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Medium-distance soil foragers dominate the *Pinus hartwegii* ectomycorrhizal community at the 3900 m Neotropical treeline

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Abstract

Pinus hartwegii is the only ectomycorrhizal host distributing at 4000 m asl in the Neotropics forming monospecific sky-island forests on the treeline. These ecosystems have unique environmental conditions like high solar radiation, dozens of soil freeze–thaw cycles per year, and high variability in daily temperatures; additionally their soils, as vitric andosols, are poor in nutrients and organic matter. In this extreme environment ectomycorrhizal fungi transform tree fine roots providing the host plant, through the plant-fungal symbiosis, with novel functions (enhanced nutrient acquisition, higher radiation and desiccation tolerance, etc.) improving tree establishment, growth, and survival. We studied the ectomycorrhizal community associated with *P. hartwegii* from three sites around 3900 m asl in the treeline of Cofre de Perote Volcano, Mexico. We dissected and characterized the mycorrhizal exploration type and morphological traits of mycorrhizae. Fungal identity and distribution were inferred by DNA sequence analysis of the ITS region. Soil conditions were determined by chemistry and macro and micronutrients contents. These fungal communities are characterized by low alpha diversity (less than ten species per site), high beta diversity (only two species shared between sites), and high dominance of Basidiomycota. Dominating genera *Tricholoma*, *Piloderma*, *Cortinarius* and *Gautieria* ectomycorrhizae belonged to the Medium-Distance exploration type, characterized by abundant external mycelia that often aggregates in mycelial cords and rhizomorphs. These forests constitute Holarctic sky-island refuges in the Neotropic where several potential endemic ectomycorrhizal fungi have evolved. Dominant ectomycorrhizal fungi are specialist soil foragers providing roots with new functions adapted to poor and harsh soil conditions.

Keywords Diversity · Treeline · Ectomycorrhizal roots · Neotropical ecozone · Sky islands · Soil

1 Introduction

The alpine-treeline ecotone is the transition zone from closed-canopy forest to treeless, alpine meadows. It consists of three zones: forest, timberline, and treeline. Treeline is the upper limit of tree growth, where trees form tree islands but are commonly reduced in growth with krummholz morphology (Hasselquist et al. 2005). The alpine-treeline ecotone is one of the most important climate driven ecological boundaries (Gerhard and Michael 2007) and is extremely vulnerable to global climate change (Wieser et al. 2010). However, in contrast to many studies of its vegetation cover, studies on its belowground biodiversity and function are scarce (Han et al. 2017). Ectomycorrhizal (ECM) fungi improve conifer survival and adaptation and are essential elements in treeline ecotone resilience (Reithmeier and Kernaghan 2013). In these ecotones young conifers experience limitations to carbon assimilation and might therefore be more critically

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affected by mycorrhizal associations than plants in other habitats (Hasselquist et al. 2005).

Alpine and subalpine ECM fungal communities have been studied predominantly in temperate or boreal regions within the Holarctic biological realm. However, tropical subalpine ecosystems are different from temperate or boreal ecosystems since they show high levels of solar radiation, low variability in the annual average temperature, high variability in daily temperatures, and dozens of soil freeze–thaw cycles per year (Cáceres et al. 2014). The combination of such environmental conditions has influenced the evolution of these high-altitude sites (> 4000 m) in tropical latitudes, characterized by native species that play a fundamental role in the structure of a community with a high degree of endemism (Cáceres et al. 2014; Sklenář et al 2014). Few studies focus on Neotropical subalpine ECM fungi diversity (Reverchon et al. 2010; Garibay-Orijel et al. 2013; Baeza-Guzmán et al. 2017), and they point out that these ecosystems have low ECM species richness in comparison to temperate or boreal communities (for e.g. Kjøller and Clemmensen 2009; Gao and Yang 2010; Han et al. 2017).

The mycelia of ectomycorrhizal fungi are the main soil exploration structures for nutrient and water transfer to ECM host trees and lastly are responsible for tree nutrition (Perez-Moreno and Read 2000; Smith and Read 2008). Up to 75% of phosphorus and 80% of nitrogen acquisition is facilitated to symbiont plants by mycorrhizal fungi (Simard et al. 2003; Hobbie and Hobbie 2006; Van der Heijden et al. 2008). Classification into exploration types is a widely used strategy to infer functional traits of ECM fungi and to assess their impact on ecosystem functioning (Agerer 2001; Hobbie and Agerer 2010; Tedersoo et al. 2012; Koide et al. 2014; Rosinger et al. 2018). However, knowledge of these functional traits is restricted to dominant ECM fungal taxa. Moreover, our understanding about functional traits and exploration type patterns on a community-level is still poor and requires further attention (Rosinger et al. 2018).

Here we studied the ECM community associated with *Pinus hartwegii* fine roots in the upper forest limit from Cofre de Perote Volcano within the Transmexican Volcanic Axis. This pine species inhabits the alpine-treeline ecotone in central Mexico with an altitude range between 3500 and 4200 m, forming monospecific forest sky-islands with adaptation to low temperatures (Perry 1991; Challenger and Soberón 2008). It is an ecologically important species since it grows at the highest elevations in the tropics and has a protective function safeguarding wildlife; it dampens environmental pollution effects and contributes as regulator of water produced by mountain glaciers meltdown (Solís and Musálem 1994). The Trans-Mexican Volcanic Belt (TMVB) is a continental magmatic arch constituted by around 8000 volcanoes (Gómez-Tuena et al. 2005); it divides Mexico in half and constitutes the physical frontier between the Nearctic and Neotropical biological realms. The volcanic activity that conformed the volcanic axis started 19 million years ago in the mid Miocene and ended around 1 million years ago in the Quaternary, however it is still active in some areas (Gómez-Tuena et al. 2005). Cofre de Perote Volcano was built in three phases, the first one 1.3 million years ago, the second 0.4 million years ago, and the third one 0.24 million years ago (Castellón et al. 2008). In consequence, it was fully built by the time of the last glaciation 0.11 million years ago. Given its complex biogeographical history, the TMVB is acknowledged as a biogeographic transition zone and diversification and endemism center (Gámez et al. 2012).

As *P. hartwegii* tree line develops in poor soils and very harsh environmental conditions, our hypotheses were that: the ECM fungal community associated to *P. hartwegii* will have low richness and will be dominated by species with abundant external mycelia specialized in nutrient transfer to their host. In consequence, our objectives were: to analyze the diversity, distribution and community structure of ECM fungi associated to *P. hartwegii* alpine forests and characterize the functions that they provide to *P. hartwegii* fine roots through its mycorrhizas. We found that these forests constitute Holarctic sky-island refuges in the Neotropic where several potential endemic ECM species have evolved. Dominant ectomycorrhizal fungi, mainly Basidiomycetes, are nutrient foraging specialists equipped with abundant external mycelium with a great capacity to explore the soil, providing roots with functions adapted to poor and harsh soil conditions.

2 Materials and Methods

2.1 Study site

Cofre de Perote is an extinct volcano 4150 m high located in the central region of the Mexican state of Veracruz (19° 29′ 39" N, 97° 08′ 53" W). Above 3000 m vegetation is composed mainly by *Pinus ayacahuite*, *P. hartwegii*, *P. montezumae*, *P. pseudostrobus* and *P. teocote* coniferous pine forests. In the highest part of the mountain (3600–4000 m), *P. hartwegii* establishes monodominant stands in vitric and humic andosol soil. Due to the harsh environmental conditions at high altitude (Vázquez-Ramírez 2014), these forests are characterized by an open canopy (Rzedowski 2006) and low tree density (670 individuals/ha). This species shows low seedling recruitment and fire dependence to regenerate its populations (Sarukhán and Franco 1981). The total dominance of *P. hartwegii* in the canopy layer is due to its characteristic high tolerance to low temperatures with extremely high solar radiation and many freezing-melting cycles throughout the year (Viveros-Viveros et al. 2007; 2009). In this region, three sampling sites were randomly selected. These sites represent the conifer treeline, where *P. hartwegii* is the only ECM host. Site 1 is 3860 m high, its slope faces N, it is composed by mature trees with some natural regeneration; the soil has a shallow O horizon. Site 2 has 3890 m high, it has a deep slope facing W and is affected by strong winds and erosion; in consequence, trees have krummholz morphology and soil lacks O horizon. Site 3 is 3620 m with a shallow slope facing E, trees are mature, tall, and soil is well developed with thick O horizon. Site 2 is 1150 m NE away from Site 1, and Site 3 is 1670 m NE away from Site 2, Site 3 is 2800 m NE away from Site 1 (Fig. S1).

2.2 Root collection

To represent 0.6 ha in each sampling site we defined 4 transects 400 m long over the same altitude separated from each other by 50 m. In each transect we established 5 sampling points every 100 m. At each sampling point, 3 soil cores were collected separated 50 cm from each other removing the thin organic layer and collecting the mineral horizon. For this, 15 cm long, sharp-ended PVC tubes with 1.5 cm diameter were used. The 3 soil samples of each point were mixed to obtain approximately 750 g. In total 60 soil samples were collected from 3 sites × 4 transects × 5 points. The samples were cooled and transported to the laboratory. Within one or two days they were sieved through a 2.0 mm mesh; then roots were washed with running water. All fine roots were examined and cleaned under a stereoscopic microscope (Stemi DV4) sorting them into morphotypes according to Baeza-Guzmán et al. (2017). One representative of each OTU by sample was placed in Eppendorf tubes with 2% CTAB at 4 ºC to preserve the DNA.

2.3 DNA Sequencing and analyses

Mycorrhizal DNA extraction, PCR amplification and sequencing followed the procedures detailed by Garibay-Orijel et al. (2013). Briefly, DNA extraction was performed using the kit XNAP (Sigma-Aldrich). PCR was performed with the Ruby Taq PCR Master Mix (Affymetrix) with ITS1F and ITS4 (Gardes and Bruns 1993). Good-quality amplicons were cleaned with ExoSAP-IT (ThermoFisher). Then, they were sequenced in both directions with same PCR primers in an ABI3100 genetic analyzer (Applied Biosystems) in the "Laboratorio de Secuenciación Genómica de la Biodiversidad y la Salud" at the "Instituto de Biología, UNAM". DNA sample sequences were edited (trimming and base call review) and forward and reverse sequences assembled into contigs; then, contig sequences were grouped into operational taxonomic units (OTUs) at 98% nucleotide similarity in Geneious 10.0.2. Only high quality (HQ > 90%) sequences at least 500 pb were considered in analyses. To assign taxonomic identity to each OTU, we conducted a BLAST search in the UNITE and NCBI databases, this results have been already published in Baeza-Guzmán et al. (2017). Sequences with more than 98% nucleotide similarity were considered conspecific. Metadata of conspecific sequences from published papers were analyzed to determine ECM fungi potential distribution and associated hosts (Bonito et al. 2010; Tse-Laurence and Bidartondo 2011; Avis 2012; Looney et al. 2016). The potential endemism of species is based on the rationale of García-Guzmán et al. (2017) as follows. We used the term "potential endemic" which includes endemic species and species with broad distributions that may not be represented in public databases. For this, we used UNITE and GenBank searches with BLAST; if the higher best match for an OTU was < 98%, we assumed that it is not common and widespread, it is not distributed in the Northern Hemisphere, and in consequence, it is potentially endemic (García-Guzmán et al. 2017).

The most representative sequence of each OTU was deposited in the nucleotide database of GenBank under accession numbers KU871223-KU871244. Once OTUs were assigned with a taxonomic identity they were treated as species.

2.4 Soil data collection

To demonstrate the low nutrient content and soil heterogeneity in the area we conducted physicochemical soil analysis in each sampling site. At each sampling point, a second soil sample was collected using 15 cm long sharp-ended PVC tubes with a 1.5 cm diameter. Samples of each transect were mixed to obtain a total of 1 kg of soil. Samples were transported in Ziploc bags, and dried them at room temperature for three days. The physicochemical properties of the soil samples (pH, soil organic matter (SOM), inorganic N, extractable P, interchangeable K, interchangeable Ca, extractable Mg, interchangeable Fe, and interchangeable Zn) were obtained for each transect in the "Laboratorio de Fertilidad del Suelo y Nutrición Vegetal" at the "Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP)".

2.5 Ecological Analyses

Below ground diversity was assessed by computing species richness and abundance and then calculating Shannon-Wiener diversity index (H') and Simpson diversity index (1/D). Computations were performed using EstimateS version 8.2.0 (Colwell 2019). To demonstrate the total ECM fungal diversity of the study site and to estimate the sufficiency of sample size, a species accumulation curve was calculated. Chao2 and Jacknife2 were used to estimate species richness. To assess ECM community structure associated to *P. hartwegii* fine roots relative abundance (species number of root tips divided by the total root tips successfully sequenced) and relative frequency (species number of transects present divided by the absolute frequency sum of all species) were computed for each ECM species. The morphological traits of ectomycorrhizae were analyzed according to Agerer (2001) and classified their exploration types as contact, short-distance, medium-distance, and long-distance exploration types and subtypes.

For each site we computed the average N, P, K, Ca, Cu, Fe, Mg, Zn, SOM content and pH using data from the four transects. As data have non-normal distribution we looked for significant differences in soil properties by sampling sites with a non-parametric Kruskal–Wallis test. Pearson correlations were conducted to assess the relationship between edaphic factors and ECM species richness; additionally a Non-Metric Multidimensional Scaling (NMDS) was performed in R to summarize differences in transect soil composition across three sites.

3 Results

3.1 *Pinus hartwegii* ectomycorrhizal fungi diversity, community structure and distribution

In total, we sequenced successfully 116 mycorrhizal root tips belonging to 22 ectomycorrhizal species associated to *P. hartwegii* fine roots. These species corresponded to 13 genera and 12 families. The families with the higher number of species were Atheliaceae (5 species) and Cortinariaceae (5 species). Site 2 showed the lowest richness with 7 species, while sites 1 and 3 had 8 and 9 species respectively. Sites 2 (H' 1.92, 4.47 1/D) and 3 (H' 2.2, 5.99 1/D) showed the highest H' and 1/D diversity indexes (Table 1). The species accumulation curve did not reach a plateau when all sampling sites were randomly resampled. Individual site accumulation curves have attenuated slopes depicting the low alpha diversity of each site (Fig. S2). Chao2 estimated a total richness of 64.2 species and Jackknife2 estimated a total richness of 41.2 species (Fig. S3). Site 1 had 7 unique species, while Site 2 had 5 and Site 3 had 8 unique species. We did not find shared species between Sites 1 and 3, and we did not find any EM fungal species present at all three sites (Table 2).

Most abundant species were *Tricholoma equestre* complex (28% of mycorrhizas), *Gautieria* sp. 1 (18%), *Piloderma* sp. 2 (13%), and *Cortinarius* aff. *colymbadinus* (10%) (Fig. 1). *Tricholoma equestre* complex was dominant at Sites 1 and 2, while *Gautieria* sp. 1 was the dominant species in Site 3. Accordingly, the most frequent taxa were *Tricholoma equestre* complex (present in 9 transects), *Piloderma* sp. 2 (8 transects), *Gautieria* sp. 1 and *Cortinarius* aff. *colymbadinus* (5 transects each) (Table 2).

Fourteen percent of the ECM fungi found associated with *P. hartwegii* have never been sequenced elsewhere (*Lyophyllum* sp. 1, *Pseudotomentella* sp. 1, and *Hygrophorus* sp. 1). DNA sequences of fourteen percent of species (*Russula* aff. *betularum*, *Hydnellum* sp. 1, and *Sistotrema* aff. *confluens*) are only known from the conifer high mountain forests of the Trans-Mexican Volcanic Belt. Other species (32%) like *Clavulina* sp. 1, *Cortinarius* sp. 1, *Gautieria* sp. 1, *Piloderma* sp. 1, 2, 3, and *Russula* sp. 1 have been found previously through North America. In contrast, most species (36%) (*Byssocorticium* sp. 1, *C. mucosus*, *C.* aff. *colymbadinus*, *C.*

Table 1 Ectomycorrhizal fungi diversity and edaphic factors in Cofre de Perote National Park *Pinus hartwegii* forests

Variable	Site 1	Site 2	Site 3	*p*-value
Shannon's diversity index (H')	1.5	1.92	2.2	
Simpson's diversity index (1/D)	3.55	4.47	5.99	
Observed richness	8	7	9	
pH in water (1:2)	3.97 ± 0.004*	4.35 ± 0.004	4.76 ± 0.004	0.035
Soil organic matter (SOM %)	23.27 ± 0.003	5.52 ± 0.003*	25.87 ± 0.005	0.046
N inorganic ($NH_4^+ + NO_3^-$) (mg/kg)	22 ± 0.24	9 ± 0.2*	17 ± 0.24	0.030
P extractable (Bray P1) (mg/kg)	59 ± 0.24	0.68 ± 0.003*	17 ± 0.23	0.05
K interchangeable (mg/kg)	389 ± 0.24	75 ± 0.31*	259 ± 0.40	0.042
Ca interchangeable (mg/kg)	1620 ± 0.24	596 ± 0.31*	2538 ± 0.37	0.052
Mg extractable (mg/kg)	160 ± 0.37	59 ± 0.20*	171 ± 0.04	0.043
Fe interchangeable (mg/kg)	62 ± 0.04	25 ± 0.037*	51 ± 0.037	0.033
Zn interchangeable (mg/kg)	5.5 ± 0.031	0*	4.9 ± 0.004	0.05
Cu interchangeable (mg/kg)	0.54 ± 0.003	0.22 ± 0.003*	0.42 ± 0.003	0.053
Mn interchangeable (mg/kg)	46.06 ± 0.04	3.4 ± 0.031*	35.06 ± 0.04	0.041

Kruskal–Wallis test comparing the physicochemical properties of the soil. Significant differences between sites according to the pairwise comparison are denoted with an asterisk

Table 2 *Pinus hartwegii* ectomycorrhizal fungi exploration type, abundance and frequency

Species	E. Type	Site 1 AAb	Site 2 AAb	Site 3 AAb	Total AAb	Total RAb	Total AFr	Total RFr
Tricholoma equestre complex	MDfe	6	27	0	33	0.28	9	0.15
Gautieria sp. 1	MDme	0	0	21	21	0.18	5	0.08
Piloderma sp. 2	MDfe	0	4	11	15	0.13	8	0.13
Cortinarius aff. *colymbadinus*	MDfe	0	12	0	12	0.10	5	0.08
Byssocorticium sp. 1	MDfe	3	0	0	3	0.03	3	0.05
Piloderma olivaceum	MDfe	3	0	0	3	0.03	3	0.05
Clavulina sp. 1	Ce	2	0	0	2	0.02	2	0.03
Cortinarius diasemospermus	MDfe	0	0	3	2	0.02	2	0.03
Cortinarius mucossus	MDfe	0	2	0	2	0.02	2	0.03
Cortinarius sp. 1	MDfe	2	0	0	2	0.02	2	0.03
Cortinarius sp. 2	MDfe	0	2	0	2	0.02	2	0.03
Hydnellum sp. 1	MDme	0	0	2	2	0.02	2	0.03
Lyophyllum sp. 1	MDfe	0	0	2	2	0.02	2	0.03
Pezizomycotina sp. 1	SDe	0	0	2	2	0.02	2	0.03
Piloderma sp. 1	MDfe	2	0	0	2	0.02	2	0.03
Piloderma sp. 3	MDfe	0	0	2	2	0.02	2	0.03
Pseudotomentella sp. 1	MDse	0	0	2	2	0.02	2	0.03
Serendipita vermifera	Ce	2	0	0	2	0.02	2	0.03
Sistotrema aff. *confluens*	SDe	2	0	0	2	0.02	2	0.03
Hygrophorus sp. 1	SDe	0	1	0	1	0.01	1	0.02
Russula aff. *betularum*	Ce	0	1	0	1	0.01	1	0.02
Russula sp. 1	Ce	0	0	1	1	0.01	1	0.02

E. Type Exploration type; *MDfe* Medium-distance fringe exploration type; *MDme* Medium-distance mat exploration type; *Ce* Contact exploration type; *SDe* Short-distance exploration type; *MDse* Medium-distance smooth exploration type; *AAb* Absolute abundance; *RAb* Relative abundance; *AFr* Absolute frequency; *RFr* Relative frequency

diasemospermus, Pezizomycotina sp. 1, *Piloderma olivaceum*, *Serendipita vermifera*, and *Tricholoma equestre* complex) are widely distributed in the Holarctic and are mainly associated with alpine and boreal forests (Table S1).

3.2 Exploration types of ectomycorrhizae associated with *P. hartwegii* fine roots

Most ectomycorrhizal fungi (68.2%) in *P. hartwegii* roots developed Medium-distance exploration type ectomycorrhizas (Fig. 2). The most common exploration subtype was Medium-distance fringe exploration represented by *Cortinarius diasemospermus, C. mucossus, Cortinarius* sp. 1, 2, 3, *Lyophyllum* sp. 1, *Piloderma olivaceum, Piloderma* sp. 1, 2, 3, *Byssocorticium* sp. 1, and *Tricholoma equestre* complex. While *Gautieria* sp. 1 and *Hydnellum* sp. 1 produced the Medium-distance mat exploration type and *Pseudotomentella* sp. 1 Medium-distance smooth exploration type. The Contact exploration type was produced by 18.2% of ectomycorrhizal fungi (*Clavulina* sp. 1, *Serendipita vermifera, Russula* aff. *betularum,* and *Russula* sp. 1). Just 13.6% of species developed Short-distance exploration type (*Hygrophorus* sp. 1, *Sistotrema* aff. *confluens,* and Pezizomycotina sp. 1) (Table 2).

3.3 Physicochemical soil properties and mycorrhizal types distribution

The Kruskal–Wallis test showed that all evaluated soil variables were significantly different between the three sites (Table 1). The NMDS analysis demonstrated that sampling sites are separated by pH ($r^2 = 0.973$, $P = 0.001$) and P extractable ($r^2 = 0.384$, $P = 0.05$) (Fig. S4). Site 1 showed higher soil concentrations of macronutrients (N, P, K) and micronutrients (Fe, Zn, Cu, Mn). Site 2 showed the lowest concentrations of all the elements evaluated. Site 3 showed the highest values of pH, SOM, Ca, and Mg. Only P was an explanatory variable negatively correlated with EMC fungal richness ($r^2 = -0.58$, $P = 0.023$). ECM of medium distance exploration type was the most abundant in sites 2 and 3 where inorganic N and extractable P are scarce, in particular it accounted for 95.9% of all mycorrhizas in site 2. In contrast, site 1 with the higher contents of

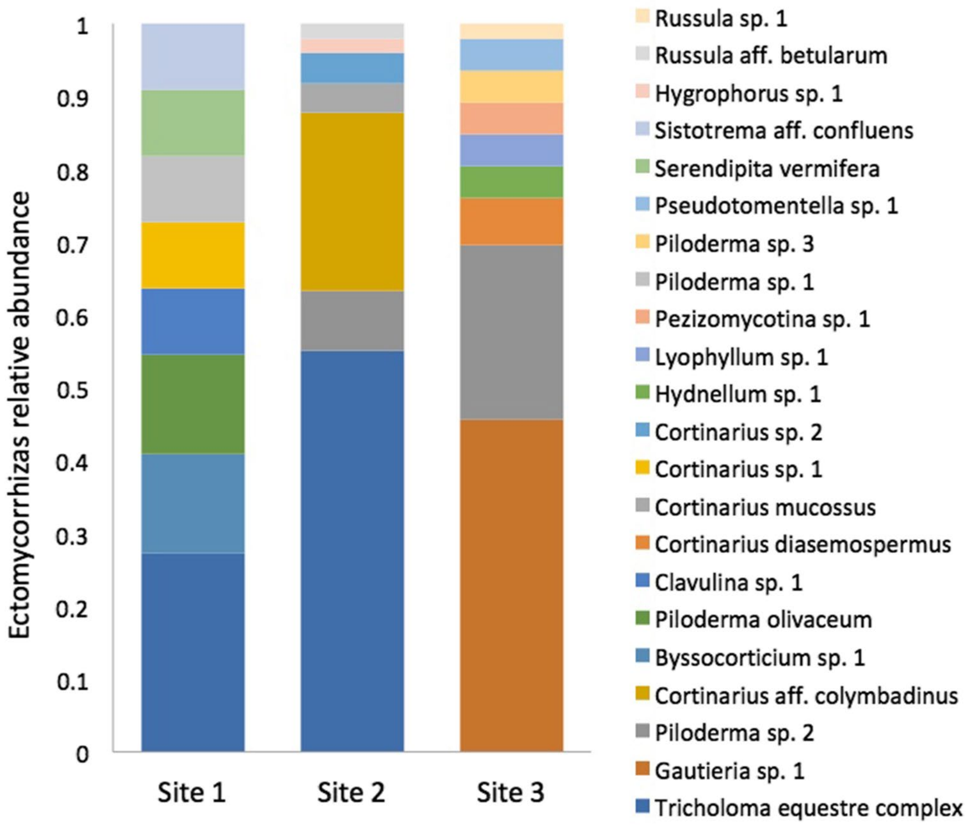

Fig. 1 Relative abundance of ectomycorrhizal fungal species associated to *P. hartwegii* fine roots in Cofre de Perote Volcano

N and P had the lowest proportion of medium distance exploration type (Fig. 3).

4 Discussion

4.1 ECM fungi diversity in alpine *P. hartwegii* forests in the Neotropic

Tropical alpine forests in the Cofre de Perote are characterized by lower ECM richness than temperate forests but high beta diversity (turnover) and potential endemism. We found just 22 ECM fungi associated with the roots of *P. hartwegii*. Even while this low richness could be a result of sampling, in fact, *P. hartwegii* forests have the lowest richness of soil fungi and mycorrhizal fungi among coniferous forests in the Trans-Mexican Volcanic Belt (Argüelles-Moyao and Garibay-Orijel 2018).

In alpine areas of North America, Europe, and Asia *Cortinarius*, *Russula*, *Lactarius*, and *Tomentella* are usually the dominant and richest ECM genera (Gardes and Dahlberg 1996; Mühlmann and Peintner 2008; Gao and Yang 2010; Koizumi et al 2018). In Cofre de Perote, *Cortinarius* and *Piloderma* had the highest richness. *Cortinarius* is a common genus, often a key component in ECM communities of boreal forests (Lindahl et al. 2010); while *Piloderma* is a mat-forming fungus common in temperate to boreal forests in western North America (Dunham et al. 2007; Trappe et al. 2012). Heinonsalo et al. (2015) and Sun et al. (2015) reported *Piloderma* as dominant in Finland forests of *P. sylvestris*, where some species such as *P. olivaceum* have a great ability to produce protease and enzymes needed to transform organic soil resources. In Neotropical mountains of Mexico, Atheliaceae also has a high abundance in *Pinus* mycorrhizal roots (Reverchon et al 2010). *Piloderma* becomes dominant after fire events (Sun et al. 2015) as its resistant propagules have specific adaptations, persistence, and tolerance to high temperatures (Glassman et al. 2016). *Pinus hartwegii* is also fire tolerant and possesses several adaptations to survive it (Rodríguez-Trejo 2001); indeed, fire is fundamental for the regeneration of its populations (Sarukhán and Franco 1981). As both symbionts are adapted to fire events, the high frequency of the *P. hartwegii* + *Piloderma* spp. mycorrhizas is likely to be a key element for the persistence of these ecosystems.

4.2 Distribution and ecology of the ectomycorrhizal fungi associated with *P. hartwegii*

The complex ECM fungal community of *P. hartwegii* in the TMVB highlands results from a sky-island dynamic characterized by geographic isolation (0.24 M years) and climate fluctuations (already conformed by the last glaciation 0.11 M years ago). This dynamic in the TMVB allows in situ population

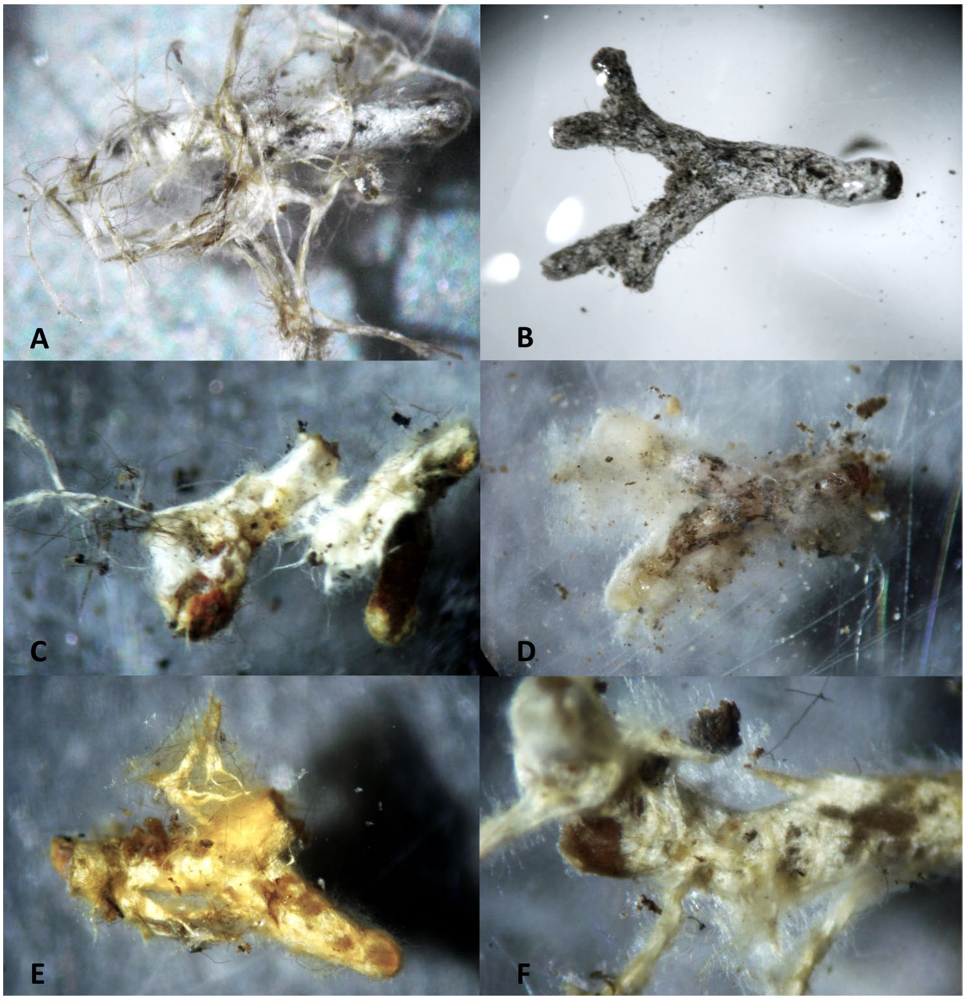

Fig. 2 Exploration types of dominant ectomycorrhizal fungi on *P. hartwegii* fine roots. **A** *Tricholoma equestre* complex Medium-distance fringe exploration type. **B** *Gautieria* sp. Medium-distance mat exploration type. **C** *Piloderma* sp. 2 Medium-distance fringe exploration type. **D** *Cortinarius* sp. 3 Medium-distance fringe exploration type. **E** *Tretomyces lutescens* Medium-distance fringe exploration type. **F** *Piloderma olivaceum* Medium-distance fringe exploration type

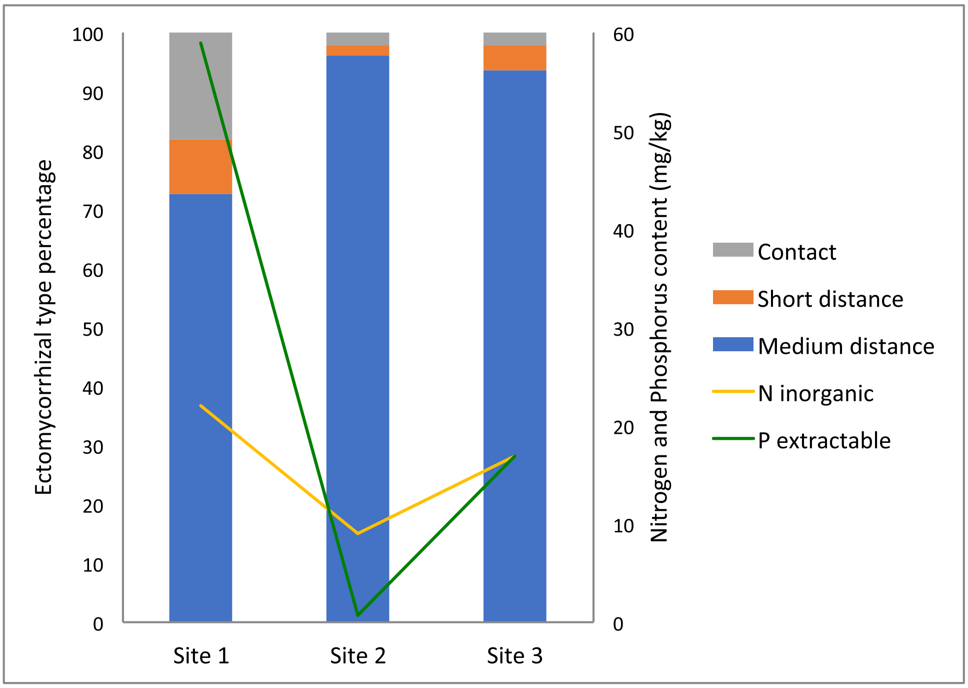

Fig. 3 Distribution of ectomycorrhizal exploration types within *Pinus hartwegii* tree line in Cofre de Perote Volcano. Left vertical axis depicts ectomycorrhizal type percentage and right vertical axis depicts inorganic N and extractable P content in (mg/kg)

persistence, particularly for boreal species, while promoting recent divergence and speciation events (Mastretta-Yanes et al. 2015). Thus, *P. hartwegii* forests constitute sky-island refuges for Holarctic ECM fungi in the Neotropic as well as a region where allopatric and parapatric speciation, driven by climatic and geological events, favors the potential endemicity of several fungal taxa. The ECM fungi associated with *P. hartwegii*, whose distribution is only known locally (Cofre de Perote volcano) or regionally (Trans-Mexican Volcanic Belt) (*Hydnellum* sp. 1, *Hygrophorus* sp. 1, *Lyophyllum* sp. 1, *Pseudotomentella* sp. 1, *Russula* aff. *betularum*, and *Sistotrema* aff. *confluens*), are only associated with pines. Their fruit bodies and/or mycorrhizae have only been sequenced in Mexican *P. montezumae* and *P. hartwegii* forests above 3000 m. Some of the continentally distributed species are particularly associated to Pinaceae, like *Clavulina* sp. 1, *Cortinarius* sp. 1, *Pilodema* sp.1, 3. Widely distributed species are generalists forming mycorrhizas with Pinaceae, Fagaceae, Salicaceae and Betulaceae; some of these like *Cortinarius* sp. 2, Pezizomycotina sp. 1, and *S. vermifera* even form arbutoid mycorrhizae. The species Pezizomycotina sp. 1, *Piloderma* sp. 1 and *S. vermifera* also establish mycoheterotrophic relationships with understory plants. Therefore available data suggest that specialist species have limited distributions being potentially endemic to the TMVB; while the widely distributed generalist Holarctic species (Koizumi et al. 2018) can establish in the TMBV highlands based on their ability to shift hosts becoming isolated in these refugees during interglacial periods.

4.3 *Pinus hartwegii* fine roots are dominated by Medium-distance exploration organic N soil forager specialists

The Cofre de Perote volcano soil is highly heterogeneous and nutrient poor. The three evaluated sites were significantly different in all macro and micronutrients (Table 1). In comparison with the normal ranges of soil nutrient content reported by Hossner (2008) Site 2 is extremely poor in all nutrients; while Site 1 and 3 are extremely acid and, even with higher concentrations, are still poor in N, P and Mg. These soils are particularly poor in N inorganic forms, with SOM being the main source of N. In these Neotropical alpine forests the genera with the most abundant ectomycorrhizas were *Tricholoma*, *Cortinarius*, *Piloderma*, and *Gautieria*; all them have Medium-distance exploration types with hydrophobic mycorrhizas and short rhizomorphs.

Overall, the ECM fungi with Medium-distance exploration type dominated the ECM community of *P. hartwegii*. Species with this exploration type accounted for 68.2% of richness, 90.5% of total abundance and 80.9% of total frequency of ectomycorrhizas (Fig. 4). This pattern was particularly evident in site 2, which is the most nutrient depleted (inorganic $N=9$ (mg/kg), extractable $P=0.68$ (mg/kg). As this site is highly eroded and lacks an O soil Horizon, organic forms of N and P are also scarce. In this site the medium distance exploration type was dominant (Fig. 3) and 71% of the ECM species present produced this specialized exploration type (Table 2). The Medium-distance is the richest exploration type present in the higher number of species across temperate and boreal forests in Europe, accounting for 43%- 51% of the ECM fungi species in *Fagus sylvatica*, *Picea abies* and *Pinus sylvestris* stands; however, in these same forests the numbers of its mycorrhizas are low with an abundance ranging from 4.7% to 5.9% (Rosinger et al. 2018). This exploration type, particularly those of *Tricholoma*, *Cortinarius* and *Piloderma*, is specialized in foraging organic N and becomes dominant in N poor forests (Trudell and Edmonds 2004; Hobbie and Agerer 2010; Lilleskov et al. 2011). Thus, the *P. hartwegii* fine roots are colonized by a set of specialized fungi that produce abundant extraradical mycelia capable of exploring poor soils and providing trees with the nutrients necessary for their development.

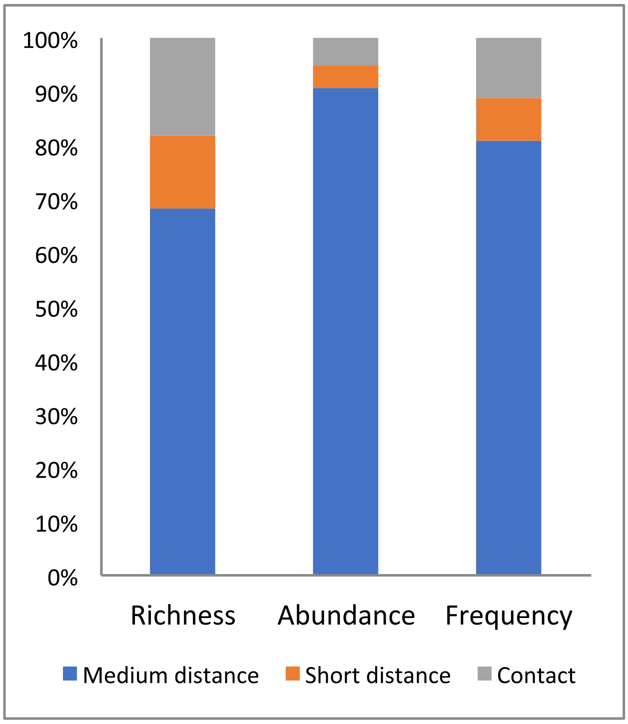

Fig. 4 Dominance of Medium-distance exploration type on the ectomycorrhizal community associated to *P. hartwegii* fine roots. Values are expressed as percentage of total richness, total abundance (number of root tips) and total frequency (number of transects)

5 Conclusions

The ectomycorrhizal community associated with the fine roots of *P. hartwegii* Neotropical alpine forest is characterized by low alpha diversity, high beta diversity (turnover), and high dominance of Basidiomycota. The fungi forming this community are a complex mix of Holarctic host generalists, North American Pinaceae specialists and potentially endemic fungi. This community is a result of a sky-island dynamic with geographic isolation and climate fluctuations that allow in situ population persistence of Holarctic species, while promoting recent divergence and speciation events. As a result, even while the ECM fungi richness of these ecosystems is low, there is a considerable unexplored fungal diversity of mushrooms with restricted distribution or potentially endemics.

As these ecosystems are constantly affected by fire and *Pinus hartwegii* and *Piloderma* spp. are fire tolerant, the symbiosis *P. hartwegii* + *Piloderma* spp. is a key element for the persistence of these ecosystems.

The Cofre de Perote volcano soil is highly heterogeneous and nutrient poor. There, *Tricholoma*, *Cortinarius*, *Piloderma*, and *Gautieria* formed the most abundant ectomycorrhizas; all them have Medium-distance exploration types. This exploration type is specialized in foraging organic N and becomes dominant in N poor forests.

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Authors' contributions RGO and RMO conceived the ideas and designed the study, YBG conducted the field and molecular work, all authors analyzed the data, YBG wrote the first draft of the manuscript; RGO, DTA and RMO commented on data analysis and interpretation. RGO prepared final version and all authors approved it.

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Data availability DNA sequences were deposited on GenBank.

Code availability Software used is cited in methods.

Declarations

Ethics approval No ethics approval needed.

Consent to participate All authors consent to participate.

Consent for publication All authors approved the final version.

Conflicts of interest Authors declare no conflicts of interest.

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