TERRA LATINOAMERICANA

Colonization Patterns by Arbuscular Mycorrhizal Fungi and Dark Septate Endophytes in a Forest Ecosystem of the Municipality of Isidro Fabela, State of Mexico

Patrones de Colonización por Hongos Micorrízicos Arbusculares y Endófitos Septados Oscuros en un Ecosistema Forestal del Municipio de Isidro Fabela, Estado de México

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SUMMARY

Arbuscular mycorrhizal fungi (AMF) and dark septate endophytes (DSEF) have such an influence on plant development and on the edaphological characteristics and nutrients available in the soil that changes in these communities can drive modifications in the biotic and abiotic variables of an ecosystem, demonstrating the synergetic between species. Given its relevance, the objective of this work was to determine the status of colonization in the roots of three representative herbaceous species of the understory of a community of *Abies religiosa* (Kunth Schltdl. et Cham.) and *Pinus harwegii* (Lindl), and to contrast the coexistence of colonizers in three conservation-disturbance scenarios that exemplify the vulnerability situations of a forest ecosystem. A tripartite AMF-HESO-plant interaction is reported by the occurrence of coenocytic hyphae, vesicles, septate hyphae, moniliform cells, and sclerotia. The values of arbuscular mycorrhization exceed 50% and are higher than those found for endophytes (15.7-64.5%). The colonization of both types of fungi, such as AMF sporulation (687 spores 50 g^{-1} soil in the reforested area), seems to be related to the microclimatic conditions resulting from soil use. The edaphological variables that allow inferring the colonization behavior correspond mainly to available phosphorus, calcium, pH, organic matter, and cation exchange capacity. It is concluded that soil properties, its use, and the type of plants are determinant in the establishment of fungal communities. However, more studies on HESO-plant interaction are needed.

Index words: *development, rhizosphere, soil, symbiosis.*

RESUMEN

Los hongos micorrícicos arbusculares (HMA) y los endófitos septados oscuros (DSEF) tienen tal influencia en el desarrollo de las plantas y en las características edafológicas y nutrientes disponibles en el suelo, que cambios en estas comunidades pueden impulsar modificaciones en las variables bióticas y abióticas de un ecosistema, demostrando la sinergia entre especies. Dada su relevancia, el objetivo de este trabajo fue determinar el estado de colonización en las raíces de tres especies

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herbáceas representativas del sotobosque de una comunidad de *Abies religiosa* (Kunth Schltdl. et Cham.) y Pinus harwegii (Lindl), y contrastar la coexistencia de colonizadores en tres escenarios de conservación-perturbación que ejemplifican las situaciones de vulnerabilidad de un ecosistema forestal. Se constata una interacción tripartita AMF-HESO-planta por la aparición de hifas coenocíticas, vesículas, hifas septadas, células moniliformes y esclerocios. Los valores de micorrización arbuscular superan el 50% y son superiores a los encontrados para los endófitos (15.7-64.5%). La colonización de ambos tipos de hongos, como la esporulación de AMF (687 esporas 50 g^{-1} de suelo en la zona reforestada), parece estar relacionada con las condiciones microclimáticas derivadas del uso del suelo. Las variables edafológicas que permiten inferir el comportamiento de la colonización corresponden principalmente al fósforo disponible, calcio, pH, materia orgánica y capacidad de intercambio catiónico. Se concluye que las propiedades del suelo, su uso y el tipo de plantas son determinantes en el establecimiento de las comunidades fúngicas. Sin embargo, son necesarios más estudios sobre la interacción HESO-planta.

Palabras clave: *desarrollo, rizósfera, suelo, simbiosis.*

INTRODUCTION

Soil is an ecosystem of vital importance for the production of 95% of food (Martínez-Hernández, Sánchez, Martínez, Núñez de Cáceres, and Octavio, 2016; Vidal *et al*., 2018). This depends on the physical and chemical characteristics of the soil, as well as the environmental-ecological parameters that determine its quality. However, the presence of materials foreign to the soil, coming from anthropogenic activities (Pedraza *et al*., 2010; FAO, 2019), contaminate and affect the processes that occur in it, such as biogeochemical cycles, negatively impacting ecosystem services and food production (Weinbauer and Wenderoth, 2002; Pedraza *et al*., 2010).

In the soil, interactions develop between plants, arbuscular mycorrhizal fungi (AMF), and dark septate endophytic fungi (DSEF); two groups of fungi that can directly influence the success of plants in a given environment (Bueno de Mesquita, Martínez del Río, Suding, and Schmidt, 2018).

Mycorrhizal-arbuscular symbiosis is established between the roots of most terrestrial plants and fungi belonging to the Phylum Glomeromycota (Di Barbaro *et al*., 2017). AMF are a critical component of the ecosystem contributing to plant nutrition and growth by providing them with access to minerals in a bioavailable form derived from the soil (Barrera, 2009; Bhantana *et al*., 2021). Plant-microorganism interactions favor plant tolerance to plant pathogenic organisms, adverse environmental conditions such as drought, salinity, potentially toxic elements, and organic pollutants (Thangavel *et al*., 2022).

Dark septate endophytic fungi are anamorphic dematiaceous root ascomycetes that occupy diverse ecosystems. The association ranges from mutualism to parasitism, and the benefits of such microorganisms to plants are debated and need to be evaluated (Giesemann *et al*., 2020).

The establishment of AMF and ESH colonization apparently depends on different factors such as plant and fungal genotype, as well as pH, electrical conductivity (EC), organic matter (OM), available phosphorus (Pi) and available nutrients in the soil, whose concentrations can drive variations in communities (Mena-Echevarría, Méndez, Ramírez, and Rojas, 2021; Méndez-Matías, Robles, and Hernández, 2021; Santos, Cesanelli, Diánez, Sánchez, and Moreno, 2021).

AMF influence soil aggregation at different scales and improve soil quality, even under adverse conditions (Lozano-Sánchez, Armbrecht, and Montoya, 2015; Thangavel *et al*., 2022). While DSEF present enzymatic activities capable of degrading organic matter (Bueno de Mesquita *et al*., 2018).

The benefits that AMF provide to rhizosphere make them useful to improve soil quality mostly in agricultural systems and within conservation, restoration, or remediation programs to return the ecosystem to a pre-disturbance state, ensuring the recovery of soil composition, structure, and functionality (Herrick, Schuman, and Rango, 2006; Barrera, 2009).

DSEF have been studied in less depth than AMF; however, their presence in limiting environmental conditions implies that the interactions in which they participate are relevant to plant survival (Santos *et al*., 2021).

Due to the importance of AMF and DSEF in plant development, as well as their participation in biogeochemical cycles and edaphological properties, the objective of this work was to determine the root colonization status of three herbaceous species representative of the understory of a community of *Abies religiosa* (Kunth Schltdl. et Cham.) and *Pinus harwegii* (Lindl), and to contrast the coexistence of the colonizing groups associated with three conservation-disturbance scenarios that exemplify the situations of vulnerability faced by a forest ecosystem located in the Municipality of Isidro Fabela, State of Mexico.

MATERIALS AND METHODS

Location of the Study Area

The study area is located in the municipality of Isidro Fabela in the northwestern region of the State of Mexico. The municipal seat is located at 19° 34′ 32″ N and 99° 25′ 48″ W (Municipality of Isidro Fabela, 2016). Three sampling sites were established at an average altitude of 3100 m, within the Otomí-Mexica Natural Protected Area and the Bosque de Agua, a region of high biological and water value (ECOBA, 2012; Villegas-Martínez, Gutiérrez, and Juan, 2019).

For the study, soil in the disturbed scenario (P), logging zone; conserved (C), and reforested (R) were considered according to their conditions. Each site was referenced with a geopositioner (GarmineTrex® 10) and a digital map was produced with ArcMap 10.8 software (Esri, 2020), (Figure 1).

Sample Collection

In each study area, three representative herbaceous species abundant in the understory were chosen: *Acaena elongata* L., *Senecio angulifolius* (DC.) H. Rob. & Brettell and *Trisetum* sp., identified through taxonomic keys (Rzedowski and de Rzedowski, 2005) and confirmed in the IZTA herbarium of the Facultad de Estudios Superiores Iztacala, UNAM and the Herbario Nacional de México (MEXU), Instituto de Biología, UNAM.

The soil of the rhizospheric area was collected at a depth of less than 20 cm and corresponding to 5 plants with an approximate separation of 20 meters between them. The soil sample was homogenized and a total of 500 g per study area was obtained and preserved in airtight polyethylene bags at 4 Degrees Celsius.

The roots were dug up at depths of 5 to 30 cm depending on the plant collected. They were stored in polyethylene bags and kept at 4 °C until processing.

Determination of Soil Physical and Chemical Properties

From each of the sampled sites, 300 g of a sample composed of five rhizospheric soil subsamples were taken according to (Avellaneda-Torres and Torres-Rojas, 2015).

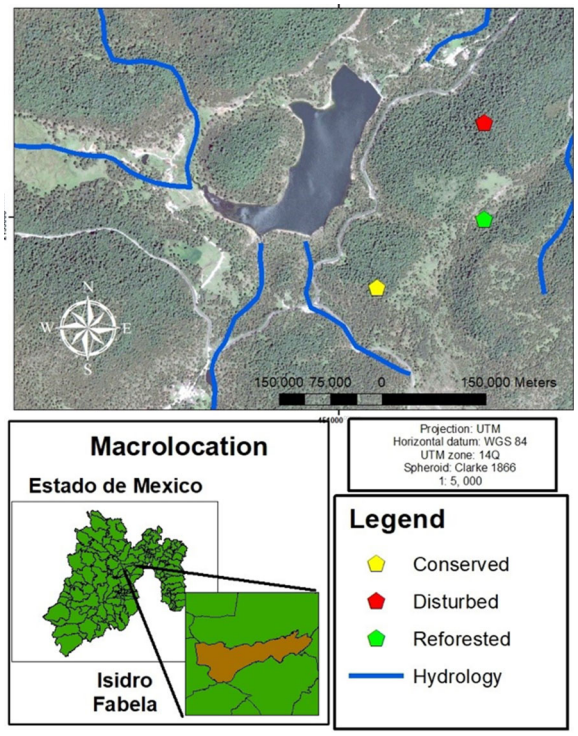

Figure 1. Location of sampling sites.

Texture was determined by the Bouyoucos (1962) method. Electrical conductivity (EC) by the conductivity meter method (Muñoz-Iniestra, Mendoza, López, Soler, and Hernández, 2002[1]). The percentage of organic matter (OM) was calculated according to the Walkley and Black (1934) method. Calcio (Ca), Magnesio (Mg), Potasio (K), Sodio (Na), by atomic absorption extraction, assimilable phosphorus by the method of Bray and Kurtz (1945), pH with a multiparametric meter with a penetration electrode (HI 9813-6N). Cation exchange capacity (CEC) using the Versenato technique and total nitrogen by the micro-Kjeldahl method (Muñoz-Iniestra *et al*., 2002). All physical and chemical parameters studied were determined according to standardized techniques (Muñoz-Iniestra *et al*., 2002; Mireles, 2009).

Phosphatase activity in soil by incubation of soil samples (1 g) with Universal Buffer (0.17 M), adjusted to pH 5.5 and 9.0, for the determination of acid (AcP) and alkaline (AlP) phosphatases, respectively (Gómez-Guiñán, 2004); 1 ml of a p-nitrophenyl phosphate solution (0.025 M) was used as substrate. To avoid dispersion of the clays and to extract p-nitrophenol (pNP) from the soil, 1 ml of CaCl (0.5M) and 4 ml of NaOH (0.5 M) were used, respectively. The mixtures were centrifuged at 3000 g/10 min and filtered through Whatman No 2 paper. The formation of pNP was determined spectrophotometrically at 400 nm (Tabatabai and Bremner 1969; Gómez-Guiñán, 2004). All parameters were quantified in triplicate.

Estimation of Colonization

To estimate the percentage of AMF and DSEF colonization, the roots were thinned with 10% KOH for one hour in a water bath (60 °C), acidified with 10% HCl/10min, stained with 0.05% trypan blue/10 min and rinsed with 10% lactic acid/10min modified from Phillips and Hayman (1970). Subsequently, 15 root fragments of 1 cm length were mounted in duplicate on slides with 50% lactoglycerol for analysis under a compound light microscope (Olympus® BX60) at 40x and 100x.

The level of mycorrhizal colonization (M%) of each root fragment and the abundance of vesicles and arbuscules (A%) were estimated. Observations of septate hyphae, melanized hyphae, vesicles, and microsclerotia of the dark septate endophytic fungal type were recorded. Calculations were made through the technique and the computer program "Mycocalc" (Trouvelot, Kough, and Gianinazzi, 1986).

Spore Quantification

The recovery and counting of AMF spores were performed by the method of wet sieving and decanting followed by centrifugation in sucrose solution, modified from Brundrett, Bougher, Dell, Grove, and Malajczuk (1996). For this, 50 g of weather-dried soil was weighed and placed in a glass container of 1 L capacity to which water was added, shaken vigorously, allowed to stand for 40 s and decanted on two sieves with mesh openings of 280 µm and 60 µm, this was repeated for five times. Only the material retained in the second sieve was transferred, shaken, and decanted once more, to be placed in centrifuge tubes and centrifuged at 2800 rpm for 4 minutes, the supernatant was decanted and passed through a 40 µm filter to be centrifuged again at 2800 rpm for 4 minutes. The supernatant was decanted and passed through a 40 µm sieve, the sample was washed to remove sucrose and placed in a Petri dish for examination under a stereo microscope (Motic® SMZ-143).

Statistical Analysis

Rhizospheric soil data from the three study areas were subjected to a one-factor analysis of variance (ANOVA). When there were significant effects, means were compared using a Tukey DHS test ($P \leq 0.05$). AMF and DSEF composition between areas was compared using the similarity index by Euclidean distances (Bautista-Cruz, Montaño, Camargo, and Pacheco, 2014) where the index equals one when there is complete similarity.

Statistical relationships between enzymatic activity, physico-chemical variables, spore abundance, AMF root colonization, and the presence of DSEF structures over the three sampled sites were determined by principal component analysis (PCA). The free software R Studio versión 4.2.2 was used for both analyses (R Core Team, 2020).

[1] Muñoz-Iniestra, D. J., Mendoza, C. A., López, G. F., Soler, A. A., & Hernández, M. M. M. (2002). *Edafología Manual de Métodos de Análisis del Suelo*. México: FES Iztacala−UNAM.

RESULTS AND DISCUSSION

Chemical and Physical Characterization of the Substrates

The soil type for the three study areas is an andosol, with a generally sandy texture, average bulk density of 0.82 g cm^{-3}, and average porosity of 57.3% (Table 1). These edaphic values coincide with those reported for andosol soil with oyamel forest cover (INEGI, 2017; Pérez-Miranda, Moreno, González, and Arriola, 2014). Of the three study sites, the conserved soil (C) is distinguished by a lower number of sand particles, a higher amount of silt, lower bulk density, lower porosity (not very different from the average), and higher electrical conductivity, which can be translated into a higher interstitial water retention area, gas exchange, permeability and better organic and mineral conditions along the soil profile (Martínez-Cruz, Carcaño, and López, 2002; Meza-Pérez and Geissert, 2003).

The chemical and nutritional traits of the studied soils share very acidic pH values (average < 5), very rich in organic matter (average 8.3%), and low cation exchange capacity (average 7.25 cmol kg^{-1}).

In particular, the preserved soil is distinguished from disturbed and reforested soils by its higher amount of organic matter, total nitrogen, inorganic phosphorus, and alkaline phosphatase activity (Table 2). These properties are typical and particular to andosol: very rich in organic content, high water retention, and high fixation of non-bioavailable phosphorus (FAO, 2015). In general, soil subjected to anthropogenic pressure will have a lower organic matter content and a lower water retention capacity, directly associated with its quality (Cruz-Flores, Guerra, Valderrábano, and Campo, 2020).

The strong phosphate fixation of andosols is associated with their richness in free Al and Fe minerals retained in high humus-aluminum complexes. These minerals originate from a weatherable silicate clay (allophane), of low activity and is very frequent in andosol textures that usually originates low cation exchange capacity (FAO, 2015), as observed in the soils studied.

Mycorrhizal Colonization

AMF structures identified on roots (Figure 2) were restricted to coenocytic hyphae and vesicles; these differed in morphology, thus, according to Di Barbaro *et al*. (2017), inferring the presence of different general of native mycorrhizal fungi. On the other hand, septate hyphae were observed, as well as numerous melanized or non-melanized microsclerotia and moniliform cells of the DSEF type. Double colonization is shown in the roots of the three herbaceous species studied, confirming a tripartite interaction between plants, DSEF, and AMF; fungal groups identified as some of the most important and widespread colonizers of plant roots (Della-Monica, Saparrat, Godeas, and Scervino, 2015).

The three herbaceous plants studied for colonization by HMA and DSEF (Figure 3) are dominant plant species in the understory of the study area, so they are responsible for the composition and structure of the Abies religiosa and Pinus hartwegii communities (Rzedowsky and de Rzedowsky, 2005; Beltrán-Nambo *et al*.,2018).

Figure 3 shows that the environment significantly influenced the colonization of the three plant species ($P \leq 0.05$). In addition to that plant identity is a factor affecting mycorrhization (Tukey $P \leq 0.05$).

Colonization values are high (> 50%), according to the criteria mentioned by Restrepo-Giraldo, Montoya, Henao, Gutiérrez, and Molina (2019), highlighting that the highest values in the three areas correspond to *S. angulifolius*, a genus considered strongly colonized (Cripps and Eddington, 2005), reaching figures of 35 to 73% (Chávez-Hernández, Barrera, Téllez, Chimal, and García, 2021). In *A. elongata*, mycorrhization close to 80% is consistent with that reported by Vázquez-Santos *et al*., (2019), while the percentage of vesicles of this species in the disturbed area (21.92%) is one of the highest recorded in the study locality.

Table 1. Physical characteristics of the soils sampled in the three study zones.

	Texture	Sand	Silt	Clay	Real density	Bulk density	Porosity	EC
		- - - - - - - - - - % - - - - - - - - - -			- - - - - - g cm^{-3} - - - - - -		%	dS m^{-1}
C	Sandy loam	51.1 c†	28.26 a	19.43 a	1.71 b	0.71 b	54.7 b	1.57 a
P	Sandy loam	67 a	18.53 b	12.37 b	2.16 a	0.84 a	62 a	0.47 b
R	Loamy silt	58 b	20.82 b	20.92 a	2.02 a	0.91 a	55.2 b	0.34 b

†Different letters indicate significant differences, according to Tukey's test ($P \leq 0.05$). NS = not significant.

Table 2. Chemical characteristics of the soils sampled in the three study zones.

	pH	MO	CICT	N tot	Pi	K	Na	Mg	Ca	AcP	AlP
	H^+	%	cmol (+) kg^{-1}	%	- - - - - - - - - - - - - -	mg kg^{-1}	- - - - - - - - - - - - - -			- - mg pNP $kgs^{-1}h^{-1}$ - -	
C	4.91 b	10.7 a	7.12 ns	0.89 a	20.72 a	500.66 a	236 a	180 a	17616 a	16.78 ns	20.43 a
P	4.46 c	5.8 c	7 ns	0.82 a	18.88 a	471.33 ab	228 ab	123.3 b	9586 b	19.38 ns	10.73 b
R	5.36 a	7.63 b	7.63 ns	0.54 b	5.51 b	392.66 b	199.3 b	88 c	480.6 c	15.69 ns	9.9 b

†Different letters indicate significant differences, according to Tukey's test ($P \leq 0.05$). NS = not significant.

According to Muñóz-Márquez *et al*. (2009), colonization levels are related to soil physical properties such as sandy loam texture, high OM level, and phosphorus and calcium ratio as promoters of root infection by arbuscular mycorrhizal fungi, so the conserved area presents the best conditions for the association.

The minimum percentage of colonization for a soil sample to serve as a biological indicator of quality and at the same time as AMF inoculum must be higher than 40% (Barriga, Visbal, and Acero, 2011; Restrepo-Giraldo *et al*., 2019). This requirement is fulfilled in the Rhizophora of the three plants at all sites.

Colonization by DSEF on herbaceous roots (Figure 3) differs from host to host in the disturbed area, while in the reforested area the difference in colonization (Tukey $P \leq 0.05$) seems to be due to species identity and microclimatic conditions related to the disturbance. Thus, the association between DSEF and hosts is a consequence of plant metabolism (Yan *et al*., 2019) and edaphic conditions (Heredia-Acuña, Alarcon, Cuevas, Ferrera, and Almaraz, 2014).

These endophytes seem to be related to altitude, as colonization has been recorded to be more intense at 3100-3200 meters of altitude. Although they are not dominant over AMF in the study area, their presence is inherent to the rigorous environmental conditions of a high mountain ecosystem. Suggesting that DSEF have an important role in the growth of plants inhabiting high altitudes, although their ecological relevance and function remain poorly understood (Heredia-Acuña *et al*., 2014).

Moniliform cells and microsclerotia have been described as storage structures that contain reserve substances and may participate in propagation and resistance in environments with adverse conditions (Heredia-Acuña *et al*., 2014; Reyes-Jaramillo, Chimal, Salmerón, Vázquez, and Varela, 2019). Although it is not possible to speak of a proportional relationship, their presence in all plants in the area disturbed by logging and in two species in the reforested area responds to the aforementioned pattern.

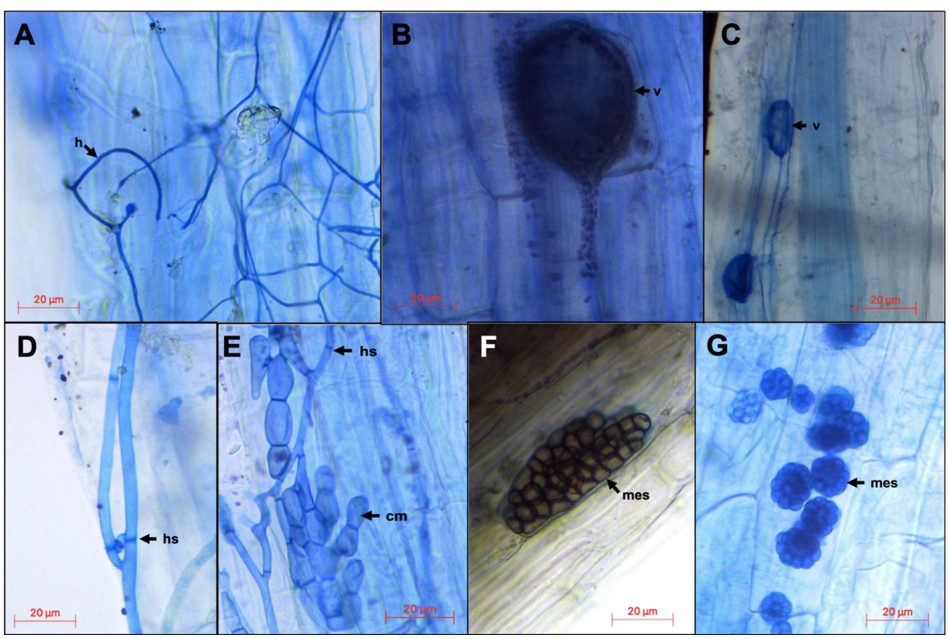

Figure 2. Root colonization structures. A-C: AMF colonization structures. H = coenocytic hyphae; v = vesicles. D-G = structures of colonization by DSEF. Hs = septate hyphae; cm = moniliform cells and mes = microsclerotia. 40x

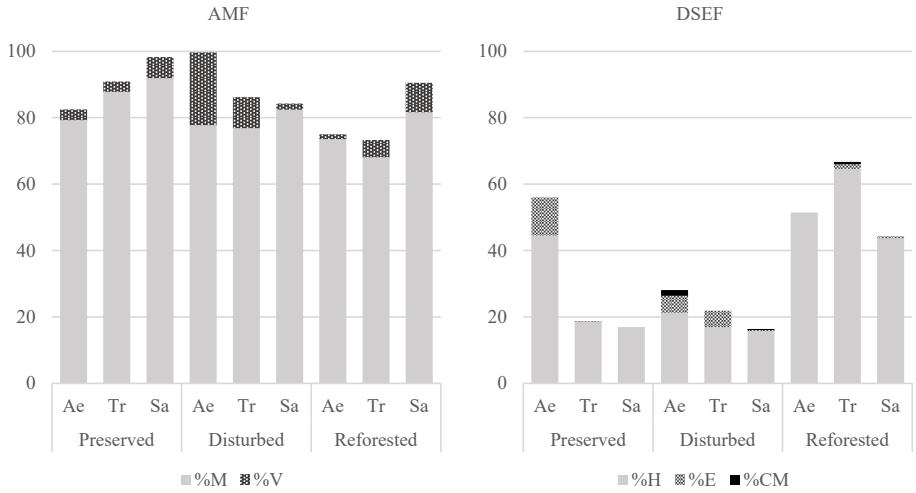

Figure 3. Intensity of mycorrhizal colonization and abundance of arbuscules by plant species and study area. Arbuscular mycorrhizal fungi (AMF), dark septate endophytes (DSEF), %M: cenocitic hyphae, %V: vesicles, %H: septate hyphae, %E: sclerotia. Ae = Acaena elongate; Tr = Trisetum sp; Sa = Senecio angulifolius.

Spore Abundance

The number of spores varied from 135 to 687 with significant differences among the environments studied. The highest number of spores was recorded in the reforested environment (Figure 4). It has been reported that AMF spore density is higher in areas with high recruitment of forest species, such as the reforested areas of this study (Figure 4), as well as in those with a certain degree of disturbance, which require the nutritional advantages offered by arbuscular mycorrhiza for the survival and establishment of plants (Olivera-Morales *et al*., 2011) and as an adaptive response by favoring the development of dormant structures of the organisms.

On the other hand, microclimatic differences in open areas of higher temperature and luminosity above the soil increase AMF spore production (Guadarrama and Álvarez, 1999).

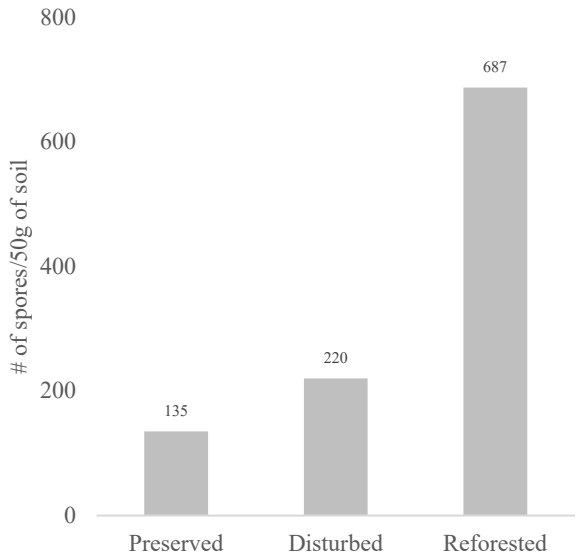

Figure 4. Average number of spores present in 50 g of soil.

Colonization Patterns

The PCA (Figure 5), whose axes explained 84.3% of the variance, indicates that colonization is related to edaphological conditions. The response of mycorrhizal symbiosis in the three plant species seems to be mediated by the proportions of Pi as also indicated by Pérez and Vertel (2010) and Vázquez-Santos *et al*., (2019), total N, Ca, and the amount of OM corresponding to the conserved area.

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The strong correlation between Pi and Ca is due to the phosphorus mineralizing capacity of mycorrhizae, through the production of phosphatase enzymes (acid and alkaline) that can solubilize calcium phosphates to a greater extent, thus contributing to the release of Pi (Peña-Venegas, Cardona, Arguelles, and Arcos, 2007; Della-Monica *et al*., 2015).

The development of AMF mycelium has mechanical effects on soil organic matter, forming aggregates of plant residues that maintain soil structure in optimal conditions (Lozano-Sánchez *et al*., 2015).

On the other hand, it is observed that other mycorrhizal structures, such as vesicles, develop in conditions conducive to aeration and moisture retention, which explains the distribution of these mycorrhizal structures (Pérez and De La Ossa, 2013), so a reduction in porosity, would negatively affect infection (Pérez and Vertel, 2010).

Since no correlation was observed between colonization percentages and the number of AMF spores, Bautista-Cruz *et al*. (2014) suggest that root infection occurred through mycelial fragments and colonized roots of adjacent plants and not only by spores.

One of the abiotic factors most related to sporulation is pH, since in strongly acidic soils, the development of these reproductive structures is favored (Peña-Venegas *et al*., 2007; Restrepo-Giraldo *et al*., 2019). Álvarez-Sánchez, Sánchez, Hernández, Hernández and Meli (2017) mention that acid soils and at the same time rich in organic matter are associated with increased sporulation, however, the results do not show such a clear coincidence for this phenomenon and this environmental variable.

In relation to plant-DSEF associations, Malicka, Magurno, and Piotrowska (2022) mention that these organisms produce a wide range of hydrolytic and oxidative enzymes that may explain their presence in difficult environments such as the reforested area. In addition, it is known that the positive, neutral, or negative behavior of endophyte colonization changes at different pH levels (Mayerhofer, Kernaghan, and Harper, 2013).

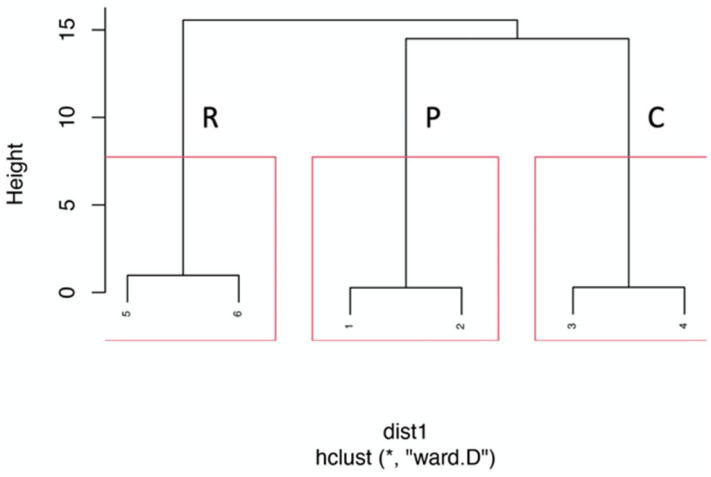

Figure 5. Similarity (Euclidean distances). Clusters according to the proportion of fungal colonization associated with the three types of land use evaluated.

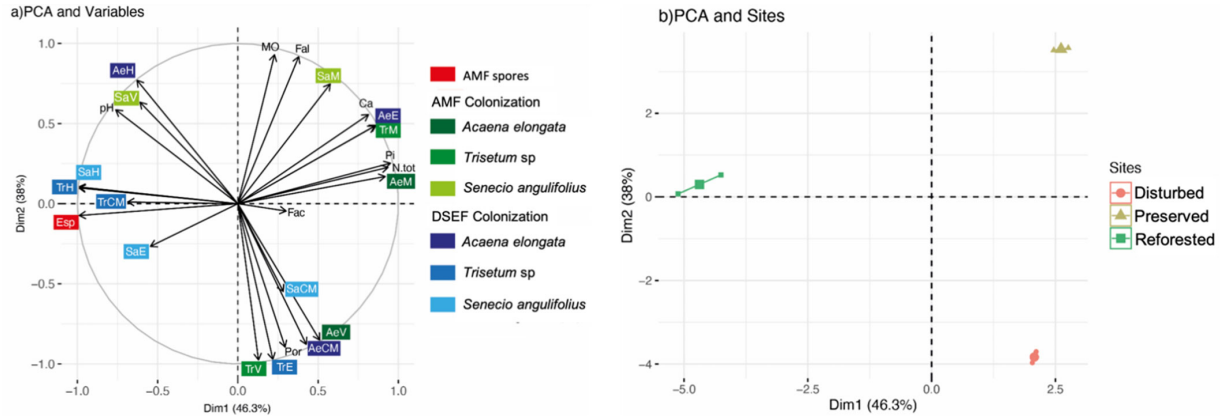

Figure 6. Principal Component Analysis (PCA). a) soil variables related to the number of spores (Esp), AMF colonization (M: percentage of mycorrhization; V: vesicles) and HESO (H: percentage of colonization; E: slrerotia and CM: moniliform cells) in each plant species and b) distribution by sampling sites.

CONCLUSIONS

The behavior of root-colonizing fungi is associated with plant species identity, soil parameters, and land use.

A dual colonization of plant roots by DSEF and AMF is recognized, however, reports showing these tripartite interactions are scarce, highlighting the need to integrate research on endophytic and mycorrhizal fungi in the rhizosphere.

These results contribute to the understanding of factors that may influence the distribution of AMF and DSEF in natural environments and may contribute to the design of management policies for conservation. The loss of ecological interactions and their functionality represents an ecological risk because the establishment and survival of forest communities would not be ensured in the long term.

ETHICS STATEMENT

Not applicable.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF SUPPORTING DATA

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FINANCING

Not applicable.

AUTHORS' CONTRIBUTIONS

Study conceptualization: A.G.M., J.L.G.F., M.J.F.G., and I.P.M.E. Development of the methodology: A.G.M., I.P.M.E., and S.C.H. Experimental validation: S.C.H., and I.P.M.E. Results analysis: A.G.M., J.L.G.F., I.P.M.E., and S.C.H. Data management: A.G.M., and I.P.M.E. Writing and draft preparation: A.G.M., and J.L.G.F. Redaction, revision and editing: A.G.M., I.P.M.E., J.L.G.F., and M.J.F.G., and S.C.H. fund acquisition: I.P.M.E., and M.J.F.G. All authors of this manuscript have read and accepted the published version of the manuscript.

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