Forest root endophytes of pioneer plants from premontane forest in Colombia

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ABSTRACT

Endophytic fungi are those that throughout or part of their life cycle colonize plant tissues forming different relationships with the host plant ranging from mutualistic to pathogenic. Some of these fungi have been found to influence the establishment of pioneer species. Using a preliminary theoretical framework, tested on a small set of host species, was developed to jump start a conversation about the trade-offs present in root-associated fungi. To this end, fungi were isolated from four plants: *Quercus humboldtii*, *Bambusa* sp, *Cecropia* sp. and *Oreopanax parviflorus*, identified molecularly and phylogenetically; functional traits important for host colonization were measured. These traits include growth rate, hyphal diameter, hyphal coloration, hyphal cytoplasmic content, mycelial color, and biomass. Finally, statistical analyses were performed to identify correlations among the traits regarding the host and habitat.

A total of 41 root endophyte cultures were obtained from the four plant species, comprising twenty-five species, all of them belonging to the Ascomycetes. All the isolates, except for *Cadophora* sp, presented similar *in-vitro* growth rates. The analysis of the hyphal coloration, which presented only a marginally significant difference among isolates, suggest an important role of melanin in the growth of these fungi. A potential non-linear relationship between hyphal diameter and growth rate was also found. Finally, there may be possible functional groups characterized by dense and sparse biomass. Our results are preliminary; however, this is a first step in understanding the functional ecology of root endophytes associated with tropical pioneer plant species.

INTRODUCTION

Habitat destruction and fragmentation are the main causes of the loss of biological diversity in the premontane forests of the Andes Mountain range (Wilcox, 1984). Ecological substitution occurs thanks to pioneer species, with a high colonization capacity but a low capacity to compete (Martínez, 1996). A study conducted by Mora et al. (2005), estimated that in the last 14 years the area covered by secondary forest in the Andes has increased approximately 4,504 hectares. In these tropical ecosystems the complexity of factors and their interaction with land use is a challenge to predict successional pathways (Norden et al., 2015). Factors such as topography, soil quality, initial species composition, and fire can influence the rates of vegetation change in successional pathways. In addition, biotic factors such as pathogens and herbivores can alter these successional processes and push communities in an unpredictable direction (Vandermeer, 2004). Therefore, understanding biotic interactions between pioneer tree species and their root inhabiting fungi is an important element to understand succession in tropical montane forests.

Endophytic fungi are those that throughout or part of their life cycle colonize plant tissues (Gaitán, 2006) forming different relationships with the host plant ranging from mutualistic to pathogenic. These fungi reside and grow inside the leaves, roots and stems of plants, and they can be vertically or horizontally transmitted (Sánchez-Fernández et al., 2013). Endophytic fungi have shown great potential in areas such as agronomy and medicine, especially its economic and environmental potential in bioremediation and ligninolytic enzyme production (Rana et al., 2019).

There are two main groups of endophytic fungi: Clavicipitaceae (C-endophytes) and Non-clavicipitaceae (NC-endophytes) (Rodriguez et al., 2009). Clavicipitaceae endophytic fungi are known to inhabit few plant groups (e.g., *Poaceae*, *Bambusoideae*, *Pooideae* and *Convolvulaceae*) and are transmitted vertically through seed colonization (Schardl et al., 2014); in some of the seeds produced by a Clavicipitaceous endophyte-infected plant contain mycelium near the embryo and in the aleurone layer; therefore, the incidence of these endophytes in natural populations of their hosts is very high (Arroyo et al. 2002). On the other hand, NC-endophytes associate with a broad range of host plants including vascular plants, ferns and conifers (Rodriguez et al., 2009). NC-endophytes are phylogenetically diverse, often colonize their host systemically, and are transmitted horizontally by colonizing their host through natural openings, wounds, or directly penetrating the host's surface (Rodriguez et al., 2009). Unfortunately, while the ecological role of C-endophytes is well understood, the details of the interaction between the NC-endophytes and their host remains largely unknown for most of the *host x endophyte* combinations. One of the main challenges is that the outcome of this interaction is highly dependent on the host genotype and the environment (Faeth & Sullivan, 2003; Faeth et al., 2006). Nevertheless, NC-endophytes are known to be ubiquitous and hyperdiverse in the tropics (Arnold et al. 2000) and are believed to represent a great percentage of the undescribed fungi but they can be everywhere, even in our own backyards; these fungi occur in niches that have not yet been investigated, as well as in known ecosystems and habitats where only the usual isolation techniques have been applied. Knowing the diversity of these fungi associated with soil, plants and insects of the tropics is not only an opportunity but also a challenge (Hawksworth & Rossman, 1997). Even though several taxonomic groups within the NC-endophytes are considered generalists, some can have host preferences and spatial heterogeneity (Abello and Kelemu, 2006, Cabezas et al. 2012).

In tropical ecosystems, we can find that leaf endophyte communities vary with the strata within the canopy, suggesting that there are differences in the composition of these communities across tree species growing in the same forest (Gaitán, 2006). Some of the host taxa in which these fungi have been studied are palms, orchids and other epiphytes, and some trees *Guarea guidonia* (Meliaceae) (Gamboa & Bayman, 2001) and *Hevea* spp. (Gazis & Chaverri, 2010) (Chaverri & Gazis, 2010); however, the number of plant species studied remains low in relation to the botanical diversity associated with tropical ecosystems.

Dark septate endophytic fungi are a diverse group of fungi with darkly pigmented and septate hyphae and are frequent intracellular root associates of plants (Piercey et al., 2004). They colonize the cortical cells and intercellular regions of the roots and occasionally form densely septate intracellular structures (microsclerotia) (Jumpponen and Trappe, 1998). The DSE group is polyphyletic, meaning it includes taxa in several unrelated orders: Pleoporales, Microascales, Xylariales, Pezizales, Dothideales, Leotiales, Chaetothyriales, Elaphomycetales, Eurotiales, Onygenales, Saccharomycetales, Neolectales, Taphrinales, Mitosporic (Jumpponen & Trappe, 1998).

These fungi participate in the maintenance of biogeochemical cycles necessary to sustain life in ecosystems. The interactions of dark septate endophytes with their host and with other microorganisms are considered as important as those of mycorrhizal fungi (Jumpponen, 2001). Santos et al. (2017) evidenced the ability of dark septate endophyte isolates to reduce the effects of water stress on Nipponbare and Piauí rice varieties. The endophytes promoted plant growth both in the presence and absence of water deficit. A decrease in the activity of antioxidant enzymes was observed demonstrating that DSE increased the tolerance of rice plants to stress caused by water deficiency.

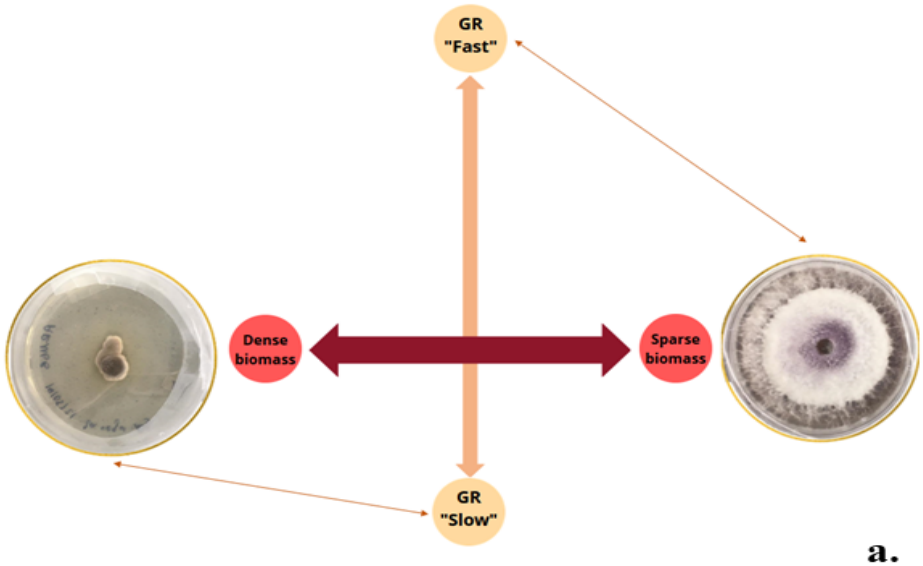
In addition to the relationship between spatial heterogeneity and the diversity of these root endophytic communities, it is expected that these fungi have some influence on the establishment of pioneer species. Currently information regarding the identity, diversity, and functional traits of root fungal endophytes is

scarce, especially from undersampled ecosystems such as tropical montane and premontane forests, where many fungi and host plant species have not yet been studied (Truong et al. 2019). Characterizing these communities and determining their ecological role can benefit important fields such as agriculture and forestry. These communities can also be used as study systems to address questions on the evolution of symbiotic relationships.

Despite the wide variety of endemic plants in Colombia, the isolation of endophytic fungi has been reported only in 12 species, including *Espeletia* spp., *Coffea arabica* and *Vanilla* spp. (Gamboa-Gaitán & Otero, 2016). Rosa (2021) tested endophytic fungi and showed that they have biocontrol abilities, plant growth enhancement, and produce bioactive compounds. Miles et al. (2012) explored the diversity and biocontrol potential of root endophytes associated with *Espeletia* species (Asteraceae) distributed in the Colombian Andean paramo and found thirty fungal endophytic strains with antagonistic activity towards plant pathogens. The industrial potential of the fungal endophytes inhabiting *Espeletia* species has also been investigated and results showed that 4 out of 100 endophytes isolates produce cellulase (Cabezas et al. 2012).

Given that tropical ecosystems are hotspots of diversity for endophytic fungi (Arnold et al. 2000) and that a high diversity of root endophytes has been found in the Colombian páramo ecosystems (Rosa, 2021), it could be hypothesized that root endophytes of trees in the tropical montane forest of Colombia are also highly diverse. In addition, pioneer plant species are an interesting study system given that they can rapidly colonize habitats with harsh abiotic conditions such as high light irradiance and low water availability. In this study we used culturing and molecular identification techniques to characterize the root endophytic communities associated with the following plant species: *Quercus humboldtii*, *Bambusa* sp., *Cecropia* sp. and *Oreopanax parviflorus* in two tropical premontane and montane forests in Colombia. We also characterized potentially important traits that can have a significant influence on the host ability to colonize highly disturbed new environments. By building a baseline knowledge about these often-overlooked microorganisms, we will be able to better understand their function and diversity patterns in Colombian ecosystems.

In addition to documenting diversity, we aim to develop a preliminary theoretical framework, tested in a small set of species, that could help start a conversation about potential tradeoffs present in root associated fungi. Kong et al., (2019) states that for an absorbing root, a higher investment in dry mass would result in a longer root life according to the cost-benefit theory (Eissenstat et al., 2000; Eissenstat at al., 1997), considering the root of a plant as a cylinder formed by several tissues, its dry mass should be a function of total diameter, and root volume increases exponentially with diameter (Kong et al., 2019). We argue here that fungal mycelia could be functionally very similar to the roots of a plant, and on many occasions, they act as a unified entity, and therefore the same framework for the “root economic spectrum” could be tested in root associated fungi. It is expected that for some functional traits we will find positive or negative correlations. For example, we expect for hyphal color to be negatively correlated with growth rate since it is believed that the darker the isolate the slower the growth given the energetic cost associated with melanin production (Fernandez et al. 2013). In addition, we expect fungi with thinner hyphae to have a higher growth rate and lower biomass since thinner hyphae are less energetically expensive to construct (Fig 1).



a.

Trait pair	Expected correlation	Rationale	P-value
GR-C	Negative	Darker cultures should grow slower than lighter ones and have a thinner hyphae	* (**0.0668**)
GR-B	Positive	Cultures with a dense biomass should have a slower growth and a thicker hyphae	n.s. (**0.634**)
GR-HD	Negative	Cultures with thicker hyphal diameter have slower relative growth rate	n.s. (**0.953**)

b.

Fig 1. a. Conceptual framework of the correlation between GR (growth rate) and B (biomass) functional traits. Based on this framework, we hypothesized (i) in a gradient from dense biomass to sparse biomass cultures their hyphal diameters would be thicker when presenting a dense biomass and (ii) a gradient from fast-growing to slow-growing cultures. Arrows indicate correlation between individual traits (see Table 1). **b.** The expected correlations are based on mathematical and ecological expected tradeoffs. P-values correspond to ANOVA tests performed for the pair of functional traits. GR for growth rate; C mycelia darkness, B mycelium biomass and HD hyphal diameter.

METHODS

Study site and sampling

Root samples of *Quercus humboldtii*, *Bambusa* sp., *Cecropia* sp. and *Oreopanax parviflorus* were collected from two sites located in the Cundinamarca province in the Colombian Andes. The first site was the Chicaque Natural Park in the eastern part of the Andean Cordillera (4°36′22″N, 74°18′17″W; altitude between 2000 and 2700 m), this study site hosts oak forest dominated by *Q. humboldtii* (Fagaceae). The weather in this area is characterized by a bimodal rainfall regime with the highest rainfall occurring between March–May and October–November and an average temperature of 15 ºC year-round (Rivera & Cordoba

1998; Ávila de Navia & Estupiñán Torres 2013). The second site was the José Celestino Mutis experimental field station, located between the towns of La Vega and Sasaima. This station is administered by the Universidad del Rosario and has 12.5 hectares of open sites, disturbed forest ecotone and secondary forest (4°95′62″N, -74°38′17″W; at and an altitude of 1300 m). The roots of *Bambusa* sp., *Cecropia* sp., and *Oreopanax parviflorus* were collected in the disturbed forest ecotone that has open vegetation with high light intensity conditions.

Isolation of endophytic fungi

Fine roots were sampled from four individuals of *Q. humbioldtii*, six individuals of *Bambusa* sp., five individuals of *Cecropia* sp., and one individual of *Oreopanax parviflorus* Lateral roots visibly connected to the trees were excavated until locating the fine roots. Root samples were placed in ziplock bags and transported to the laboratory for processing inside a cooler.

For the isolation of endophytes, approximately 5 root segments of 2 cm long were selected at random from each plant. These root fragments were sterilized by immersing them in 70% ethanol for 5 minutes and then in 5% sodium hypochlorite for 5 minutes and finally washed three times with sterile distilled water. The fragments were then transferred to potato dextrose agar (PDA) culture medium and kept at room temperature (approx. 20 °C) under dark conditions.

To isolate individual strains, a week after roots were plated in PDA, mycelium growing from the cut ends of the root segments showing distinctive characteristics were transferred to new PDA plates and maintained in the dark at room temperature (approx. 20 °C). Isolates were then transferred at least two times to new plates before DNA isolation and growth rate assays.

Molecular identification of endophytic fungi

Genomic DNA was extracted from approximately 50 mg of fresh mycelium scraped from the surface of the cultures and stored in 2x CTAB, following Gardes and Bruns (1993). DNA amplification was carried out using taq DNA Polymerase (New England Biolabs) following the manufacturer's instructions.

The Internal Transcribed Spacer (ITS) was amplified using the ITS1F and ITS4 primers (Gardes & Bruns 1993). PCR conditions were as follows: 95 °C for 1 min, then 35 cycles of 95 °C for 30 s, 52 °C for 30 s to 1 min, and 72 °C for 1 min, with a final extension time at 72 °C for 10 min. PCR amplicons were visualized on a 1% agarose gel stained with SYBR Green. Positive products were cleaned with ExoSap-IT (Affymetrix, Santa Clara, CA, USA) and sent to Macrogen Korea (Seoul, South Korea) for Sanger sequencing.

Alignment and phylogenetic analysis

Sequences of the ITS region were cut-off the flanking regions and primers using UGENE version 37.0 and Geneious version R10 (Kearse et al., 2012). Then, using the UNITE database, we searched and downloaded sequences with a threshold of 3.0% of similarity to be used in the phylogenetic trees per genera. Sequences were aligned using MUSCLE. The resulting alignment was edited using Gblocks 0.91b to exclude misaligned or ambiguous positions.

The resulting dataset was analyzed using the maximum likelihood (ML) approach. For ML analysis, the alignment was loaded as a fasta file in Cipres Science Gateway (Miller et al. 2010) and analyzed using RAxML-HPC BlackBox (8.2.12) (Stamatakis, 2014) under the GTR GAMMA + I model with 1000 bootstrap iterations.

To facilitate taxonomic placement of our specimens, we constructed several phylogenetic trees, one for all the Classes in the Ascomycete and one for each of the following genera: *Botrytis, Cadophora, Clonostachys, Colletotrichum, Dactylonectria, Diaporthe, Fusarium, Ilyonectria, Metapochonia, Mycoleptodiscus, Neodeigthonia, Nigrospora, Penicillium, Trichoderma* and *Xylaria*. Species belonging to the same family of our samples, were used as outgroups. Sequences were downloaded from GenBank. We used the following outgroups for each tree: For the genus *Botrytis* the outgroup was *Dicephalospora sessilis*, for *Cadophora* it was *Discinella margarita*, for *Clonostachys* it was *Bulbithecium hyalosporum*, for *Colletotrichum* it was *Glomerella graminicola*, for *Dactylonectria* it was *Fusarium* sp, for *Diaporthe* it was *Mazzantia galii*, for *Fusarium* it was *Cosmospora fomiticola*, for *Ilyonectria* it was *Fusarium* sp, for *Metapochonia* it was *Claviceps tenuispora*, for *Mycoleptodiscus* it was *Magnaporthiopsis agrostidis*, for *Neodeigthonia* it was *Botryosphaeria dothidea*, for *Nigrospora* it was *Trichosphaeriales* sp, for *Penicillium* it was *Petromyces alliaceus*, for *Trichoderma* it was *Protocrea illinoensis* and for *Xylaria* it was *Poronia erici*.

Measurement of species functional traits

From 24 selected isolates we measured the following functional traits: 1) growth rate, 2) hyphal diameter, 3) hyphal coloration, 4) hyphal cytoplasmic contents (spores, septate or presence of fibulae), 5) color of mycelia in culture, 6) biomass and 7) darkness. To measure the growth rate, five-millimeter diameter plugs were taken from the isolates and grown in 90 mm Petri dishes with at least 3 replicates per isolate in an incubator (Thermo Midi 40 CO2 incubator 3403 Scientific) at 25 ˚C for 23 days. The total area of the colonies was measured every 2 days by photographing the cultures and then analyzed using the software ImageJ (Schneider et al. 2012) to calculate the changes in total cover and mycelia coloration darkness. To calculate each species' relative growth rate (r) we used the values of the occupied area at the beginning and the end of the growth period for each of the three replicates and used the following formula: *LN (final area)-LN (initial area)*. Then we calculated an average r value using estimates from all the three replicas.

A logarithmic model was used to model growth using the following equation:

$$P1 = P0 * EXP(r * (1 - (P0/K)$$

Where *P0* is the initial value of the plug (5 mm), *r* is the average relative growth rate and *k* is a constant value representing the carry capacity or total amount resources available for the population corresponding to the total area of the petri dish (38.48, calculated based on the circle area with a diameter of 7 cm). Hyphal functional traits (diameter, hyphal coloration, cytoplasmic contents, presence of fibulae and septation) were measured in the same cultures. Microscopic observations were made in 10% KOH (potassium hydroxide) in a Leica dm750 microscope, under a 100x objective using a micrometer. In some cases, congo red was used in the samples that were hyaline and for the measurement of biomass, this was done qualitatively using the photos of each of the crops.

Based on the taxonomic classification of each of the isolates, we performed a search for ecological information about each of the identified species in the UNITE database (Nilsson et al., 2019) and the FugalTraits database (Põlme et al., 2020). We registered information about their ecology, other sources of isolation and functional groups (such as: ectomycorrhizal, pathogens, saprotrophs and endophytes of leaves, roots, stems, etc.).

Statistical analysis

To test our initial hypothesis about potential tradeoff between relative growth rate and hyphal diameter, darkness and biomass we performed Analysis of variance (ANOVA) using R software (RStudio Desktop

1.4.1717). We also calculated means, standar deviations and dispersion plots between growth rate, diameter, hyphal coloration, hyphal cytoplasmic contents, color of mycelia in culture, biomass and darkness intensity with EXCEL software.

Correlation plots, boxplot and 3D plots were also made with the data of functional traits with R, using the scatterplot3d package, the plot and boxplot package and the rgl package (RStudio Desktop 1.4.1717, Ligges et al., 2018).

RESULTS

Diversity and species composition of fungal isolates

Root collections were made between 2019 - 2021, four individuals of *Q. humboldtii*, six individuals of *Bambusa* sp., five individuals of *Cecropia* spp., and one individual of *Oreopanax parviflorus*. A total of 41 isolates of endophytic fungi were obtained from the roots of the four plant species (see Annex1). We obtained seven cultures from *Q. humboldtii*, 11 from *Bambusa* sp., 18 from *Cecropia* sp. and five from *Oreopanax parviflorus*. The isolates represented four Classes of Ascomycetes: Leotiomycetes, Eurotiomycetes, Dothideomycetes and Sordariomycetes (Fig 2). From these cultures a total of 25 species were identified (Table 1), belonging to eighteen genera (Fig 3). Detailed information about each species is included as Annex 3.

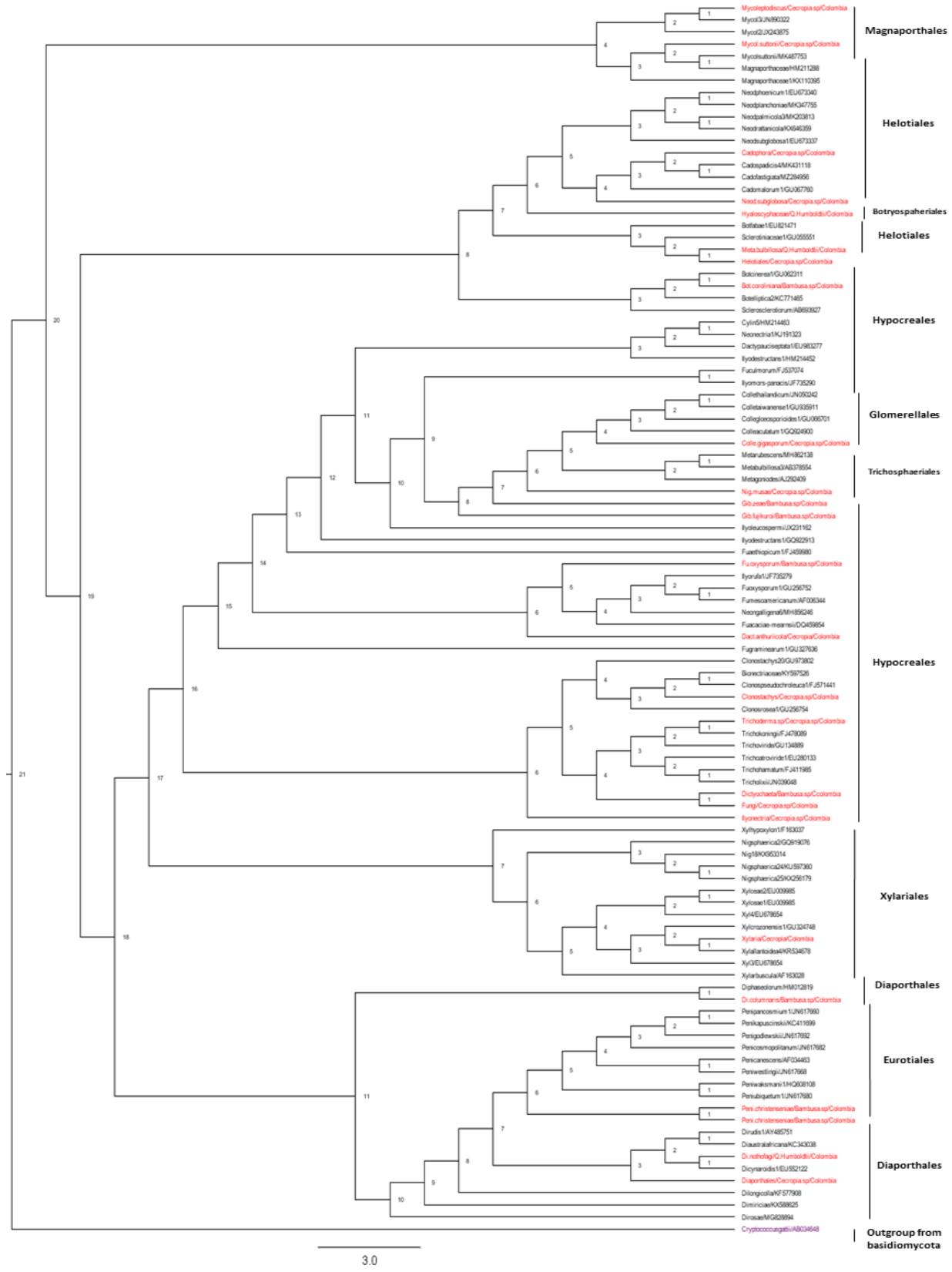


Fig 2. ML phylogram of the 12 Ascomycota orders in our dataset based on the ITS region. Branch numbers indicate bootstrap values. Isolates recovered in this study are in red, and host and country are included.

The isolates recovered belonged to nine orders: Helotiales, Diaporthales, Hypocreales, Eurotiales, Trichosphaeriales, Magnaporthales, Xylariales, Glomerellales and Botryosphaeriales. Diaporthales and Hypocreales were the dominant orders with 25.71% of all the species recovered, followed by Helotiales with 20%. We found thirteen families Dermateaceae, Hyaloscyphaceae, Dermataceae, Clavipitaceae,

Aspergillaceae, Sclerotiniaceae, Trichosphaeriaceae, Magnaporthaceae, Xylariaceae, Glomerallaceae, Botryosphaeriaceae, Nectriaceae and Diaporthaceae being the last two the most abundant with 22.85% and 20% of all species, respectively.

Table 1. Total fungal endophyte species isolated from four host plant species (*Quercus humboldtii*, *Bambusa* sp., *Cecropia* sp., *Oreopanax parviflorus*) with host information and their functional traits scores for hyphal diameter, hyphal coloration, hyphal cytoplasmic contents, presence of spores, septa or fibulae, color of mycelia in culture, and biomass.

		Hyphal traits			Culture traits						
Isolate code	**Species/Family**	**Host**	**Diameter (µm)**	**SD**	**Color**	**Darkness (Cd)**	**SD**	**Growth rate (cm2/day)**	**SD**	**Biomass**	**Observations**
QH3	Hyaloscyphaceae	*Quercus humboldtii*	1,27	0,47	Hyaline	173,18	53,648	3,574	0,781	Dense	With asexual spores, not septate, no fibulae
QH4A	*Diaporthe nothofagi*	*Quercus humboldtii*	1,67	0,72	Dark	78,181	53,648	3,556	0,781	Dense	With fibulae, without spores, septate hyphae
QH4C	*Metapochonia bulbillosa*	*Quercus humboldtii*	1,23	0,44	Hyaline	208,515	53,648	3,979	0,781	Dense	Without fibulae, with spores
Ba1	*Penicillium christenseniae*	*Bambusa* sp.	1,31	0,48	Light	291,263	53,648	4,05	0,781	Dense	With spores, non-septate, non-fibulae
Ba2	*Diaporthe columnaris*	*Bambusa* sp.	1,93	0,80	Dark	189,586	53,648	3,825	0,781	Dispersed	Guttulate (dark contents), septate, no fibulae
Ba3	*Gibberella fujikuroi*	*Bambusa* sp.	3,15	0,69	Hyaline	191,111	53,648	3,95	0,781	Dispersed	Septate, asci and ascospores, asexual spores, guttulated
Ba4	*Penicillium christenseniae*	*Bambusa* sp.	1,38	0,51	Hyaline	208,260	53,648	0,702	0,781	Dispersed	Spore-bearing, guttulate/nucleate, septate (thin)
Ba6	*Dictyochaeta* sp.	*Bambusa* sp.	1,2	0,42	Hyaline	208,066	53,648	4,092	0,781	Dense	Not septate, no spores
Ba9	*Botrytis caroliniana*	*Bambusa* sp.	3,23	0,73	Hyaline	156,028	53,648	3,979	0,781	Dispersed	With spores, without fibulae, not septate
Ba10	*Gibberella zeae*	*Bambusa* sp.	1,3	0,48	Hyaline	270,827	53,648	4,247	0,781	Dispersed	With spores, without fibulae, non-septate

C4M3	Ba11	C6M10	C6M4	C5M2	C4M8	C4M5	C4M1	C3M3	C3M1	C1M7B	ARM8	ARM6B	ARM6A
Colletotrichum gigasporum	*Fusarium oxysporum*	*Diaporthales*	*Neodeightonia subglobosa*	*Clonostachys* sp.	*Helotiales*	*Mycoleptodiscus* sp.	*Xylaria* sp.	*Fungi*	*Trichoderma* sp.	*Nigrospora musae*	*Mycoleptodiscus suttonii*	*Cadophora* sp.	*Dactylonectria anthuriicola*
Cecropia sp.	*Bambusa sp.*	*Cecropia* sp.	*Cecropia* sp.	*Cecropia* sp.	*Cecropia* sp.	*Cecropia* sp.	*Cecropia* sp.	*Cecropia* sp.	*Cecropia* sp.	*Cecropia* sp.	*Oreopanax parviflorus*	*Oreopanax parviflorus*	*Oreopanax parviflorus*
3,2	1,25	1,36	3,31	2,07	1,55	1,64	1,38	1,4	1,33	3,73	1,18	1,8	1,25
0,68	0,45	0,50	0,95	0,80	0,52	0,63	0,51	0,52	0,49	0,88	0,40	0,77	0,45
Dark	Light	Hyaline	Dark	Light	Hyaline	Hyaline	Hyaline	Light	Hyaline	Dark	Light	Dark	Hyaline
Not measured	Not measured	185,890	190,789	173,908	206,570	91,538	194,104	203,964	182,885	162,652	154,608	232,896	65,422
Not measured	Not measured	53,648	53,648	53,648	53,648	53,648	53,648	53,648	53,648	53,648	53,648	53,648	53,648
Not measured	Not measured	3,713	3,983	4,096	4,245	3,989	4,102	4,144	4,122	4,137	3,919	2,482	4,169
Not measured	Not measured	0,781	0,781	0,781	0,781	0,781	0,781	0,781	0,781	0,781	0,781	0,781	0,781
Not measured	Not measured	Dispersed	Dispersed	Dense	Dispersed	Dense	Dispersed	Dispersed	Dispersed	Dispersed	Dispersed	Dense	Dense
Septate, with spores, with fibulae	With spores (many spores), without fibula, non-septate	Spores small, septate (thin)	Spore-bearing, septate, with fibula, guttulate	With spores, guttulated, septate	With spores, guttulate (balls in heaps), septate	With spores, not septate, not fibulae	septate, spore-bearing, fibulate	Many spores, septate, guttulated	With spores, not septate, not fibulae, not guttulated	Without fibulae, septate, spore-forming (asexual)	With spores, septate, no fibulae	Without fibulae, with spores, septate, guttate/cores	With spores, not septate, no fibulae

C4M10	*Ilyonectria* sp.	*Cecropia* sp.	3,87	0,83	Light	Not measured	Not measured	Not measured	Not measured	Not measured	With spores, not septate, not fibulae

Relative abundance of each fungal genera was calculated for each host plant species. *Rhizoderma* was the most abundant genus in *Q. humboldtii* roots (40%), *Penicillium* and *Gibberella* (30%) in *Bambusa* sp., and *Diaporthe* in *Cecropia* sp. and *Oreopanax parviflorus, with 20% and* 40%, of the recovered isolates respectively (Fig 3). Phylogenetic trees of all the genera identified in the study are presented in Annex 4. The only genus present in all hosts was *Diaporthe* (Fig 3).

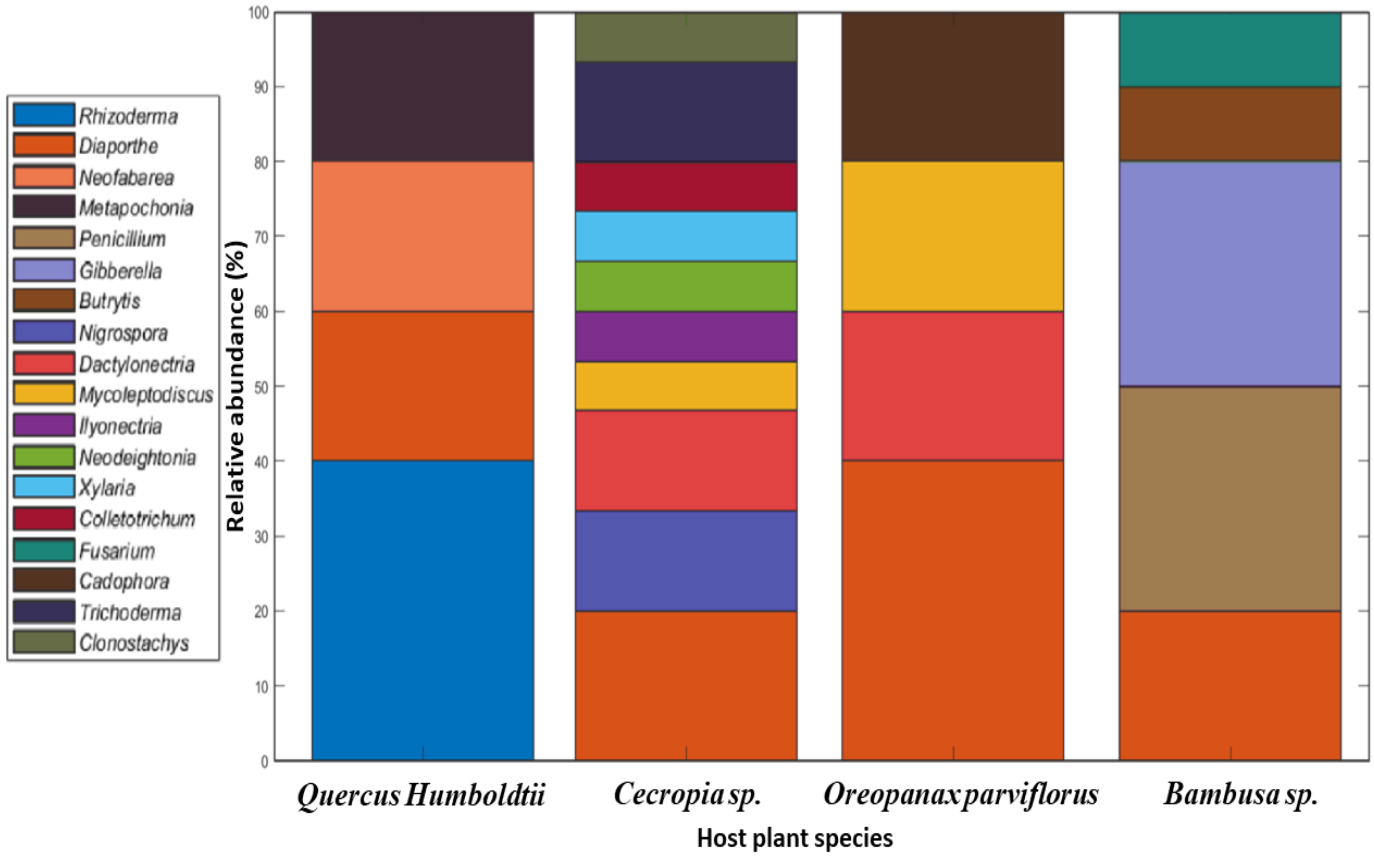


Fig 3. Relative abundance of the genera identified for each of the host sampled (*Quercus humboldtii, Bambusa* sp., *Cecropia* sp., *Oreopanax parviflorus*).

From the publicly available datasets, we recovered information about functional guilds for 25 species (see Annex 1), representing five functional groups: ectomycorrhizal (33.33%), foliar endophyte (33.33%), root endophyte (12.5%), saprotroph (16.66%) and plant pathogen (4.16%) (Fig 4).

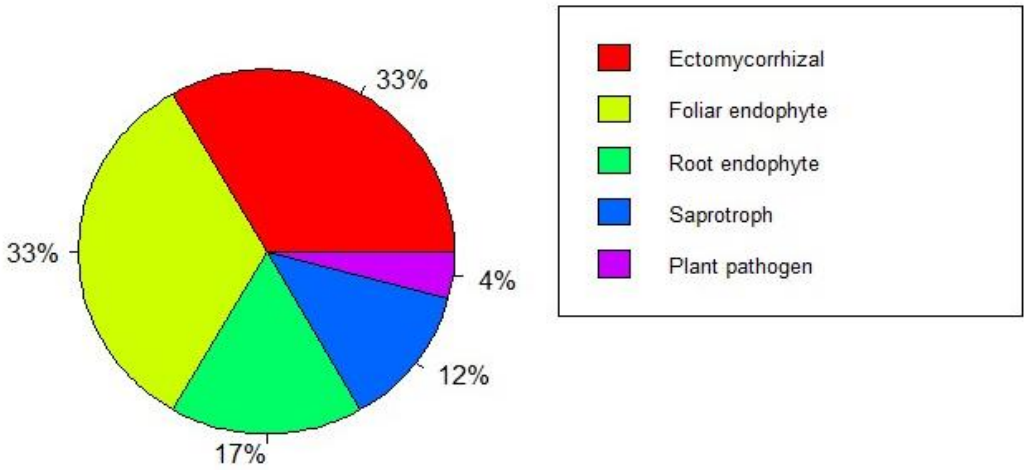


Fig 4. Distribution of isolated fungal endophytes by functional groups (ectomycorrhizal, foliar endophyte, root endophyte, saprotroph and plant pathogen). Isolates were recovered from four plant host species: *Quercus humboldtii*, *Bambusa* sp., *Cecropia* sp., and *Oreopanax parviflorus*.

Functional traits of fungal isolates

The relative *in vitro* growth rate of the selected isolates ranged between 2.482 and 4.461 (Table 1). Culture growth based on the logarithmic model showed that all the species have fast growth, except for *Cadophora* sp. (ARM6B) isolated from the host *Oreopanax parviflorus* (Fig 5).

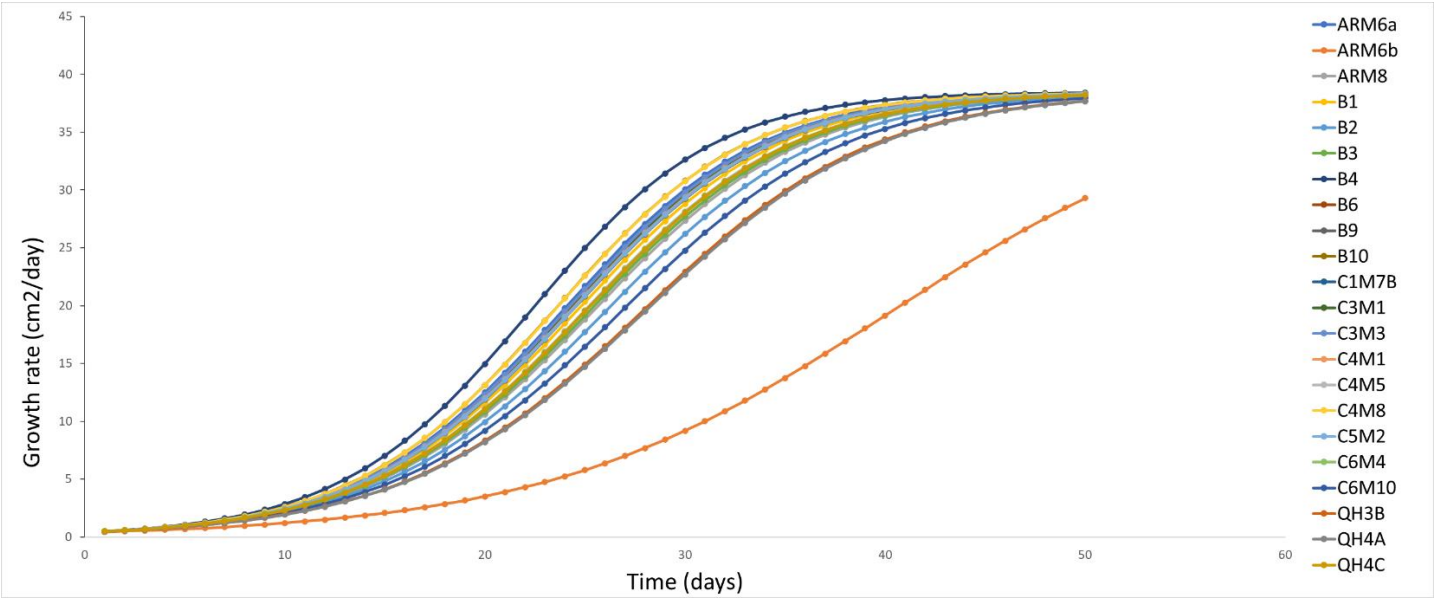


Fig 5. Logarithmic model of the growth of fungal isolates projected to 50 days.

When correlating the growth data and the average hyphal diameter of each culture, a potential non-linear relationship was found (p-value = 0.953, Fig 1), in this case an exponential relationship between these two variables (Fig 6) shows that as the growth rate of each of the samples increases, the diameter of their hyphae also increases (Table 1).

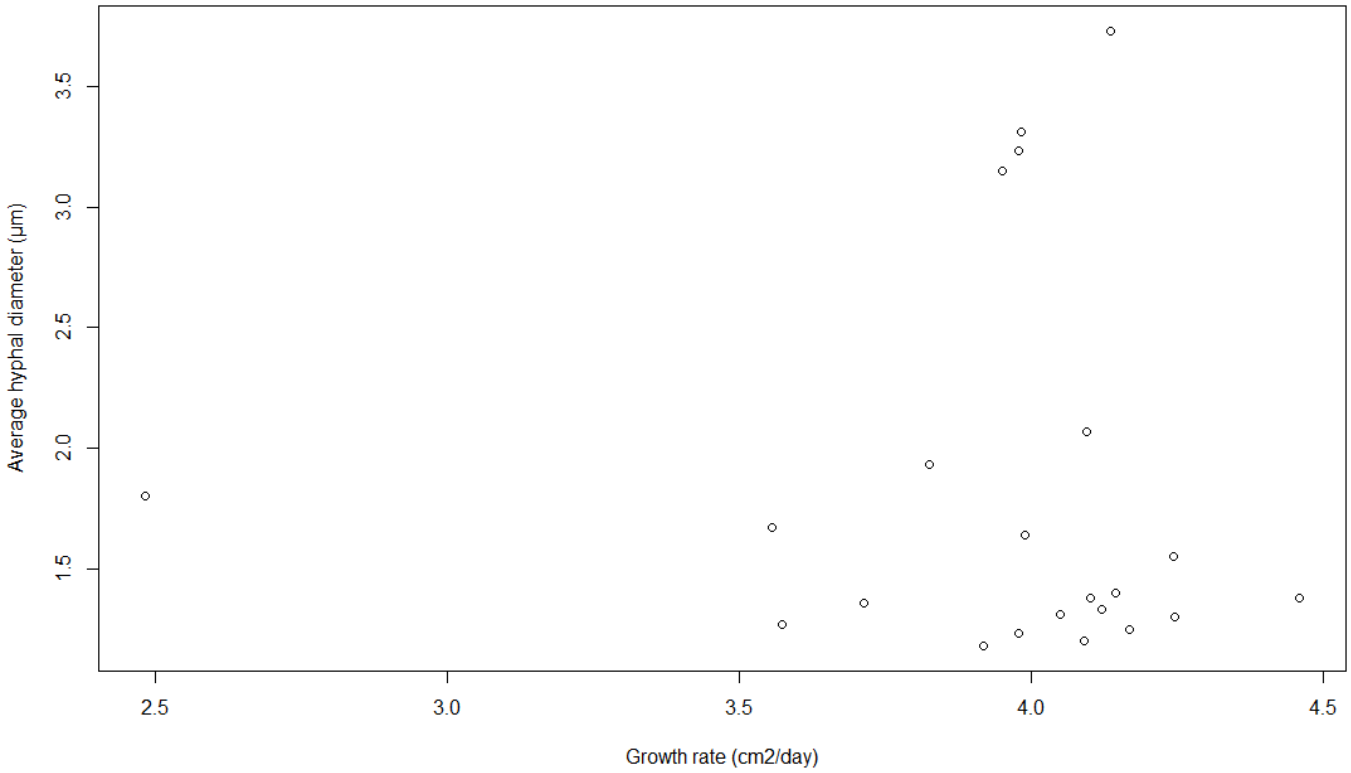


Fig 6. Scatter plot of relative growth rate and average hyphal diameter for all 25 fungal isolates.

We found marginally significant differences in the relative growth rate of cultures based on their hyphal color (p-value = 0.0668, Fig 1), contrary to our expectations based on our hypothesis (the darker the culture, the slower its growth rate) (Fig 7).

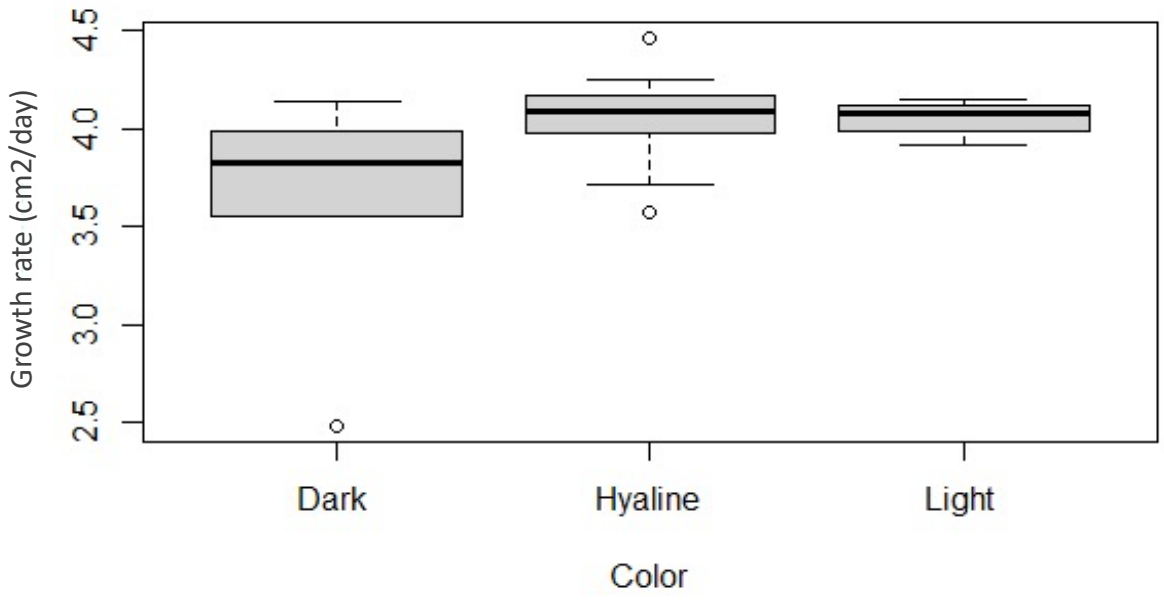


Fig 7. Boxplot of correlation between hyphal color and growth rate.

It is observed that both, color and darkness data, are quite scattered against the growth rate, supporting previous observations that these two traits are not correlated, regardless of whether the culture is dark, hyaline or light colored (Fig 8).

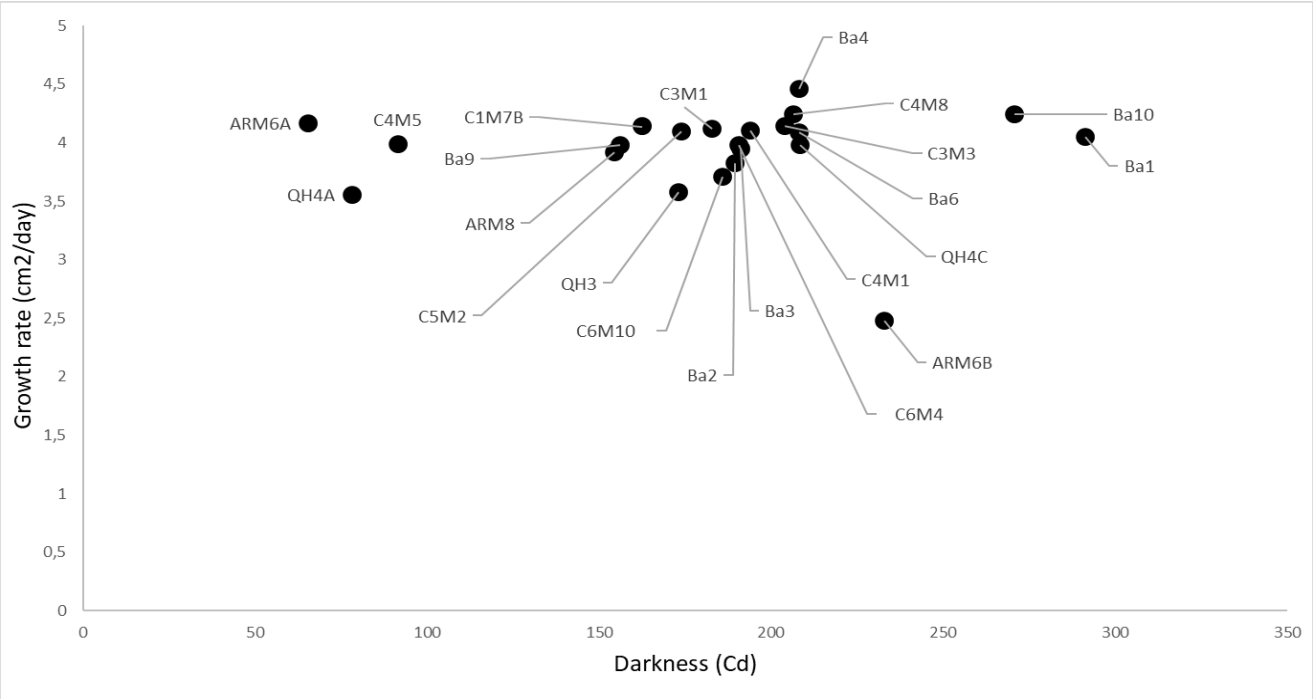


Fig 8. Plot for culture darkness vs relative growth rate.

We didn't find significant differences on the relative growth rate between isolates with high or low biomass (p-value = 0.634, Fig 1). By relating three functional traits (hyphal diameter, growth rate, and darkness intensity) we observed a clustering of species. However, again the plot supports a non-linear relationship between hyphal diameter and growth rate and none of the other traits explain the grouping (Fig 9).

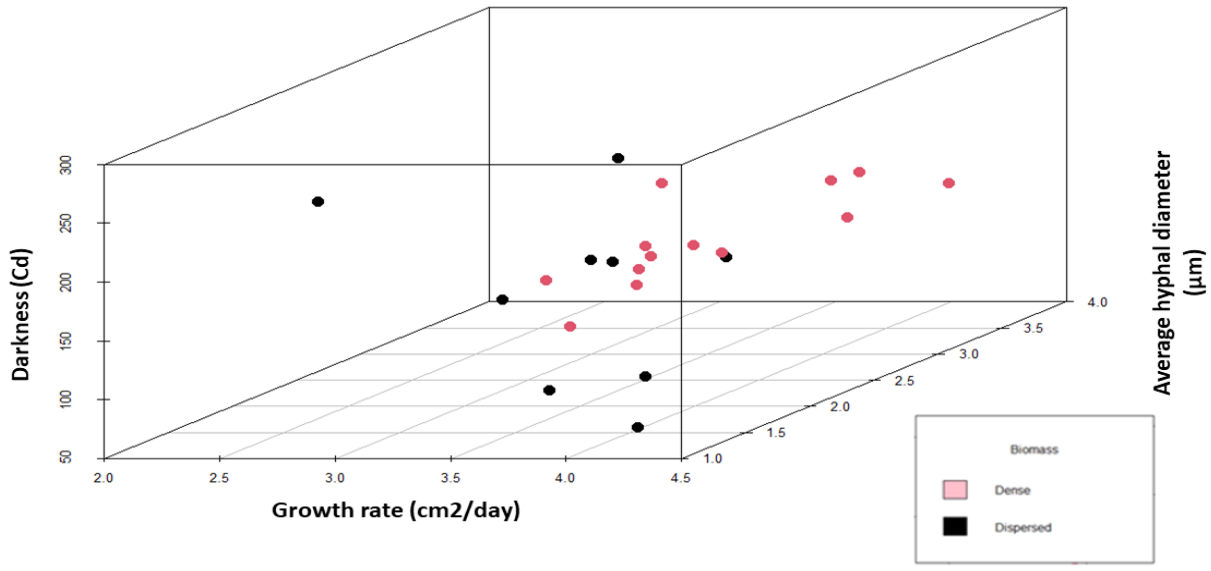


Fig 9. 3D plot of the relationship between growth rate, culture darkness and biomass of root endophyte cultures.

DISCUSSION

Our study is the first one to characterize the root fungal endophytic communities inhabiting ecologically important pioneer tree species in Colombian tropical montane forests. It also represents the first effort in establishing a conceptual and hypothesis driven framework to study these communities. Here, we establish a

baseline working protocol to encourage studies of functional ecology of root associated fungi. Although the first report of tropical endophytic fungi on plants was in made in 1981 (Petrini and Dreyfuss), over the years these fungi, especially root endophytes, have remained understudied and knowledge on these communities have not increased significantly. One reason could be because, compared to leaf endophytes, root endophytes present great difficulty for insolation (Blackwell, 2011). Despite finding only 25 species of root endophytes in these four hosts, this study represents an important contribution to tropical fungal ecology.

Although studies on root endophytic fungi are scarce, the study by Sikes et al. (2016) suggests that these fungal communities should be considered when evaluating plant community structure and the success of pioneer and exotic plants. It is possible that these root endophytic fungi may have an impact of great magnitude at the time of their introduction, in the practice of restoration, or in biological control. For example, some or all the 25 species found in this study may influence the processes of restoration and ecological substitution in tropical premontane forests; therefore, knowing their role and their interaction with their hosts and soil microbiota could help towards a comprehensive understanding on the ecology of the secondary sucession (Sikes et al., 2016).

Despite the lack of studies that have measured functional traits of fungal grown in vitro, it was observed that the growth of *Cadophora* sp. (ARM6B) was low compared to the rest of the species (Fig 5). In a study by Yakti et al. (2019), the potential of *Cadophora* sp. growing against tomato soil pathogens (*Rhizoctonia solani*, *Pythium aphanidermatum* and *Verticillium dahliae*) was evaluated, showing an antagonism suggesting that this species can suppress the growth of plant pathogens. It can be observed in their experiment (looking at the photos of their crops after 4 weeks in PDA) that the growth rate of this isolate is as slow as that of our isolate, which could be related to its potential to produce bioactive secondary metabolites and enzymes to decompose organic matter. Likewise, positive effects of this fungus have been reported mainly related to the improvement of plant nutrition (Newsham 2000; Yakti et al. 2018; Yakti et al. 2019). For example, Newsham (2011) obtained that this fungal species presented significant effects on plant biomass when nitrogen was supplied in organic form (Redman, Dunigan & Rodriguez, 2001; Mandyam & Jumpponen, 2015).

We found a nonlinear relationship between the isolate growth rate and hyphal diameter (Fig 6). Comparative results were found by Kong et al. (2019) when analyzing a dataset of global traits of absorbing roots that included more than 800 plant species, finding a nonlinear relationship between root tissue density and root diameter. In comparison with our results, an increase in hyphal diameter with increasing culture growth rate was observed, suggesting a potential role of growth rate in determining fungal hyphal diameter also meaning that hyphal diameter will influence growth rate.

Caveats of this study include that the experiments were performed under *in vitro* conditions and included only a small number of species. Nevertheless, we consider that these experiments and protocols represent suitable tools for the study of the mechanisms involved in the study system. Potato Dextrose Agar (PDA), the culture medium used for this study, is composed of dehydrated potato and dextrose that promotes fungal growth, with the addition of antibiotics that inhibit the growth of bacteria in the culture and allow isolation of fungi. Culture conditions, including the type of artificial nutrient medium must be considered, since it is not yet known how the fungi may behave in other type of media, perhaps their growth rate is higher or lower; and if their growth will be similar to *in planta* natural conditions.

We also found a marginally significant relationship between relative growth rate and hyphal pigmentation and colony color (Table 1 and Fig 7). Fernandez et al. (2013) evaluated the effect of melanin as an important functional trait when fungi were grown under stress conditions. These authors found that melanin inhibition had negative effects on the growth of the ectomycorrhizal fungi *Cenococcum geophilum* isolates when

exposed to water stress conditions, but not under control conditions. Therefore, since melanin is indeed an important factor fungal stress response, it could be hypothesized that in our results as shown in Fig. 7 and Table 1, melanin has some significant effect on the growth of these root fungi by the significant differences observed between fungi with hyaline coloration and fungi with dark coloration. However, culture color or pigmentation and growth rate are very plastic traits that could change depending on culture conditions. For example, Méndez-Zavala et al. (2007) evaluated the growth rate and pigmentation of *Monascus* and *Paecilomyces* sp. fungi growing in nine different culture media. They found that pigment production (tonality and intensity) was influenced by the type of media. These results suggest that the fungi cultivated in our study could express different values for functional traits regarding their growth rate and pigmentation under different culturing conditions.

Unfortunately, we can't compare our findings about the lack of association between growth rate and isolate biomass (Fig 1), because there are no studies focused on biomass of root endophytic fungi. However, our analysis shows a potential clustering of functional groups of root endophytic fungi based on their biomass density (dense vs. sparse) (Fig 9).

Although in plants studies that evaluate trait characters of root endophytic fungi are essentially nonexistent, we can make parallels with studies that address the same research topic in plants. For example, Aguilar-Trigueros & Rillig (2016) found that when fungal root endophytes were inoculated in roots of *Festuca brevipila* (70% plant cover) there were positive effects in regard to host growth. Inoculated plants were able to compensate for low nutrient levels, and furthermore, host responses to the presence of three fungi were different from those of only one, suggesting that endophyte-endophyte interactions may play an important role in the structuring of plant communities (Aguilar-Trigueros & Rillig, 2016). As with the other functional traits targeted in the study, it has been found that they influence plant biomass when inoculated. In the study by Vannier et al. (2020), tested the hypothesis that the abundance of species in each plant space could leave an imprint on its community of root endophytic fungi, ultimately impacting its biomass. The plant *Medicago truncatula* was used to describe its endophytic fungal community, and it was shown that the fungal community of a plant is largely determined by its neighboring plants, suggesting that plant-plant interactions may be mediated by changes in the root endophytic fungal community (Vannier et al., 2020).

Root-associated functional ecology is a promising field of study. However, many theoretical and methodological questions remain unanswered, in particular about fungal ecological niches and plasticity of functional traits. Our results are preliminary and include a small data set; moreover, it is important to consider that the values of species functional traits measured here might be different from those expressed when fungal strains live within host tissue. However, from this study, conceptual frameworks of the correlations between the functional traits of these endophytic fungi could be generated (Fig 1) taking as an example studies such as Bergmann et al. (2020) where this conceptual framework is used. This is a first step in understanding the functional ecology of root endophytes associated with pioneer tropical plant species and in the future may serve as a guide to better understand the functioning of these fungi inside and outside their host plants.

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