

Ecology

Soil culturable microbial diversity in an undisturbed montane cloud forest of Oaxaca, Mexico

Diversidad microbiana cultivable del suelo en un bosque mesófilo de montaña prístino en Oaxaca, México

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Abstract

Montane cloud forests are among the most threatened ecosystems globally. These forests face several stressors, such as deforestation and climate change, jeopardizing their functional sustainability. Although microbial communities act as key regulators of the soil nutrient cycles, microfungus and bacterial diversity remains largely unknown in this ecosystem. We evaluated cultivable soil microbial diversity associated with the soil below iconic plant taxa (Cyatheaceae and Juglandaceae) in a pristine montane cloud forest of Mexico, and explored small-scale ecological patterns linked to edaphic biogeochemical variables. Our findings revealed the copious occurrence of entomopathogenic fungi such as *Tolypocladium geodes* and potentially phosphate solubilizer bacteria such as *Pseudomonas* and *Bacillus* spp. We observed a strong association between edaphic microbial assemblages and environmental variables such as soil C:N:P availability. This close relationship with the physical setting should be considered for the development of management and *in situ* conservation strategies aiming to preserve microbial functions.

Keywords: Microbial edaphic diversity; C:N:P stoichiometry; Spatial heterogeneity; Phytopathogenic fungi

Resumen

El bosque mesófilo de montaña se encuentra entre los ecosistemas más amenazados a escala mundial. Actualmente, enfrenta diversos disturbios de origen antrópico, tales como la deforestación y el cambio climático, que comprometen su sostenibilidad funcional. A pesar de que las comunidades microbianas funcionan como reguladoras de los ciclos de nutrientes en el suelo, su diversidad permanece desconocida en gran medida para el bosque mesófilo. En el presente estudio evaluamos la diversidad y la estructura de comunidades bacterianas y fúngicas asociadas con el suelo

circundante a especies vegetales icónicas (Cyatheaceae y Juglandaceae) en una localidad prístina de bosque mesófilo de montaña en México, y exploramos patrones ecológicos a una escala geográfica fina vinculados con variables biogeoquímicas edáficas. Nuestros resultados revelaron una alta prevalencia de especies de hongos entomopatógenos como *Tolypocladium geodes* y de bacterias potencialmente solubilizadoras de fosfato como *Pseudomonas* y *Bacillus* spp. Observamos una fuerte asociación entre la composición de las comunidades fúngicas y bacterianas con variables ambientales clave, tales como la disponibilidad de C:N:P en el suelo. Estos resultados deben ser considerados para el desarrollo y aplicación de estrategias de conservación *in situ* con el objetivo de preservar las funciones microbianas.

Palabras clave: Diversidad edáfica microbiana; Estequiometría C:N:P; Heterogeneidad espacial; Hongo fitopatógeno

Introduction

Montane cloud forests (MCF) cover 0.26% of the Earth's surface, and less than 1% of the Mexican territory (Bubb et al., 2004). This ecosystem is characterized by a persistent cloud immersion (Rosas Rangel et al., 2019), occurring as patches at elevations of 600-3,200 m asl (Alfonso-Corrado et al., 2017; Ochoa-Ochoa et al., 2017; Santillán et al., 2020). It hosts a number of macroscopic endemic species (12% of the overall American mammal, bird and amphibian species; Hamilton, 2009; Karger et al., 2021), being recognized for its notorious levels of fungal diversity even at the small scale (Velez et al., 2021). The MCF provides vital ecosystemic services such as carbon capture, erosion control, as well as climate regulation, soil fertility, water supply and quality (Bazzaz, 1998; Bruijnzeel et al., 2010, 2011; Martínez et al., 2009). However, this unique biome ranks among the most threatened ecosystems globally, facing several stressors such as deforestation (Leija-Loredo & Pavón, 2017), climate change (Alfonso-Corrado et al., 2017), reduction of humidity (Santillán et al., 2020), increments in temperature (Foster, 2001), among others.

Microbial communities constitute important soil components (De Long et al., 2019) that fulfill key roles in edaphic nutrient cycles, serving as a sink and source of nutrients due to their remarkable ability to immobilize and release carbon (C), nitrogen (N), and phosphorus (P) in different chemical forms (Zak et al., 2003). This group includes several taxonomic assemblies (e.g., fungi, bacteria, virus, archaea and protists) of organisms smaller than 100µm (Wagg et al., 2018). Among these taxa, bacteria and fungi (hereafter referred to as a microbial assemblage, *sensu* Nemergut et al., 2013) are the largest and most diverse components comprising up to 90% of the overall microbial biomass in soils (Rinnan & Bååth, 2009). Hence, the understanding of this imperceptible, yet large component of soil diversity in MCFs represents a fundamental element for conservation.

Data from microcosm and field studies have demonstrated that microbial diversity and community

composition influence soil ecosystem process rates (McGuire & Treseder, 2010). In this sense, bacteria and fungi collaborate in the decomposition and mineralization of organic remains (Romaní et al., 2006; Tapia-Torres & García-Oliva, 2013), driving the development of edaphic stable and labile pools of C, N and other nutrients, which facilitate the subsequent establishment of plant communities (Schulz et al., 2013). In forest systems, bacteria carry out the hydrolysis and mineralization of organic matter through the biosynthesis of exoenzymes, followed by the release and uptake of nutrients (PO_4^- and NH_4^+) from the soil solution. Emblematic taxa with these capacities include members of *Pseudomonas*, *Burkholderia*, *Escherichia*, *Serratia*, *Bacillus*, *Enterobacter*, *Nostoc*, *Caulobacter*, *Sinorhizobium*, *Mesorhizobium*, and *Corynebacterium* (Horwath, 2017; Idriss et al., 2002).

Additionally, edaphic fungi perform several ecological roles as pathogens, saprotrophs, and symbionts (Nguyen et al., 2016). These osmotrophs play essential roles in nutrients turnover (Zanne et al., 2020), depolymerizing recalcitrant lignin and cellulose molecules contained in leaf and wood litter through the production of extracellular enzymes (de Boer et al., 2005). Furthermore, fungal pathogenic taxa act as biological control agents, being implicated in plant diversity maintenance (Brown et al., 2011). To the best of our knowledge, soil microfungi diversity in Mexican MCFs includes members affiliated to *Alternaria*, *Aspergillus*, *Bipolaris*, *Chaetomium*, *Cladosporium*, *Cordana*, *Curvularia*, *Chalara*, *Dictyochaeta*, *Fusarium*, *Gyrophthrix*, *Humicola*, *Monodictys*, *Myrmecridium*, *Penicillium*, *Periconia*, *Pestalotiopsis*, *Sporidesmium*, *Stachybotrys*, *Talaromyces*, *Trichoderma*, and *Virgaria* (Arias & Heredia-Abarca, 2014, 2020; Heredia-Abarca et al., 2011). Also, several ectomycorrhizal fungi have been linked with roots of Juglandaceae species (Corrales et al., 2021).

The generation and amalgamation of diversity data at different scales is fundamental to develop a broad understanding of ecosystems (Oda et al., 2019). At the large scale, MCFs have been extensively investigated, reporting high heterogeneity and diversity levels

(Williams et al., 2013). Though, small-scale studies have received less attention with respect to larger macroscale explorations. Pioneer efforts analyzing biogeochemical data have demonstrated an environmentally heterogeneous setting, with enzymatic activities suggesting distinctive small-scale soil patterns (Velez et al., 2021). Nevertheless, microbial diversity patterns remain poorly understood at the small scale in this environment, hampering the robust view of ecosystem functioning as small-scale processes may be masked by larger scale features (Mori et al., 2018).

In view of MCFs vulnerability to anthropogenic stressors, and the lack of knowledge on soil microbial diversity and its relationship with ecosystem processes (e.g., nutrient cycling) at different scales, herein we evaluated cultivable soil microbial diversity and community structure associated with the soil below 2 iconic plant taxa (endemic, relict, and endangered species) in a pristine location of Mexican MCF at the small spatial scale. We predict that our approach will lead to the predominant isolation of saprotrophic fungi and potentially phosphate solubilizer bacteria; in addition, we hypothesize that the small-scale distribution of microbial assemblages will be strongly associated with environmental variables such as soil phosphorous availability.

Materials and methods

The fieldwork was conducted in the MCF locality of El Relámpago (17°35'30.4" N, 96° 23'57.1" W; at 2,219 m asl), within the municipality of Santiago Comaltepec, in the mountainous system of northern Oaxaca (del Mar Delgado-Serrano et al., 2015). This forest harbors high numbers of endemic vertebrate species and a genetically diverse population of *Oreomunnea mexicana*, due to its good conservation status (del Mar Delgado-Serrano et al., 2015; Ponce-Reyes et al., 2012, 2020). The climate is usually temperate-humid with rainfall in summer (INEGI, 2010), an annual average temperature of 16-20 °C, and average annual precipitation of 2,000-4,500 mm (Trejo, 2004). The main soil type is Acrisol, which is strongly acidic, with a subsurface horizon of clay accumulation and low nutrient retention capacity (Alfaro-Sánchez, 2004; Krasilnikov et al., 2013). Velez et al. (2021) described the biogeochemical characteristics of the study site, highlighting high edaphic heterogeneity at the small spatial scale, abundant total carbon (TC) and dissolved organic carbon (DOC), as well as high polyphenol oxidase (POX) activity values.

We employed a triangular sampling method as proposed by Bąk (2014). Therefore, 3 sampling plots were set up along a 10 m-triangular transect, considering different elements of MCF for a greater representation of

the microbial community: the first plot was established adjacent to an individual of *O. mexicana* (17°35'0.36" N, 96°23'36.6" W), a representative and endangered species of Mexican MCF (Alfonso-Corrado et al., 2017; Rzedowski, 1996); the second plot was settled next to an individual of *Alsophila salvinii* (17°35'18.4" N, 96°23'57.7" W), a conspicuous fern in the region (Rzedowski & Palacios-Chávez, 1977); and the third plot corresponded to the area under a fallen wooden log (17°34'51.83" N, 96°24'5.17" W). Subsequently, in each plot a 1 m-equilateral triangular subplot was traced (Fig. 1). In total, 9 soil cores (3 per plot) were sampled in the first 10 cm of soil (excluding litter) using sterile Falcon tubes and transported in a cooler to the laboratory within the next 48 h for processing.

During the fieldwork we detected a visibly sick population of *A. salvinii* (presence of dark spots and blights on fronds), including the individual within our sampling plot (Fig. 2). So, despite that this was not part of the objectives of this study, and given the importance of prompt disease detection in threatened ecosystems in order to mitigate outbreaks, samples (consisting of sick fronds individually placed in Zip-lock® plastic bags) were collected to characterize the etiological agent. All the material was immediately stored and transported at 4° C in the dark to the laboratory and processed within the next 48 h.

Fungi and bacteria were isolated using the dilution plating method (Warcup, 1960) on Potato Dextrose Agar (PDA; Fluka Analytical, Sigma-Aldrich), Corn Meal Agar (CMA; Fluka Analytical, Sigma-Aldrich), Luria Bertani Agar (LB; Lennox Agar, Invitrogen), and LB⁻ (2-fold diluted LB). Dilution plates were prepared using 1 g of soil sample, at 10⁻¹ - 10⁻⁶ dilutions in test tubes with sterilized distilled water. Three replicates for each dilution of each sample were plated (1 ml aliquot). Petri dishes were incubated at laboratory room temperature (22-25 °C), with a 12 h photoperiod, and examined periodically for up to 2 weeks. During this period, different colony morphologies from each medium were transferred and maintained on PDA and LB plates for fungi and bacteria respectively.

Fronds from *A. salvinii* were initially washed in running tap water. Next, surface sterilization was attained by sequential solutions of 70% ethanol (1 min), 2.6% sodium hypochlorite (3 min), and 70% ethanol (1 min). Small pieces (0.5 × 0.5 cm) from sections including dark spots and blights on leaves were placed in Petri dishes containing PDA, and incubated at 25 °C for 10 to 15 days. Following incubation, fungal axenic isolates were obtained and subsequently transferred to PDA for maintenance.

Fungi were identified by evaluating morphological characteristics, combined with the analysis of the ITS1-5.8S-ITS2 rDNA, hereafter referred to as the ITS region.

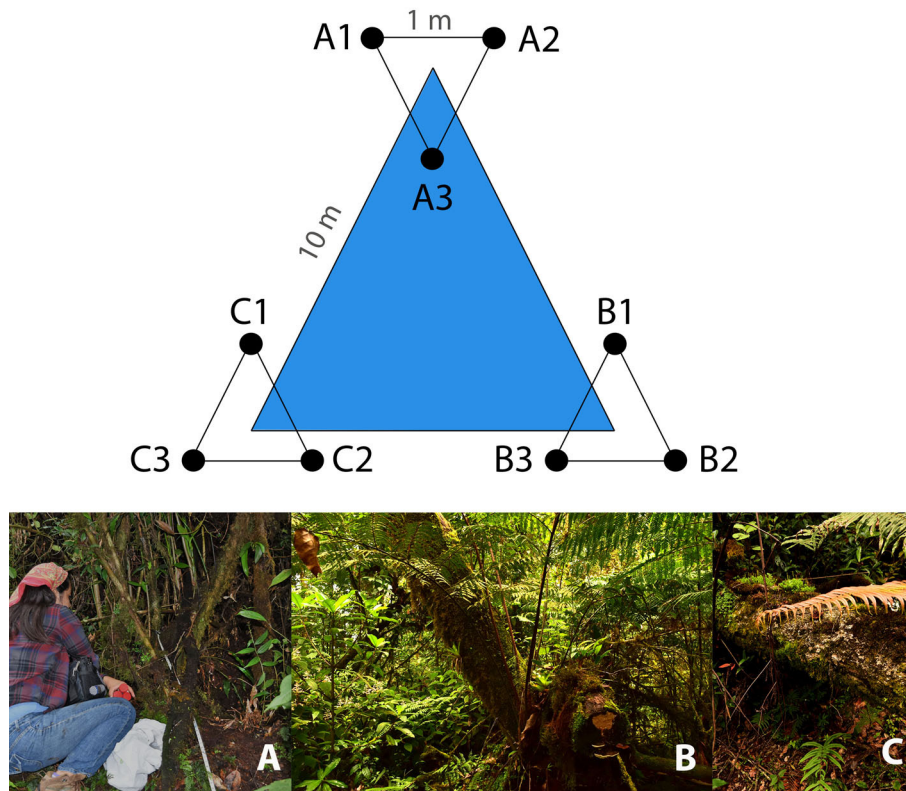


Figure 1. Triangular sampling design where 3 plots were established in: (A) at the base of *Oreomunnea mexicana*, (B) at the base of *Alsophila salvinii*, and (C) at the base of a fallen decaying tree; subsequently in each plot a 1 m-equilateral triangular subplot was traced for soil sampling.

So, genomic DNA of the axenic isolates was extracted using the protocol described by Doyle and Doyle (1987). The ITS region was amplified by using primers set ITS1 and ITS4 as reported by White et al. (1990). The bacterial genomic DNA from axenic cultures was isolated using Dneasy Blood & Tissue Kit®. The 16S ribosomal DNA region was amplified with primers 27F and 1492R (Lane, 1991). The PCR products were sequenced in both directions using a 3730xl DNA Analyzer (Applied Biosystems™) at LANABIO, Biology Institute, National Autonomous University of Mexico (UNAM). Cultures and total DNA are stored in the culture collection of the Laboratory C-121, Biology Institute, UNAM, headed by Dr. Patricia Velez, and are fully available for research upon request.

Quality assessment and assembly of the ITS region and 16S Sanger sequences from fungal and bacterial isolates was performed using the finishing tool Consed version 29.0 (Ewing & Green, 1998; Ewing et al., 1998; Gordon et al., 2001). For the taxonomic assignment, sequence homology was evaluated through the comparison against the UNITE database for fungi (Kõljalg et al., 2020; Nilsson

et al., 2019), and sequences from type material of the National Center of Biotechnology Information GenBank database using the BLAST algorithm for bacteria through a BLAST search (Abarenkov et al., 2010; Kõljalg et al., 2013). Sequence similarity for defining OTUs was set with a cut-off value of 98-100% for presumed species, 94-97% for genus level and 80-93% for order level. For conflicting hits, the lowest common rank level was used (Peršoh et al., 2010; Table 1). The sequences were deposited in GenBank under the accession numbers MT108978-MT109012 for fungi (Table 1), and MZ048754-MZ048770 for bacteria (Table 2).

Statistical analyses

We evaluated the relationship between microbial community structure and the following biogeochemical data retrieved from Velez et al. (2021; synchronously collected from the exact same sampling sites): 1) soil physicochemical properties: pH, NH_4^+ , TC, total nitrogen and phosphorus (TN and TP respectively), DOC, dissolved nitrogen and phosphorus (DON and DOP respectively)



Figure 2. Fronds of *Alsophila salvinii* showing disease symptoms such as the presence of dark spots and blights, indicated by the arrow.

and forms of carbon, nitrogen, and phosphorus contained in microbial biomass (Cmic, Nmic and Pmic respectively), and 2) 6 soil exoenzymes: β -1,4-glucosidase (BG), cellobiohydrolase (CBH), β -1,4-N-acetylglucosaminidase (NAG), phosphomonoesterase (AP), phosphodiesterase (APD), and POX. The raw data matrix was normalized using Z scores. We evaluated clustering patterns among sampling sites based on the culturable microbial community and environmental variables with the “hclust” function in ade4 v1.7-13 package in R (Dray & Dufour, 2007). A Principal Component Analysis (PCA) and a Spearman correlation test between soil biogeochemical variables and enzyme activities were conducted to select the variables for the subsequent multivariate analysis aiming to elucidate the relationships between biological assemblages of species and their environment. For the PCA, we considered as informative the components that

represented at least 85% of the accumulative variance; whereas for the Spearman correlation matrix, we defined an uncorrelated model by using a threshold of 0.85 (Booth et al., 1994). These analyses were computed in R software 3.6.0 (R Core Team, 2018) using FactoMineR version 2.1 (Lê et al., 2008). Next, a Canonical Correspondence Analysis (CCA) with the selected biogeochemical variables (pH, DOC, DON, DOP, NH_4^+ , POX, NAG and AP) and species data was calculated using the R package vegan (Oksanen et al., 2009).

Results

Overall 101 axenic fungal isolates were obtained from the 9 soil subsamples, clustering into 35 OTUs. The OTUs belonged to the phyla Mortierellomycota (*Mortierellaceae* sp. and *Mortierella turficola*), and Ascomycota (33 OTUs). The Ascomycota represented the most abundant and diverse phylum in our samples; affiliated with 8 orders: Capnodiales (1 OTU), Diaporthales (3 OTUs), Dothideales (1 OTU), Eurotiales (3 OTUs), Glomerellales (1 OTU), Hypocreales (14 OTUs), Magnaporthales (1 OTU) and Pleosporales (6 OTUs). At the genus level, isolates of the Ascomycota belonged to 21 genera: *Aspergillus*, *Aureobasidium*, *Beauveria*, *Cladosporium*, *Clonostachys*, *Diaporthe*, *Furcasterigmium*, *Fusariella*, *Gaeumannomyces*, *Ilyonectria*, *Mariannaea*, *Metarhizium*, *Parapyrenochaeta*, *Parengyodontium*, *Penicillium*, *Phomopsis*, *Setophaeosphaeria*, *Talaromyces*, *Tolypocladium*, *Trichoderma* and *Wojnowiciella* (Table 1). Among these, the dominant component was *Tolypocladium geodes*.

We isolated 170 bacterial strains out of which representative isolates with distinctly unique morphologies were identified based on the homology of the 16S rRNA gene region towards reference sequences from the NCBI database. A total of 17 OTUs were delimited within the Actinobacteria (*Arthrobacter* and *Microbacterium*), Firmicutes (*Bacillus*) and Proteobacteria (*Pseudomonas*). The most abundant elements were *Pseudomonas* and *Bacillus* representatives.

The distance dendrogram showed a sparse clustering among sampling sites (Fig. 3). Likewise, the PCA confirmed a considerable heterogeneity in the soil environmental data, with the first 2 ordination axes explaining 61.81% of the total variation (Fig. 4). The variables that most contributed to the first component were TC, TN, AP, NH_4^+ and TP; whereas for the second component BG, DON, Pmic and DOP showed the top contribution (Fig. 5). Spearman analysis showed significant correlations among biogeochemical variables, such as: NH_4^+ , DOC and DOP, as well as AP, NAG, APD and POX (Supplementary material, Tables 1, 2).

Table 1

Fungal isolates obtained from soil samples collected in a pristine location of Mexican cloud forest. * Isolated from sick fronds of *A. salvinii*.

Isolate	ITS1-5.8S-ITS2				
	OTU	Reference accession numbers	% Identity	e-value	Accession NCBI
N10	<i>Aspergillus inflatus</i>	MH859900			
		MH859521			
		MH859519			
		AJ608959	99	0	MT108998
		AF033393			
T8	<i>Aureobasidium pullulans</i>	MT882127			
		MH864403			
		JX188099	100	0	MT109010
		EU272483			
M22_T13	<i>Beauveria</i> sp.	MF062189			
		AY532003			
		HQ880820			
		MH865206	99	0	MT108987
N3_T2_T4_T9_M6	<i>Cladosporium</i> sp.	MH862139			
		HQ880819			
		MN543985			
		MN543962			
		MN543951	100	0	MT109002
M5_2A	<i>Clavicipitaceae</i> sp.	MN521809			
		MN518420			
		MN905773.1			
		HM030580			
		MH864652.1	100	0	MT108996
M19_Tube 12	<i>Clonostachys rosea</i> *	MH859547.1			
		AB709835.1			
		MN511326			
		KX421414			
		HM052817	99	0	MT109000
T19_N16	<i>Diaporthaceae</i> sp.	KM265525			
		MH859090			
		MH864503.1	99	0	MT108997
		MH299960.1			
		MH299958.1			
M20_M26	<i>Diaporthe</i> sp.	MH020798.1			
		FN597586.1			
		MF435154			
		MF435146			

Table 1. Continued

Isolate	ITS1-5.8S-ITS2				
	OTU	Reference accession numbers	% Identity	e-value	Accession NCBI
M10_N11_M28	<i>Didymellaceae</i> sp.	MF435133	100	0	MT108986
		MF435132			
		MF435131			
		MH861244			
		MN077427			
M23	<i>Dothidiomycetes</i> sp.	JF817335	99	0	MT108978
		JN207257			
		MF435134			
M13	<i>Furcasterigmium furcatum</i>	MN421894			
		MN421889			
		MN421869	99	0	
		KX640595			
M2B	<i>Fusariella</i> sp.	KX640594			
		MH859660			
		MH856099			
		AJ608973	99	0	
M25	<i>Gaeumannomyces californicus</i>	JF311914			MT108980
		LR590130			
		EU687056	96		
		KF800481	94		
		FJ820737	94	0	
T14	<i>Ilyonectria</i> sp.	MH859784	94		MT108986
		MH860688	93		
		NR_155135.1	98.149		
		NR_155133.1	97.799		
		KX306490.1	98.149	0	
Tube 8_Tube 9	<i>Mariannaea</i> sp.	KX306480.1	97.799		MT108990
		KX306482.1	97.269		
		KP761750	100	0	
		MK164179			
		KF895008			
Tube 8_Tube 9	<i>Mariannaea</i> sp.	MF101382			MT109005
		LC133803			
		MH863675			
		MH862153			
		KM231758	100	0	
Tube 8_Tube 9	<i>Mariannaea</i> sp.	KM231757			MT109001
		KF767354			

Table 1. Continued

Isolate	ITS1-5.8S-ITS2				
	OTU	Reference accession numbers	% Identity	e-value	Accession NCBI
N14	<i>Metarhizium anisopliae</i>	MH864642 MH483803 KY786031 EU307915 AF137059	99	0	MT109000
M34	<i>Metarhizium carneum</i>	MK164228 MK164227 HQ392598 MK387968 EU553292	98	0	MT109012
M9	<i>Mortierellaceae</i> sp.	MH860437 MH860436 MH860435 MH860122 MH860121	99	0	MT108997
M17	<i>Mortierella turficola</i>	EF521229 AM292200 EU240043 JX976025 JX975952	99	0	MT108982
T11	<i>Nectriaceae</i> sp.	KP265346 MT534189 HQ897787 HQ897787 AM410602	97 96 96 96 96	0	MT109004
N13	<i>Parapyrenochaeta acaciae</i>	KX228265 KF673765 MK441755 KX147607 KX147606	100 99 95 98 98	0	MT108999
M14	<i>Parengyodontium album</i>	MK834516 MW187752 MW077094 MT672589 MT626052	99	0	MT109008
M1A_M1B	<i>Parengyodontium album</i>	MK719933 MH860372 LC092885	100	0	MT108985

Table 1. Continued

Isolate	ITS1-5.8S-ITS2				
	OTU	Reference accession numbers	% Identity	e-value	Accession NCBI
N30_T5	<i>Penicillium</i> sp.	LC092884			
		LC092882			
		NR_077153			
		MN515068			
		MN511336	100	0	MT109003
M18_M3	<i>Phomopsis</i> sp.	MN371392			
		MT872087			
		MF185326			
		EU002915			
T22	<i>Pleosporales</i> sp. 1	MF185359	99	0	MT108983
		MF185341			
		MF185334			
		FM178244	99		
		FM178246	98		
M24	<i>Pleosporales</i> sp. 2	MK066907	99	0	MT109008
		MH931265	99		
		MH844084	99		
		MH935005	100		
		KY367514	99		
Tube 6	<i>Pleosporomycetidae</i> sp.	KT309810	100	0	MT108989
		MH861839	99		
		KY940787	99		
		KJ591760	96		
		KY454761	96		
M11	<i>Setophaeosphaeria hemerocallidis</i>	LT623218	96	0	MT108992
		KF811432	95		
		JQ388267	95		
		KJ869161	99		
		KX515692	97		
M28	<i>Talaromyces wortmannii</i>	KX515688	97	0	MT108979
		KX515679	97		
		KX515674	97		
		MK020174	100	0	MT108991
		KF984826			
KF984825					
KF984824					
KF984823					

Table 1. Continued

Isolate	ITS1-5.8S-ITS2				
	OTU	Reference accession numbers	% Identity	e-value	Accession NCBI
M35_M27_M30_M33_T20_M15_M32	<i>Tolypocladium geodes</i>	MH859919			
		KU556539 JX507694	99	0	MT108995
T16B_T17	<i>Trichoderma</i> sp. 1	MN516473 MN516472			
		MN186861 MN186859 MK871069	100	0	MT109006
		MN518401 MN516457			
		MN516456 MN516454 MN516452	100	0	MT109011
T6	<i>Trichoderma koningii</i>	Z79628 X93983			
		MN516479 MN516476 MN516475	100	0	MT109010
M29	<i>Wojnowiciella dactylidis</i>	LT990661 LT990659			
		LT990658 MK442631 KF800363	99	0	MT108993

Table 2

Bacterial isolates obtained from soil samples collected in a pristine location of Mexican cloud forest.

Isolate	16S				
	OTU	Accession NCBI	% Identity	e-value	Accession NCBI
28.P	<i>Arthrobacter</i> sp.	MW227493.1	100		
		NR_133969.1	98.45	0	MZ048754
		JX949648.2	98.01		
		MN080869.1	98.01		
		NR_170399.1	98.01		
C4	<i>Bacillus</i> sp.	CP009692.1	100		
		NR_113990.1	100		

Table 2. Continued

Isolate		16S			
	OTU	Accession NCBI	% Identity	e-value	Accession NCBI
N10	<i>Microbacterium</i> sp.	AM747229.1	100	0	MZ048770
		NR_115993.1	100		
		NR_036880.1	100		
		MK424288.1	100		
		NR_042263.1	100		
		MT760166.1	99.47	0	MZ048756
		NR_117603.1	99.47		
47.P	<i>Planococcaceae</i> sp. 1	MT760185.1	97.87		
		NR_113837.1	99.78		
		NR_029233.1	99.78		
		X68415.1	99.78	0	MZ048755
		NR_113752.1	99.57		
N12	<i>Planococcaceae</i> sp. 2	MT760068.1	99.57		
		NR_025628.1	98.22	1.00E-165	
		NR_025627.1	98.22	1.00E-165	MZ048757
		NR_109749.1	97.63	2.00E-162	
		NR_041521.1	97.63	2.00E-162	
N13	<i>Planococcaceae</i> sp. 3	NR_118296.1	97.33	1.00E-160	
		NR_025627.1	97.93	0	
		NR_025628.1	97.67	0	MZ048758
		NR_025029.1	95.61	4.00E-175	
		NR_041521.1	95.09	9.00E-172	
46.P1	<i>Planococcaceae</i> sp. 4	NR_144702.1	94.07	2.00E-164	
		NR_116601.1	97.86		
		CP016539.2	97.69	0	MZ048763
		CP013659.2	97.69		
		LC379145.1	97.69		
50.P2	<i>Planococcaceae</i> sp. 5	NR_113814.1	97.69		
		KU886574.1	94.93		
		NR_171442.1	94.93	0	MZ048764
		NR_134133.1	94.8		
		CP016534.2	94.74		
T2	<i>Planococcaceae</i> sp. 6	CP016539.2	94.75		
		NR_113752.1	99.77		
		MT760068.1	99.77	0	MZ048769
		MT757992.1	99.77		
		NR_113837.1	99.08		
T11	<i>Pseudomonas</i> sp. 1	NR_036942.1	99.08		
		LT629778.1	100		

Table 2. Continued

Isolate		16S			
	OTU	Accession NCBI	% Identity	e-value	Accession NCBI
29.P1	<i>Pseudomonas</i> sp. 2	CP062253.1	99.69	0	MZ048759
		CP029608.1	99.54		
		KT321658.1	99.54		
		CP062252.1	99.54		
		MT027239.1			
N8	<i>Pseudomonas</i> sp. 3	NR_103934.2	99.66	0	MZ048760
		NR_148295.1			
		NR_134795.1			
		LK021121.2			
40.P	<i>Pseudomonas</i> sp. 4	NR_148295.1	99	0	MZ048761
		MW111151.1	98.67		
		LR134290.1	98.01		
		LC507444.1	97.84		
C1	<i>Pseudomonas</i> sp. 5	NR_134795.1	97.84	0	MZ048762
		LT629790.1	99.32		
		LC409077.1	99.01		
		MZ099645.1	99.01		
C2	<i>Pseudomonas</i> sp. 6	LC409075.1	98.94	0	MZ048768
		NR_025102.1	98.86		
		JX545210.1	99.51		
		LC595308.1	99.51		
N251	<i>Xanthomonadaceae</i> sp.	MK680061.1	99.18	0	MZ048765
		MG719526.1	99.18		
		CP009533.1	99.18		
		NR_121739.1	98.73		
T17	<i>Xanthomonadaceae</i> sp.	LC500864.1	100	0	MZ048766
		LC548100.1	99.86		
		KX186943.1	99.86		
		KX186942.1	99.86		
		KX186936.1	99.86		
T17	<i>Xanthomonadaceae</i> sp.	CP007597.1	98.73	0	MZ048766
		MW629800.1	98.73		
		KY020782.1	98.36		
		NR_028930.1	98.37		
T17	<i>Xanthomonadaceae</i> sp.	NR_121739.1	99.47	0	MZ048766
		CP007597.1	99.47		
		MW629800.1	99.47		
		NR_028930.1	99.47		
T17	<i>Xanthomonadaceae</i> sp.	AJ293463.1	99.47	0	MZ048766

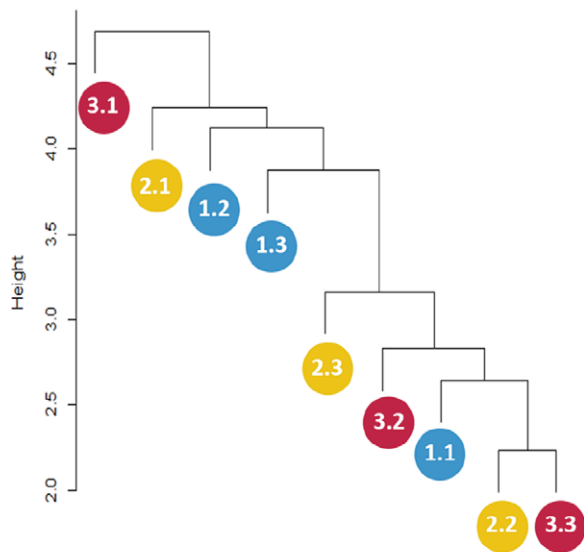


Figure 3. Distance dendrogram showing sparse clustering patterns among sampling sites based on the culturable microbial community. Blue circles represent sampling subplots at the base of *Oreomunnea mexicana* (1.1, 1.2, and 1.3), yellow circles represent sampling subplots at the base of *Alsophila salvinii* (2.1, 2.2, and 2.3), and red circles represent sampling subplots at the base of a fallen decaying tree (3.1, 3.2, and 3.3).

The CCA data suggested that the distribution of fungal and bacterial assemblages in the soil is strongly associated with key environmental variables (Fig. 6). For instance, we detected 5 relevant associations: 1) *T. geodes* and DON; 2) *Penicillium* sp., *Diaporthaceae* sp., and DOC; 3) *Pleosporales* sp. M24, *Xanthomonadaceae* sp. N251, and POX; and 4) *M. turficola*, *Dothideomycetes* sp., *Metarhizium carneum*, *Wojnowiciella dactylidis*, and NH_4^+ (linked to the samples collected near the fern *A. salvinii*).

Discussion

Soil microbiota, including bacteria and fungi, plays central roles in soil fertility and promotes plant health via complex cross-kingdom interactions. Nonetheless, microbial diversity in soils remains poorly understood at different spatial scales. Herein we report 52 microbial OTUs that represent several edaphic functional guilds at the small-scale. This culture-based approach provides the opportunity for a posteriori studies and the possibility of ex situ preservation of genetic resources in face of MCF imminent threats.

Compared with culture-dependent studies of soil fungal diversity in cloud forests at the large-scale (e.g.,

90 samples across coffee plantations and MCF yielding to 415 species in Arias and Heredia-Abarca [2014]; and 20 samples from 4 forest fragments reporting 233 species in Arias and Heredia-Abarca [2020]), our results suggested moderate culturable diversity levels within a $10 \times 10 \times 10$ m-transect. Remarkably, the occurrence fungal OTUs such as *Trichoderma koningii* and species of the genera *Beauveria*, *Cladosporium*, *Penicillium* and *Trichoderma*, agree with former reports on these taxa from conserved and fragmented cloud forest sites (Arias & Heredia-Abarca, 2014, 2020). In terms of prokaryotic diversity, the most abundant genera were *Pseudomonas* and *Bacillus* (both potentially phosphate solubilizer bacteria) in agreement with former work in the Santuario del Bosque de Niebla, a protected area of MCF in Veracruz State (Reverchon et al., 2019, 2020).

The obtained fungi included ubiquitous soil saprobes, as well as potential pathogens of insects, plants, and fungi. In this sense, the abundant isolation of entomopathogenic fungi such as *T. geodes*, *Beauveria*, *Metarhizium*, and *Trichoderma* members, agrees with previous reports from remnants of the original cloud forest in Mexico (Arias & Heredia-Abarca, 2014; Zarza et al., 2022) and may indicate strong antagonistic processes in soil communities (Zimmermann, 1993). In addition, these taxa may be implicated as an important component of edaphic nitrogen dynamics, by mobilizing nitrogen from hosts (e.g., insects) to the soil, resulting in increased nitrogen availability (Behie et al., 2012), in accordance to the observed high values of β -1,4-N-acetylglucosaminidase —chitinolytic enzyme involved in C and N-acquiring microbial activities that is highly correlated with fungal biomass (Miller et al., 1998; Parham & Deng, 2000; Sinsabaugh & Findlay, 1995).

Given the importance of prompt disease detection and identification of ethological agents in phytopathology, particularly for endemic species inhabiting fragile ecosystems (such as *A. salvinii*), as a marginal result we present the first report of *Clonostachys rosea* as a possible phytopathogen of *A. salvinii*. This fungus has been identified as a phytopathogen of numerous hosts including faba bean (Afshari & Hemmati, 2017), *Gastrodia elata* (Lee et al., 2020), soybean (Bienapfl et al., 2012) and the fern *Sphaeropteris lepifera* (Guu et al., 2010); remarkably causing disease under environmental conditions similar to MCF. In this work, we detected the occurrence of *C. rosea* in soil samples and on sick fronds of *A. salvinii*, alerting about a possible emerging disease that should be further monitored.

Overall, we did not detect sharp spatial patterns at the small-scale in the analyzed microbial communities, resembling previous observations on the marked spatial heterogeneity at this scale in soil communities (e.g.,

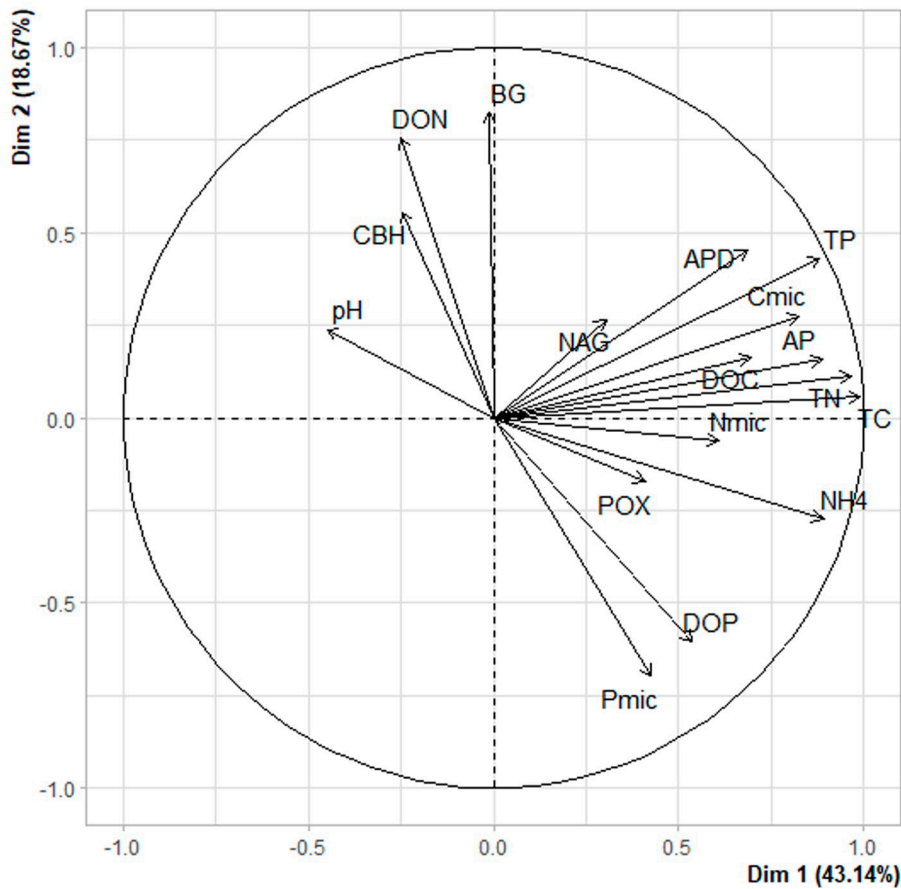


Figure 4. Principal component analysis of environmental variables published in Vélez et al. (2021), including: β -1,4-glucosidase (BG); cellobiohydrolase (CBH); β -1,4-N-acetylglucosaminidase (NAG); phosphomonoesterase (AP); phosphodiesterase (APD); polyphenol oxidase (POX); ammonium (NH_4^+); TC, TN and TP are total carbon, nitrogen and phosphorus; DOC, DON and DOP are dissolved organic carbon, nitrogen and phosphorus; Cmic, Nmic and Pmic are carbon, nitrogen and phosphorus in microbial biomass.

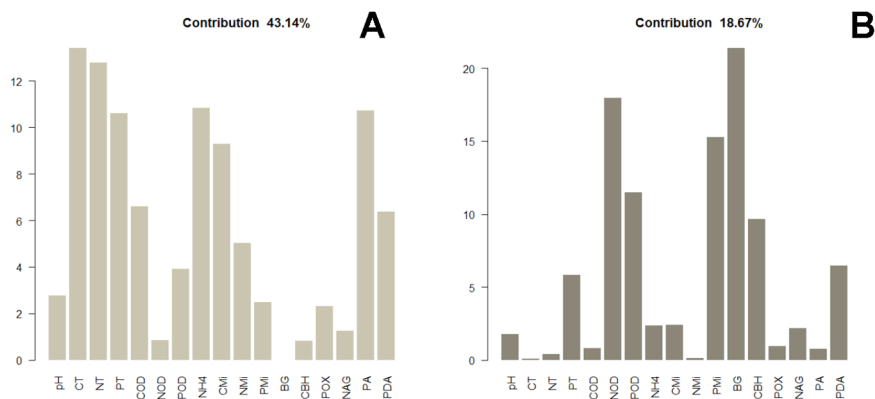


Figure 5. Individual contribution of variables to the first (A) and second component (B) of the PCA, where β -1,4-glucosidase (BG); cellobiohydrolase (CBH); β -1,4-N-acetylglucosaminidase (NAG); phosphomonoesterase (AP); phosphodiesterase (APD); polyphenol oxidase (POX); ammonium (NH_4^+); TC, TN, and TP are total carbon, nitrogen and phosphorus; DOC, DON, and DOP are dissolved organic carbon, nitrogen and phosphorus; Cmic, Nmic, and Pmic are carbon, nitrogen and phosphorus in microbial biomass.

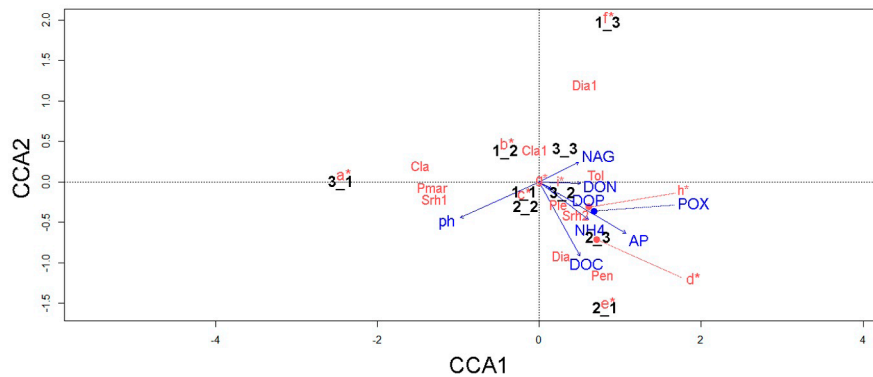


Figure 6. Canonical correspondence analysis showing the relationship between environmental variables, 9 subsamples, and microbial key taxa. Nomenclature of biogeochemical variables is as follows: β -1,4-N-acetylglucosaminidase (NAG), phosphomonoesterase (AP), polyphenol oxidase (POX), ammonium (NH_4^+), dissolved organic nitrogen (DON), dissolved organic phosphorous (DOP), and dissolved organic carbon (DOC). Microbial names are indicated as follows: a = *Arthrobacter* sp., *Furcaterigmium furcatum*, *Phomopsis* sp., *Clonostachys rosea*, *Ilyonectria* sp., and *Trichoderma* sp.; b = *Pseudomonas* spp., *Fusariella* sp., and *Mortierellaceae* sp.; c = *Didymellaceae* sp., *Talaromyces wortmannii*, and *Parengyodontium album*; d = *Mortierella turficola*, *Dothideomycetes* sp., *Metarhizium carneum*, and *Wojnowiciella dactylidis*; e = *Beauveria* sp., *Gaeumannomyces californicus*, *Aspergillus inflatus*, *Metarhizium anisopliae*, *Nectriaceae* sp., *Pleosporales* sp., and *Trichoderma koningii*; f = *Microbacterium* sp., *Planococcaceae* spp., *Setophaeosphaeria hemerocallidis*, *Parapyrenochaeta acacia*, and *Aureobasidium pullulans*; g = *Pseudomonas* sp., *Planococcaceae* sp., and *Bacillus* sp.; h = *Pleosporales* sp., and *Xanthomonadaceae* sp.; i = *Planococcaceae* sp., and *Parengyodontium album*; Cla = *Cladosporium* sp., Cla1 = *Clavicipitaceae* sp., Dia = *Diaporthaceae* sp., Dia1 = *Diaporthe* sp., Pen = *Penicillium* sp., Ple = *Pleosporales* sp., Pmar = *Planococcaceae* sp., Srh1 = *Xanthomonadaceae* sp., Srh2 = *Xanthomonadaceae* sp., Tol = *Tolypocladium geodes*.

Nielsen et al., 2010). Particularly, the CCA results for bacteria depicted no particular influence of the tested biogeochemical factors on bacteria. This highlights the need of understanding how small-scale environmental heterogeneity underlies microbial species richness in MCF. Nonetheless, in accordance with our hypothesis, we observed that some microbial players were strongly associated with particular soil biogeochemical variables. For example, *T. geodes* (entomopathogen) was associated with DON. This is relevant, as *Tolypocladium* members are known as key players in denitrification processes (Jirout, 2015). Furthermore, the association between *M. turficola*—plant growth promoting fungus (Ozimek & Hanaka, 2021)—, *M. carneum* (entomopathogen), and *W. dactylidis*—potentially phytopathogenic (Marin-Felix et al., 2019)—, with NH_4^+ in samples collected near *A. salvinii* might indicate their contribution to the regulation of edaphic inorganic N in the proximities of this fern.

The copious isolation of antagonistic OTUs such as *T. geodes*, *Metarhizium* spp., and *M. turficola*—taxa secreting siderophores, a peptide with potential for biological control of fungi and bacteria (Ozimek & Hanaka, 2021)—, agrees with former metabarcoding data (Velez et al., 2021), and

suggests imperative in situ biotic regulatory processes of ecosystem functioning, which should be further confirmed by experimental work. In this sense, research on microbial interactions should shed light into building models to predict the outcome of community alterations and the effects of perturbations (Faust & Raes, 2012).

Soil microbial community in the examined pristine Mexican MCF was dominated by potentially entomopathogenic fungal taxa such as *T. geodes*, and theoretically phosphate solubilizer bacteria such as *Pseudomonas* and *Bacillus* spp. In accordance with our hypothesis, microbial assemblages were associated with soil biogeochemical variables such as DON, DOC, POX and NH_4^+ . The lack of small-scale community structure patterns coupled to a strong environmental heterogeneity, even at the small-spatial scale, is of vital significance and should be considered for the development and application of *in situ* conservation strategies. This is the first report of *C. rosea* as a phytopathogen of *A. salvinii*, which could pose a threat to the communities of this emblematic plant species. In the future, the possible utilization of the herein isolated native microbial genetic resources ought to be probed to evaluate their response to shifting environmental conditions, as well as for their relationship with plant hosts.

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