia and Honduras, which together contribute 71.9% of the global arabica coffee volume; however, for Robusta coffee, Vietnam, Brazil and Indonesia account for 77.6% of the global production. The beneficiaries of the world coffee trade today are large corporations despite the cultivation and production of coffee being carried out by small producers [1]. Data from Fairtrade International [2] indicate that approximately 125 million people globally depend on coffee cultivation for their livelihood and that for most of those people, coffee cultivation does not provide a reliable livelihood.

Coffee plantations are cultivated at elevations ranging from 300 to almost 2,000 meters above sea level and in areas with diverse climates, soils, and vegetation; however, coffee plantations

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develop better between 600 and 1,200 meters above sea level and are found mainly in hilly and mountainous areas on the slopes facing the Gulf of Mexico and those facing the Pacific. The coffee growing areas coincide with very rich and diverse regions; coffee cultivation areas play a very important role in the dynamics of the basins and in the conservation of soils since they help prevent the loss of soil on the slopes [3]. The structure of shaded coffee plantations is like that of natural ecosystems; thus, such plantations function as refuges for innumerable species of plants and animals and as sources for germplasms of saprobic and mycorrhizal fungi [4, 5, 6, 7, 8, 9, 10].

Despite the coffee production areas in the state of Veracruz being extensive, they face various problems; for example, there are issues related to the soil and its composition, as the soil is acidic and deficient in nutrients. To address these issues, producers resort to the application of fertilizers; however, the excessive use of chemical fertilizers creates other problems, such as eutrophication, soil degradation and subsequently the loss of soil biodiversity [11]. In the soil, interactions occur between microorganisms, including mycorrhizae, which are symbiotic associations established between fungi and plant roots [12].

Arbuscular mycorrhizal fungi (AMF) have been shown to be a sustainable alternative for optimizing resources since they offer multiple benefits, such as a high tolerance to abiotic stressors [13], the ability to contribute to bioremediation following contamination by heavy metals [14], and bioprotection [15], as well as the ability to improve soil structure and aggregation properties [16]. Furthermore, AMF provide plants with nutrition [17]. Sustainability has become a requirement of coffee production among the largest consumer groups, such as Europeans, and is necessary for marketing. To fulfil this requirement, alternative systems have been developed; these systems are referred to as sustainable, organic, and special systems, because they aim to conserve biodiversity.

Among the approaches for producing, marketing and consuming coffee, organic coffee is considered a holistic production management system that promotes and improves the health of agroecosystems, and particularly the health, biological cycles, and activity of soil. These achievements are reached through practices that avoid the use of chemical products as well as genetically modified organisms, sewage, sweeteners, and synthetic preservatives [18]. The application of AMF as bioinoculants to crops can minimize the use of chemical fertilizers in agricultural practices and guarantee agricultural sustainability by improving symbiotic associations with plants. However, given their biotrophic nature, these bacteria cannot be propagated in artificial culture media in the laboratory. This has complicated the development of large-scale production methods, limiting their commercial exploitation. Hence, the use of host trap plants with favorable sporulation and mycorrhizal colonization qualities is highly relevant for the propagation of native AMF [19]. Another important factor is the use of native or local AMF strains, which provide better results without the introduction of allochthonous organisms that compete with or lead to an imbalance of native organisms and are local resources typical of the coffee growing areas.

2. Materials and Methods

Study sites. The study sites were established in three locations in the center of the state of Veracruz, Mexico. Five shade-grown coffee farms that used conventional or traditional management of *Coffea arabica* var. costa rica were selected (Figure 1). The characteristics of each of the sites are shown in Table 1.

Sampling. Soil sampling was carried out on five coffee farms. On each farm, five sampling points were established, each separated by a distance of 50 m to ensure that they were independent. At each point, a coffee plant was considered the center, from which two axes measuring 1 m were defined—one north–south and another east–west. At the end of each axis, a 250 g soil sample was taken at a depth of 0-15 cm. The soil was dried at room temperature, after which physicochemical analysis was performed.

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Figure 1. Coffee plantations in the center of the state of Veracruz: a) San Isidro, b) Los bambus y c) Tuzamapan.

Table 1. Geographic location, elevation, mean annual precipitation, mean annual temperature and management type of the study sites.

Site	Precipitation (mm)	Latitude	Longitude	Elevation (masl)	Temperatute (°C)	Management type	Description
San Isidro	1636	19°36'42.74''	96°56'16.01''	1230	19.4	Traditional polyculture	Compost and NPK fertilizers applied 2 times/year. Weed management by manual removal.
Los bambus	1636	19°36'38.07''	96°55'40.57''	1350	19.4	Traditional polyculture	Compost and NPK fertilizers applied 3-4 times/year. Weed management by manual removal.
La barranca	1636	19°36'12.15''	96°54'44.91''	1295	19.4	Traditional polyculture	Compost and NPK fertilizers applied 1time/year. Weed management by manual removal.
Tuzamapan	1125	19º.38'43.01''	96º84'82.25''	650	27	Traditional polyculture	Fertilizers are not applied. Weed management by manual removal.
San Marcos	1361	19°25'34''	96°58'08''	1099	21	Traditional polyculture	Fertilizers are not applied. Weed management by manual removal.

Propagation test in Sorghum (*Sorghum vulgare*) trap plants. Soil from the coffee farms was used for the AMF propagation test in the trap plants, and five pots were prepared with soil from each of the farms and sterile sand at a 50:50 ratio. *S. vulgare* was used as the trap plant, and individuals were planted in 5 kg pots that were kept in a greenhouse for 120 days. Irrigation was carried out every third day with Hewitt nutrient solution without phosphorus. At the end of the experiment, the irrigation was suspended, the aerial biomass of the plants was measured, the spore morphotypes were isolated and counted, and the percentage of mycorrhization was quantified.

Isolation and counting of spore morphotypes of arbuscular mycorrhizal fungi. The AMF spores were separated by wet sieving and decantation [20]. A total of 50 g of soil was placed in a flask with 250 mL of water, and the sample was vigorously shaken for 10 minutes. Subsequently, the flask was left to rest for 10-15 minutes. The supernatant was passed through a series of Tyler sieves with 750, 250, 150 and 50 µm apertures. The supernatant from the last sieve was placed in 50 mL Falcon tubes,

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which were subsequently centrifuged at 2000 rpm for 5 minutes (Thermo Ice Centra CL2 Centrifuge). Once the samples were centrifuged, they were decanted, and a 70% sucrose solution was added. After vigorously shaking the sample, it was centrifuged again at 2500 rpm for 1 minute. The supernatant was passed through a 50 µm sieve, washed under running water and placed in plastic bottles. The samples were subsequently placed in Petri dishes and observed under a stereoscopic microscope (Carl Zeiss). The spores were grouped according to their morphological characteristics, such as size, color, and presence of supporting hyphae. The abundance of the spores was quantified, and the spores were mounted in permanent preparations in polyvinyl alcohol with or without Melzer solution for observation under a compound microscope (Carl Zeiss).

Thinning and staining of the roots of trap plants (*S. vulgare*). The roots were washed under running water, cut into small pieces, and placed in test tubes. The roots were stained following the thinning and staining technique [21], in which 10% KOH was added until the roots were completely covered, after which they were left to rest for 30 seconds and then placed in a water bath for 15 minutes. The roots were then washed with running water to remove the KOH. Next, 10% HCL was added, and the mixture was left for 3 minutes. The mixture was washed again with running water until the remaining HCL was removed. Then, 0.05% trypan blue was added, and the roots were placed in a water bath for 10 min. Finally, the stained roots were placed in lactic acid for observation.

Measurement of the percentage of mycorrhization. To determine the percentage of root colonization, the technique described by Giovannetti and Mosse [22] was used. The stained roots were placed in a square Petri dish (1x1 cm) filled with lactic acid, and the fungal structures in the roots were observed under a compound microscope. The quantification of the percentage of mycorrhization was carried out according to the methods described by McGonigle et al. [23]. A simple percentage count of the stained roots was carried out; each time a longitudinal portion of a root was observed with hyphae, vesicles or arbuscules that also touched the axis of the reticule that crossed the root, the result was considered positive; otherwise, the result was considered negative. The counts are expressed as percentages and were calculated as the difference between the number of positive intersections and the total number of intersections (set at 100).

Physicochemical analysis of soils. Physicochemical analyses of the soil from the coffee farms were carried out in accordance with NOM 021-RECNAT-2000 [24]. The organic matter (OM) and the organic carbon (CO) were quantified by the modified Walkley-Black method [25], the pH was measured by the electrometric method, the cation exchange capacity (CEC) was determined with 1 N ammonium acetate (pH 7.0), the total nitrogen was determined by micro-Kjeldahl [26], the available phosphorus (P) was measured by the Bray Kurtz1 method [27], and the retained phosphorus was quantified by the Blakemore method [28]. The analyses were carried out in the Soil, Plant and Water Analysis Laboratory of the Institute of Ecology, AC (Table 2).

Table 2. Physicochemical characteristics of the coffee plantations evaluated: pH, Available phosphorus (P), Retained P, organic matter, organic carbon, Cation exchange capacity (CEC), Field capacity (FC), Bulk density, Clay, Silt, Sand, texture, Total carbon (C), Total nitrogen (N) and soil type.

Coffee	pH	Available P	Retained P	Organic	Organic	CEC	FC	Bulk density	Clay	Silt	Sand	Texture	C	N	Soil
plantations	1:2 H2O	(mg/Kg)	(%)	matter(%)	carbon	1NpH7	% humidity	g/cm3	(%)				%		type
San Isidro	4.09	5.2	87.35	12.46	7.23	27.09	31.72	0.893	29.8	30.56	39.64	Clay loam	8.5	0.72	Andisol
Los bambus	5.24	14.35	89.8	3.93	2.28	20.88	22.69	1.016	45.8	22.56	31.64	Clay	2.9	0.27	Andisol
La barranca	4.81	13.56	81.63	4.72	2.74	21.51	21.62	0.994	49.8	28.56	21.64	Clay	3.5	0.27	Andisol
Tuzamapan	5.34	11.72	86.94	7.15	4.15	14.31	21.9	0.918	41.8	26.56	31.64	Clay	4.7	0.42	Vertisol
San Marcos	4.97	6.62	88.72	9.99	5.79	24.12	29.06	0.899	49.8	27.56	22.64	Clay	5.9	0.51	Luvisol

Statistical analysis. Spore abundance was expressed as the total number of spores found in 100 g of soil. Species-abundance distributions were plotted (Whittaker plots) to elucidate AMF dominance patterns in each one of samples. Distributions were obtained by plotting the abundance of AMF (from most abundant to least abundant species). To identify differences in spore abundance between the initial count in the coffee-growing soil and the final count after propagation in the

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sorghum trap plants, we conducted one-way analysis of variance was performed after determining whether the data met the assumption of a normal distribution and homogeneity of variance using the Kolmogorov–Smirnov test and Bartlett test, respectively).

Percentage of root colonization were compared between samples using one-way ANOVAs (with 5 replicates). When the effects of factors were significant in the ANOVAs, a Tukey's HSD post hoc test was run to test for pair-wise mean differences at P = 0.05. These analyses were conducted using Statistica 12.0 software. To estimate the relationships between the physicochemical variables of the soil and abundance of spores, Pearson's correlation analysis was performed with a significance level of $p \leq 0.05$.

3. Results

Abundance of arbuscular mycorrhizal fungal spores

The abundance of spores increased significantly ($p < 0.05$) between the initial count in the coffee-growing soil and the final count after propagation in the sorghum trap plants. This was observed for all the sites analyzed. On the San Isidro farm, the number of spores increased from 136 to 918; on the Los bambus farm, the number of spores increased from 89 to 867; on the La Barranca farm, the number of spores increased from 130 to 801; on the Tuzamapan farm, the number of spores increased from 195 to 990; and on the San Marcos farm, the number of spores increased to 809. The greatest increase was detected on the Los bambus farm (9.7 times), and the smallest increase was detected on the San Marcos farm (3.87 times) (Table 3).

The results demonstrate the presence of morphotypes of the *Glomus* and *Acaulospora* genera in all the coffee plantations and a large increase in these genera through propagation in trap plants of *S. vulgare*. It is important to highlight that the abundances of the morphotypes belonging to the genus *Glomus* (*Glomus* sp3 and *Glomus* sp1) increased more than the abundances of the morphotypes of the genus *Acaulospora*. Within the genus *Acaulospora*, the number of *A. scrobiculata* spores increased on the trap plants. The genus *Gigaspora* was represented by a single morphotype (*Gigaspora* sp1), and the number of spores from this morphotype did not increase in the *S. vulgare* trap plants. This result could be due to the low number of spores detected at the beginning of the experiment or poor compatibility with the trap plant.

Table 3. Morphotypes and spore abundance of arbuscular mycorrhizal fungi in 50 g of coffee plantation soil (initial) and after 120 days (final) with trap plants (*S. vulgare*) in the greenhouse.

Morphotypes	San Isidro		Los bambus		La barranca		Tuzamapan		San Marcos	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Glomus sp1	16	123	16	140	33	149	63	220	82	247
Glomus sp2	3	40	3	144	2	104	2	119	3	162
Glomus sp3	54	328	22	163	69	233	18	114	35	162
Glomus sp4	10	81	19	84	4	82	25	214	20	79
Rhizophagus clarus	31	163	8	196	5	28	20	151	8	27
Rhizophagus	1	2	1	3	1	68	1	38	2	17

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Acaulospora scrobiculata	15	124	19	103	13	132	43	127	61	106
Acaulospora sp1	1	20	0	0	1	1	26	2	1	3
Acaulospora sp2	3	14	2	34	1	2	1	3	1	5
Gigaspora sp1	3	23	1	1	1	2	1	2	2	1
TOTAL	136	918	89	867	130	801	195	990	209	809
*Mean ± SE	45.6±5.5b	306±47.15a	30.3±2.08b	289.0±42.03a	43.3±1.52b	207±21.65a	67±10b	330±2.64a	71.66±2.5b	269.66±3.78a

* Value corresponds to average of five replicate ± standard deviation. Different letters between pairs of columns indicate no significant differences of spore abundance of morphotypes AMF between initial and final samples $p<0.05$).

Dominance of arbuscular mycorrhizal fungal morphotypes in trap plants

The dominance of the morphospecies is illustrated by Whittaker plots (Figure 2). A In total, 10 spore morphotypes were observed in the soil from the coffee plantations in both the initial and final samples. During the initial sampling, the *Glomus* sp3 morphotype was dominant on the San Isidro farm, the Los bambus farm and the La barranca farm. The *Glomus* sp1 morphotype was dominant on the Tuzamapan and San Marcos farms; however, after propagation in the trap plants, *Glomus* sp3 and *Glomus* sp1 were dominant. At the Los bambus farm, *Rhizophagus clarus* was the dominant morphotype.

Mycorrhizal colonization of trap plants (*S. vulgare*)

Characteristic structures of AMF (Figure 3), such as hyphae, vesicles, arbuscules and spores, were observed within the roots of the trap plants (*S. vulgare*) 120 days after sowing. The percentages of mycorrhizal colonization did not significantly differ among the trap plants inoculated with soil from different coffee farms ($p> 0.05$). The mycorrhization percentages ranged from 71-80% (Figure 4). However, the differences between the values were not significant, and the highest colonization rate was observed on the San Isidro farm (80%). Kormanic and McGraw [29] defined 5 degrees of mycorrhizal colonization: null (0%), low (1-25%), moderate (26-50%), high (51-75%) and very high (76-100%). According to these categories, the extent of mycorrhizal colonization was classified as very high.

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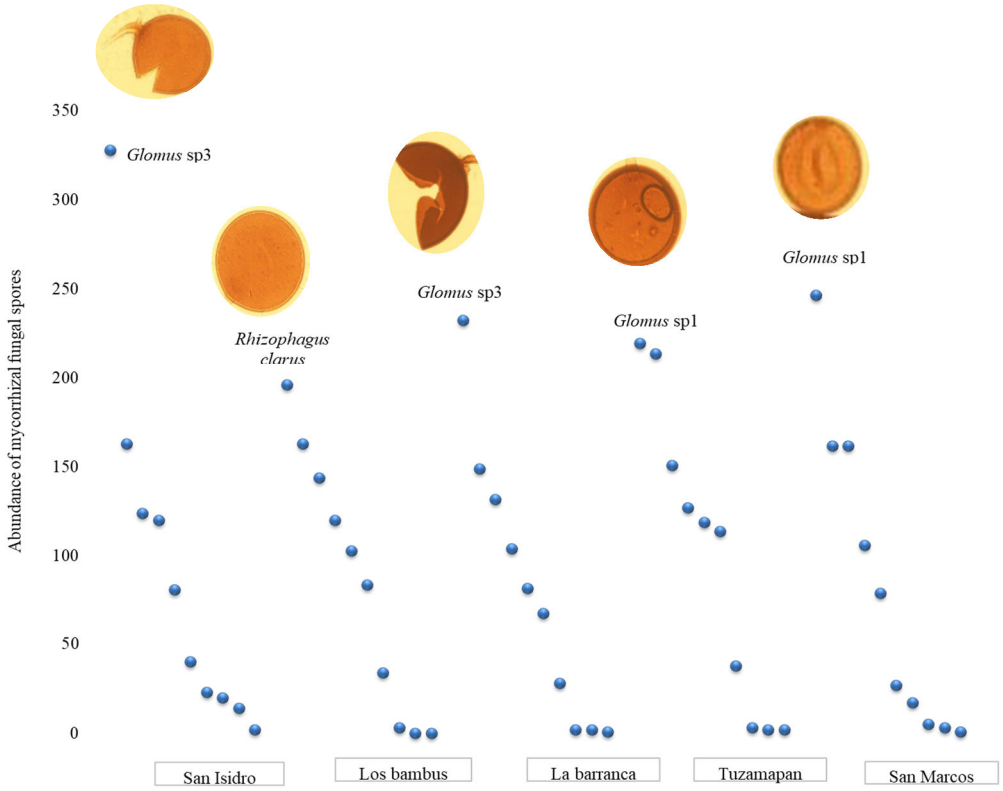

Figure 2. Whittaker plots (rank/abundance plots) illustrating dominant morphotypes of arbuscular mycorrhizal fungi in trap plants (*S. vulgare*) inoculated with coffee plantation soil after 120 days in the greenhouse. Morphospecies are plotted in sequence from highest to lowest abundance along the X axis. The Y axis represents the total spore abundance of each morphospecies.

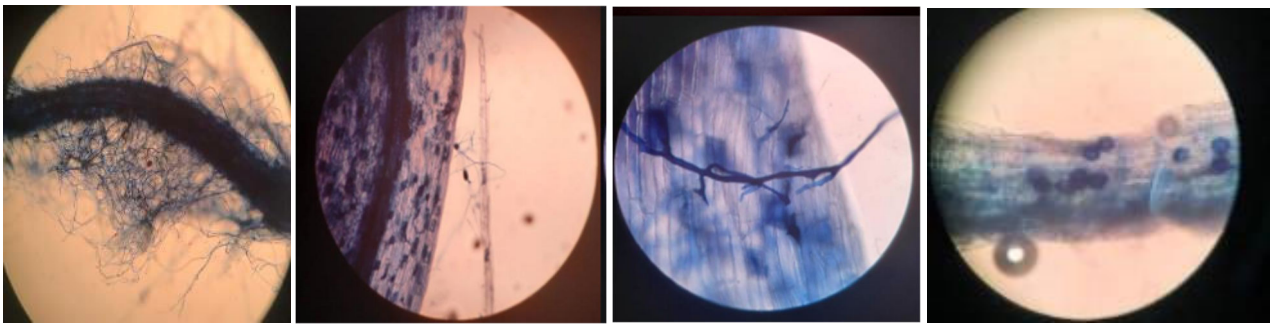

Figure 3. Secondary roots of Sorghum trap plant (*S. vulgare*) with hyphae colonization.

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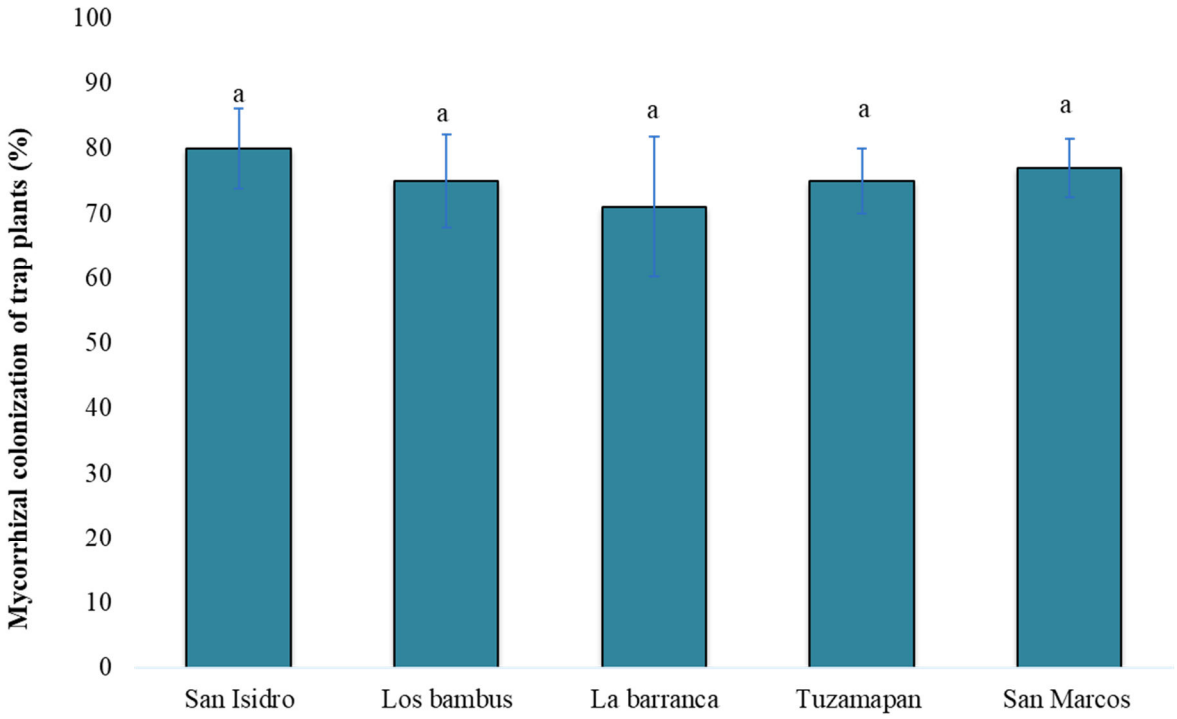

Figure 4. Mycorrhizal colonization of trap plants (*S. vulgare*) inoculated with soil from coffee plantations. Value corresponds to average of five replicate ± standard deviation. Shared letters in a column indicate no significant differences between samples $p<0.05$).

4. Discussion

The *Glomus* sp1 and *Glomus* sp3 morphotypes were dominant in the coffee plantations analyzed in this study. Arias et al. [6] indicated that the genera *Glomus* and *Acaulospora* were dominant in coffee plantations in Veracruz under different management practices; in particular, *Glomus* sp. and *G. clarum* were the dominant morphospecies at the regional level. This work highlights the greater abundance of the *Gigaspora* morphotype on San Isidro Farm. Compost is applied exclusively on this farm, resulting in a higher organic matter content; thus, it is important to highlight that various agroecological practices, such as the use of compost, can favor the presence and abundance of spores. Based on a study of coffee plantations in Chiapas, Bertolini et al. [30] report that there may be specific AMF consortia associated with different levels of P and soil acidity. In addition, they noted that various species of *Acaulospora* and *Glomus* could be common in the environmental conditions in which coffee is grown. They even suggested that functional compatibility studies be carried out before the application of biofertilizers.

The physicochemical characteristics of the soil determine the distribution of these microorganisms, and additional studies of different types of vegetation can help improve the understanding of the ecological preferences of AMF species. Species belonging to the genus *Glomus* adapt to almost any edaphoclimatic condition; likewise, *Acaulospora morrowiae* and *Acaulospora scrobiculata* have been reported to exist at a wide range of pH values (between 3.8 and 8.0) and to adapt to various levels of fertility [31].

In the present study, the soil pH (4.09) and clay content (29.8) at the San Isidro farm favored high abundances of *Acaulospora* sp1, *Glomus* sp2 and *Gigaspora* morphospecies. Peña-Venegas et al. [32] argue that the pH affects the number of spores in the soil because a slight increase in pH changes the level of aluminum saturation, causing a decrease in this parameter; therefore, in clays, for example, an increase in pH improves the cation exchange capacity of the soil, favoring higher population densities of microorganisms.

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The genera *Scutellospora* and *Entrophospora* are highly diverse in the tropics [31]; however, in altered agroecosystems, the genera *Scutellospora* and *Gigaspora* are less common [33]. Arias et al. [6] concluded that various factors related to management practices, such as weed control, soil conditions and chemical fertilization, interfere with the abundance and composition of AMF in coffee plantations.

In coffee-growing soils in the study area, the use of *S. vulgare* plants is recommended for promoting the spread of AMF spores (801-918 in 50 g of soil) and supporting high rates of mycorrhization (71-80%). This high colonization rate could be due to the high level of compatibility with the trap plant used. In a study by Chaiyasen et al. [34] in soil from coffee plantations in Vietnam, *Zea mays* was selected among different herbaceous plants (*Zea mays, Plantago* spp., *Oryza sativa*, *Bidens pilosa* and *Pensacola bahia*) due to its ability to support a larger increase in AMF spores (3690 spores/100 cm3 and 65% mycorrhizal colonization). The International Collection of (Vesicular) Arbuscular Mycorrhizal Fungi (INVAM [35], sorghum plants are routinely used in germplasm banks, and these plants are considered excellent hosts for a wide range of AMF under greenhouse conditions; however, these plants may not behave as optimally in all geographic regions because each host plant has particularities or characteristics that can affect or limit the spread of AMF spores in a trap crop [36]. Thus, it is important to choose the species with the best performance when carrying out this type of work.

There are different methods for propagating AMF, such as monosporic culturing [37, 38], solid substrate culturing [39, 40], aeroponic culturing [41] and hydroponic culturing [42]. However, storing propagated AMF spores requires technical skills and specialized knowledge, making this task difficult for farmers. One of the common practices for the propagation of spores is the trap culture method. In the production of inoculum at a commercial level, host plants such as *Allium cepa, Cenchrus ciliaris, Panicum maxim, Paspalum notatum, Sorghum halepense, Trifolium subterraneum* and *Zea mays*, which require 3-4 months for development, are used [43, 44]; however, it is recommended that evaluations be carried out with other plants that could be compatible. Substrates and host plants influence the propagation of AMF species or AMF consortia, so it is extremely important that these factors be considered for successful propagation. Likewise, the dominant species should be considered since the effectiveness of propagation varies depending on the plant-AMF interaction.

The application of AMF requires careful selection of viable species that are effective for propagation since one of the problems in the use of commercial mycorrhizae is that they are delivered in inoculum in low concentrations, and their composition is generally not revealed by the manufacturer, reducing their effectiveness in the field [45]. Thus, it is necessary to expand what is known about the multiplication and characterization of viable AMF spores to produce AMF inoculum within a specific host plant to improve plant growth and guarantee sustainable agricultural practices [46]. It is important to note that both a host plant and a consortium of host plants can benefit the propagation of AMF spores, as demonstrated by Yao et al. [47], who found that cocultures of certain host plant species were more effective than monocultures for propagating AMF spores. Recently, a coculture of corn and sorghum was shown to have a greater capacity as a host consortium of trap crops than individual trap plants [48]. Trejo and Bañuelos [49] recommend the rotation of host plants after every four cycles of trap cultivation to maintain the original diversity of AMF. The production of native AMF inoculum provides several benefits, thus enabling the production of viable and low-cost inoculum; additionally, the use of native AMF inoculum is environmentally friendly and can facilitate the characterization of the propagated AMF inoculum.

5. Conclusions

The propagation of AMF spores from soils in coffee-growing areas using *S. vulgare* as a trap plant promoted efficient spore growth and high rates of mycorrhizal colonization. This host plant was able to maintain the AMF community present in the soils. However, it is suggested that experiments with plant consortia be performed to increase the efficiency of AMF propagation. In general, the *Glomus* sp1 and *Glomus* sp3 morphotypes are considered the dominant species of coffee plantations and could be used in the formulation of biofertilizers. This technique of propagating

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native AMF of coffee plantations by using trap plants offers an alternative to sustainable coffee production and the possibility of obtaining an additional coffee product for consumption or sale.

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