Use of native soil as inoculum for the restoration of soil microorganisms in *Juglans neotropica*

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Abstract

Juglans neotropica is an Endangered tree species threatened mainly by declines in habitat and its high commercial value for its timber and nut production, and therefore, it is urgent to find conservation strategies for the species in South American ecosystems. *J. neotropica* is known to form symbiotic relationships with root-associated fungi, including arbuscular mycorrhizae (AMF), which plays a fundamental role in seedling survival by improving nutrient uptake (Mortier et al. 2020). Normally, the soil microbiome is totally forgotten when it comes to ecosystem conservation and restoration plans, even though we know its importance for the development and survival of plants. In our study, we implemented three types of native soil inoculum of *Juglans* in nursery seedlings to observe the influence of this on seedling growth. We found that the seedlings of *J.neotropica* are positively influenced in their growth when they are planted with inoculum of native soil from conspecific adult trees, which could contain a microbiome that can improve their yield, likewise, we found a high abundance of phytopathogens within the samples corresponding to seedlings that were planted with commercial sterile peat. Our results highlight the importance of including the soil microbiome in propagation processes to increase seedling growth, health, and survival.

Key words

Juglans neotropica, Arbuscular mycorrhiza, Root-associated fungal communities, soil microbiome, Tropical montane forest, Ecological restoration

Introduction

The loss of biodiversity currently facing Earth's key ecosystems and its associated species requires urgent efforts to avoid the massive disappearance of species. Most conservation efforts around the world are focused on protecting above-ground biodiversity and therefore a big conservation gap exists for belowground species (Busby et al. 2022). Particularly soil microorganisms are fundamental for maintaining the ecosystem's biogeochemical cycles and ensuring plant productivity. Within the soil microbiota of tropical forests, root-associated fungi, particularly arbuscular mycorrhizal (AMF) fungi, play a prominent role by establishing symbiotic relationships that facilitate efficient nutrition, benefiting numerous tree species and ensuring their survival (Harrison 1997; Arteaga Cuba 2020).

Currently, different strategies are being studied to integrate the microbiome of trees into forest conservation and restoration plans. Reforestation plans depend on the survival of the trees that are planted or the seedlings that are generated naturally in ecosystems. It is known that the seedling state is the most vulnerable stage of the tree life cycle and it needs its symbionts to be able to successfully survive both biotic and abiotic stress (Werner D 1992; Busby et al. 2022).However, in the approach to ecological restoration and rehabilitation strategies, the microbiome associated with trees is not considered and in many cases ignored. The restoration of the soil and root microbiome can influence the plant's ability to respond to different pathogens and abiotic stressors in addition to influencing their development, growth, and resilience (Busby et al. 2022).

Juglans neotropica, a plant native to South America, exhibits a wide ecological distribution, thriving in pre-montane humid forests, montane dry forests, and very humid low montane forests. This plant is currently classified as endangered according to IUCN due to overexploitation for timber purposes and habitat loss (IUCN red list 1998; Toro Vanegas & Roldán Rojas 2018; Nieto & Rodríguez 2002). *Jugans* spp. forms vital symbiotic associations with many root-associated fungi including arbuscular mycorrhizal fungi (AMF), which play a fundamental role in the uptake of nutrients and development (Mortier et al. 2020). However, to our knowledge, the root symbiotic associations of *Juglans neotropica* haven't been previously studied.

Root-associated fungal communities of *Juglans* spp. are likely different from many other plant species, given the presence of a biochemical compound in their roots known as juglone, which has an allelopathic function (Rietveld 1983; Hejl et al. 1993). Juglone can restrict the germination, development, reproduction, and even the behavior of other plant species growing nearby and therefore play an important role in competition between

individuals for resources such as water, nutrients, and space (Rietveld 1983; Hejl et al. 1993). Several studies have shown that arbuscular mycorrhizal fungi could play a role in the transport and movement of juglone through the soil, the long-lasting interconnections of mycorrhizal fungi can speed up the spread of juglone in the soil (Achatz & Rillig 2014; Achatz et al. 2014). In a study conducted by Achatz et al. (2014) they found that the presence of a hyphal network amplifies the transport of juglone, resulting in a decrease in the growth of tomato plants, reducing their root and shoot biomass. They also found that the presence of juglone and mycorrhizae had a significant impact on soil available phosphorus and plant phosphorus content (Achatz et al. 2014). This is because the hyphal connections of mycorrhizal fungi can act as fast conduits connecting different plant species underground, thus expanding the bioactive areas of allelopathic compounds (Achatz et al. 2014). One of their experiments showed that the level of extractable juglone in the soil in the tomato root zone increased by 271% when the soil was interconnected with the nut litter through AM hyphae (Achatz & Rillig 2014; Achatz et al. 2014).

Previous research has focused on the plant-mycorrhizal interaction of commercial walnut species (*Juglans regia*) mostly in agroforestal systems. A study by Mortier et al. (2020) highlights the fundamental role of the symbiotic relationship between walnut trees and AMF in nutrient absorption. Their research reveals that nutrient uptake capacity is particularly related to first- to third-order roots, particularly fibrous roots that depend on AMF. In the context of agroforestry systems, walnut trees emerge as important reservoirs of AMF. However, it should be noted that the dependence of AMF may be subject to environmental factors, such as shade and phosphate availability, which influence the overall effectiveness of this symbiotic relationship.

Williams & Ginzel (2022) also evaluated how the soil microbiome of natural forests and plantations of black walnut (*Juglans nigra*) could suppress the infection of *Geosmithia morbida* (a well known walnut pathogen) and other rhizosphere pathogens in seedlings. They found that the natural forest floor microbiome suppressed *Fusarium spp*. and indirectly suppressed *G. morbida*, resulting in a decreased damaged area compared with seedlings growing in sterile soil. Also Inoculation with *G. morbida* induced changes in fungal community composition in the rhizosphere, and the magnitude and direction of the change depended on soil treatment.

This study aims to evaluate the effect of inoculation with live forest soil from conspecific vs a traditionally used propagation substrate on nursery grown *Juglans neotropica* seedlings performance and diversity of their root-associated fungi. We used a nursery experiment and eDNA Illumina sequencing to 1) Evaluate the influence of nursery propagation methods and seed origen on seedlings' growth rate and other above ground functional traits and 2) Evaluate the influence of nursery propagation methods on the root mycobiome of the those same seedlings to determine the alpha and beta diversity patterns of the root-inhabiting fungal communities. For this, we collected wild seedlings and soils and established a nursery inoculation experiment that was monitored for six months, at the Amoya sun-basin in the department of Tolima, Colombia.

Materials and methods

Study sites

This study was developed in the Amoyá sub river basin, specifically in the village of La Alemania, located in township An José de las hermosas, in the municipality of Chaparral in the South of Tolima Department, Colombia (Fig 1). The study site is located in a humid and very humid premontane forest. This experiment was developed as part of a conservation and restoration project of *J. neotropica* directed by the WildLife Conservation Society (WCS) called " Conservation and Restoration of Colombian Walnut *(Juglans neotropica)* and Other Native Threatened Tree Species from Andean Forests".

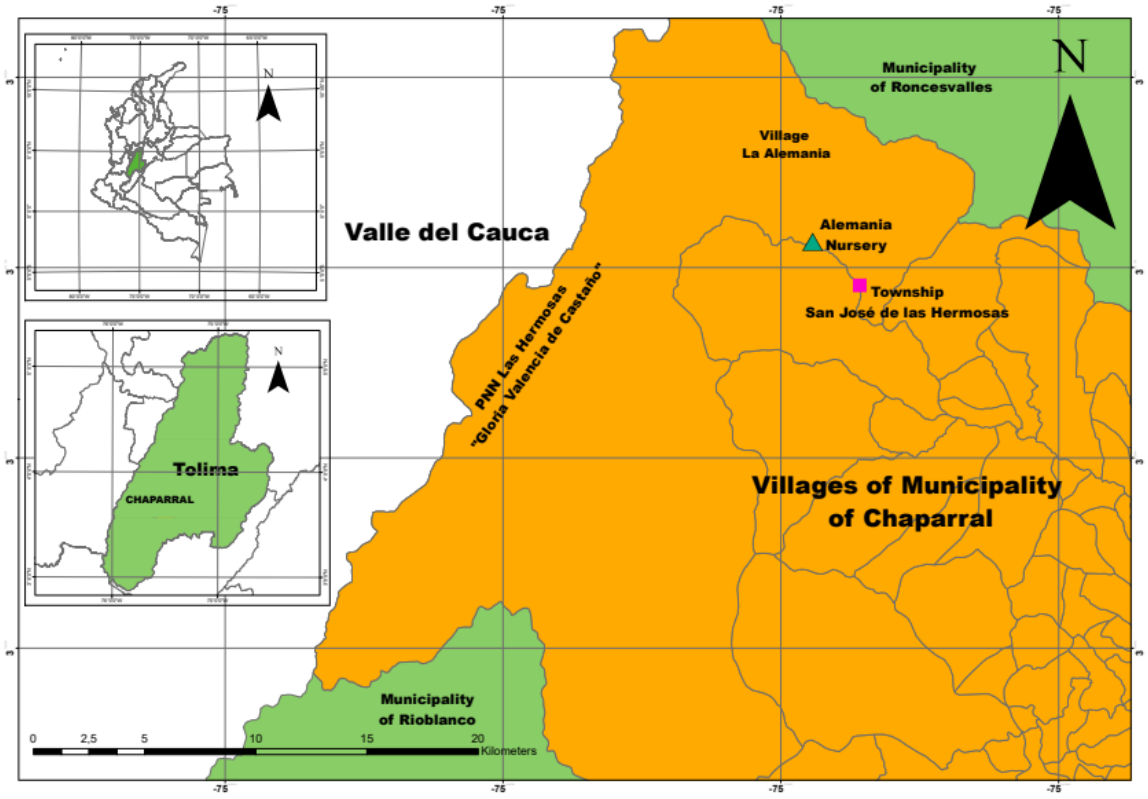

Fig.1 Geographic location of area of study, Amoyá sub-basin Municipality of Chaparral, Township San José de las Hermosas. Village La Alemania 2100 -2300 meters above sea level

Greenhouse experiment

For the greenhouse experiment we used *J. neotropica* seeds from two different populations in Colombia. One population was located in the Amoya river sub-basin in the Tolima department and the other one in the municipality of Sogamoso in the Boyacá department. A pre-germination treatment was applied to all seeds using a deep cleaning process with

metal brushes and an imbibition in water at room temperature for 72 hours, after which the seeds were sown immediately in the seedbeds. Around 100 seeds were germinated for three months in seedbeds containing commercial sterile peat (PINDSTRUP PLUS PEAT SUBSTRATE, blonde peat).

Afterward germination, 60 seedlings were transplanted individually in 20 x 15 cm bags into two different treatments. For the first treatment, 30 seedlings were sown in a mixture of traditional nursery substrate composed of local soil (not collected under *Juglans*), earthworm humus, and sterile rice husks with sprinkler irrigation every 4 days (from now on will be called "vivero" (V) treatment). For the second treatment, another 30 seedlings were sown with traditional nursery substrate and 6% *J. neotropica* inoculum consisting of forest soil collected near wild adult individuals (from now on will be called "nativo" (N) treatment). We collected inoculums from under three different *J. neotropica* adult trees growing at least 100 mt apart, 2 of the adults were inside the forest and 1 was in the pasture area. All inoculum were used independently to control for individual effects and were added as a central cylinder of soil surrounding the seedling root system that accounted for approximately 6% of the total volume of the bag.

The seedlings were transplanted in the bags with the inoculum treatment in July 2022 and were grown for 7 months until February 2023 (inoculum source 1: 13 seedlings, inoculum source 2: 9 seedlings and 3: 8 seedlings, no inoculum (0): 30 seedlings). All seedlings were randomly moved in the nursery every two weeks to avoid growth bias due to their location. All seedlings were monitored once a month and some non-destructive measurements were taken to observe changes in their growth. We recorded the total height of each seedling (measured from the base of the stem to the terminal bud), the diameter of the stem (measured at the base of the stem), and the total number of leaves and leaflets. We calculated the Relative growth rate using the formula: (final data-initial data)# days elapsed.

At the end of the experiment, we harvested 20 seedlings to measure their functional traits (inoculum source 1: 4 seedlings, inoculum source 2: 5 seedlings, inoculum source 3: 4 seedlings and no inoculum (0): 7 seedlings). The leaves, stem and root were dried separately to obtain their dry weight. Then we calculated the leaf mass ratio (LMR), root to shoot ratio (RSR), leaf area ratio (LAR), specific leaf area (SLA) and root mass ratio (RMR) following (Poorter H & Van der Werf 1998; Qi Y et al. 2019; Sebastiani L et al. 2004) using the formulas: *LMR*= Total aboveground biomassleaf biomass, RSR=Root biomassShoot biomass, LAR=Total leaf areaTotal aboveground biomass, SLA=Total leaf areaTotal leaf dry mass, RMR=Root biomassTotal plant biomass.

Characterization of wild seedlings and soils mycobiome

To establish a baseline of the community composition of natural fungal communities associated with *J. neotropica* in this area, we collected roots from 6 wild seedlings

growing in natural forests near the greenhouse and native soils under three *J. neotropica* adults (sources for the inoculum #1, 2 , and 3 of the greenhouse experiment).

As a negative control, we collected roots of 5 seedlings germinated in the sterile peat to identify potential contamination and fungal species present in initial plant material used for the experiment in this substrate, these are the same samples that we use for the treatment of seedling germinated in sterile peat (TE).

Root collection DNA extraction, PCR and DNA sequencing

After the end of the nursery experiment 20 of the 60 seedlings were collected (inoculum source 1: 4 seedlings, inoculum source 2: 5 seedlings, inoculum source 3: 4 seedlings and no inoculum (0): 7 seedlings), then stored in ziplock bags and sent refrigerated to the Ecology laboratory at Universidad del Rosario in Bogotá. Once in the laboratory the entire root system of the 20 seedlings was extracted, and then cleaned with sterile distilled water, then 10 pieces of root of 2cm of each seedling were stored in a 2ml tube filled with 2X CTAB buffer and put at -20°C until DNA extraction. A tube with distilled water was processed along with the samples to be used as negative control during PCR.

For the DNA extraction we used the cetyl-trimethylammonium bromide (CTAB) 2x method (Gardes & Bruns 1993), then we quantify the quality and concentration of the extraction using a Nanodrop microvolume spectrophotometer. The DNA was sequenced on an Illumina HiSeq2500 PE250 using fungus-specific primers ITS5-1737F (GGAAGTAAAAGTCGTAACAAGG) and ITS2-2043R (GCTGCGTTCTTCATCGATGC) at Novogene (Bioinformatics Technology Co. Ltd, Beijing, China).

Bioinformatics

We used the LotuS2 software (Özkurt et al. 2022) to process the raw reads and obtain operational taxonomic units (OTUs) using the following parameters: -ref DB UNITE, -amplicon_type ITS2, -L CA_idthresh 97,95,93,91,88,78,0, -tax_group fungi, -tax Aligner blast, -clustering vsearch, -derep Min 10:1,5:2,3:3, -id 0.97. To give more detail, first we filtered sequences using fastqc. The ITS2 region was extracted using the ITSx software (Bengtsson-Palme et al. 2013) and then used Uchime for chimera removal (Edgar et al. 2011). We used a dereplication step to eliminate very rare OTUs. Clustering of sequences was done using a UPARSE (Edgar et al. 2013) with a 97% similarity threshold. Blast and the UNITE databases (version 9.0 from 2023/07/18) were used to assign taxonomy. Finally we generated a *phyloseq* object to be used in downstream diversity analysis using R software. We assigned the primary lifestyle to the characterized OTUs using the FungalTraits database (Põlme et al. 2020).

Statistical analyses

The statistical analyses corresponding to the seedlings growth were performed in JASP v0.18.0 using one-way ANOVA, two-way ANOVA, T-student tests and U-Mann-Whitney for non parametric data, including Shapiro-Wilk normality test.

All the fungal community diversity and composition analyses were also done in R v4.3.1 (R core Team, 2023). Alpha diversity was compared between treatments by calculating the Chao1, Shannon and Fisher diversity indices using the R package Phyloseq v1.44 (McMurdie et al. 2013). We also made abundance barplots for the taxonomic orders of family and genus taking into account the treatments and also for the primary lifestyle of the characterized OTUs.

The R package Vegan v2.6-4 was used to calculate the species accumulation curves for each of the treatments (Oksanen et al. 2019). Beta diversity was analyzed using Multidimensional scaling (MDS) to visualize the fungal communities and compare them among treatments. In addition, an ADONIS test was carried out where 200 permutations were used to observe significant differences between the treatments (Anderson et al. 2011; Oksanen et al. 2008).

The abbreviations of the treatments used in the analysis are as follows: Native soil (SN), seedling germinated in sterile peat (TE), Native seedling (N), Seedling grown in nursery with seed origin Amoyá (AV), Seedling grown in nursery with native inoculum with seed origin Amoyá (AN), Seedling grown in nursery with seed origin Boyacá (BV) and Seedling grown in nursery with native inoculum with seed origin Boyacá (BN).

Results

Response of height growth rate to inoculum and origin of the inoculum

Overall we found that seedlings subjected to inoculation with native soil exhibited a statistically significant higher height growth rate compared to those that grew in traditional nursery soil ($p= 0.049$, Fig 2A).

In addition, we found that different inoculum sources produced different responses. For example, overall the native soil of origin 2 proved to be more effective at inducing seedlings growth compared with inoculum sources 1 and 3 ($p= 0.03$ Fig 2B). We also found differences in the response of seedlings from different origins to the inoculum treatments. Specifically, seedlings from Amoyá showed a significant positive response to the overall addition of native inoculum ($p= 0.017$, Fig 2C) while seedlings from Boyacá showed a very similar growth rate between the individuals planted in traditional nursery soil and soil with native inoculum ($p= 0.78$, Fig 2D). Also, we found that the Amoyá seedlings sown with inoculum 2 show greater growth than with the other inocula used ($p= 0.042$, Fig 2E) while the results were not significant for the Boyacá plants ($p=0.783$, Fig 2F).

Also, we found that the Amoyá seedlings presented a significantly higher change in their number of leaflets with inoculum number 2 compared with the other inocula used (p= 0.019, Fig 3). Boyacá seedlings did not show a significant response to a specific type of inoculum (p= 0.08, Fig 3). Together, these results indicate that seedlings from Amoyá (local) appear to be more receptive to the benefits of native inoculum in terms of promoting further growth. In contrast, Boyacá seedlings appear to be less sensitive to native inoculum and show similar growth regardless.

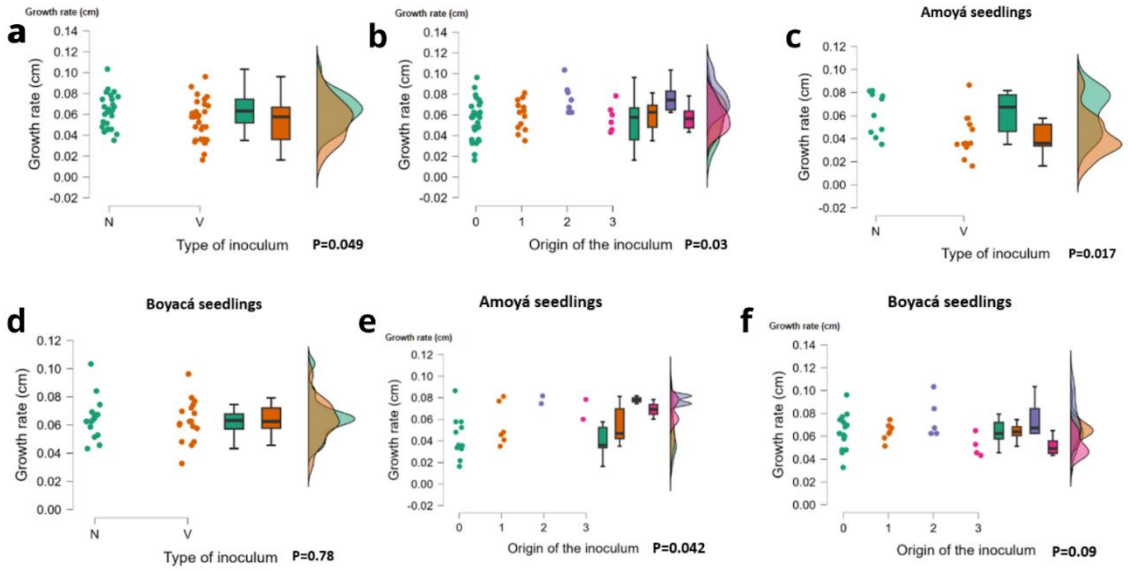

Fig.2 Comparison of the height growth rate of *J. neotropica* seedling. **a.** Seedlings grown with and without native inoculum, where N represent seedlings sown with native soil inoculum and V seedlings without native inoculum. **b**. a comparison of the height growth rate of seedlings with native inoculum collected from three different *J. neotropica* adult trees (1, 2 and 3) and without native inoculum (0). **c**. Height growth rate of seedlings from Amoyá with native soil inoculum (N) and without inoculum (V). **d**. Height growth rate of seedlings from Boyacá with native soil inoculum (N) and without inoculum (V). Comparison of origin of the inoculum and growth rate of seedlings **e**. Seedlings from Amoyá seed origin for the origin of the inoculum, 0 corresponds to seedlings without inoculum and 1,2 and 3 correspond to the three inocula sources used. **f.** Represents the same as **e** but for the Boyacá seedlings

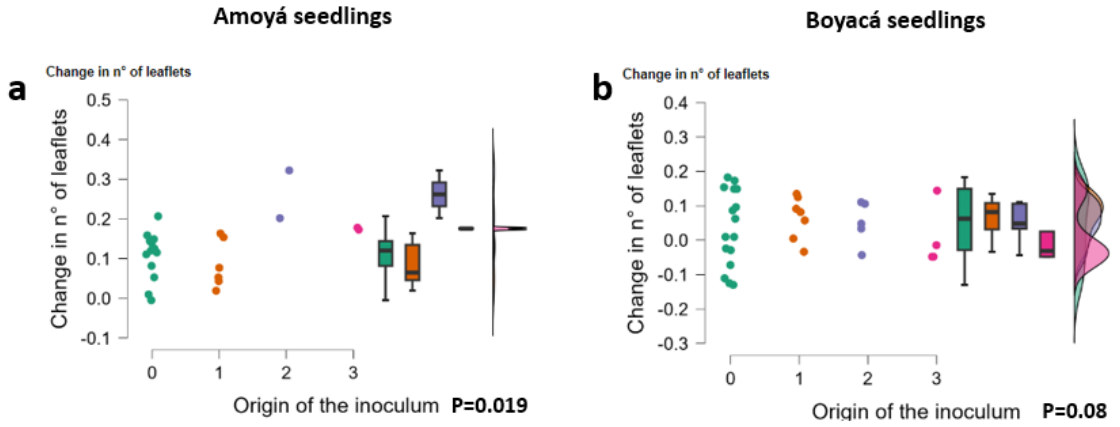

Fig.3 Origin of inoculum and the change in number of leaflets of Amoyá and Boyacá seedlings. **a**. Change of leaflets for the Amoyá seedlings with respect to the origin of the inoculum, where 0 corresponds to seedlings without inoculum and 1,2 and 3 correspond to the 3 inocula used. **b**. Represents the same but for the Boyacá seedlings

Comparison of seedlings functional traits by seed origin and inoculum treatment

We observed significant differences in seedling's height growth rate depending on their seed origin independently of the inoculum treatment. Specifically, seedlings from Boyacá exhibited a notably higher height growth rate compared to those from Amoyá, with a mean difference of approximately 0.02 cm/day (0.6 cm per month). This disparity in growth indicates the influence of genetic factors related to seed origin on seedling development (Fig 4A).

When analyzing the rate of leaflet production we found that the Amoyá seedlings showed a significantly greater production of leaflets in comparison with Boyacá regardless of the inoculum treatment. This result suggests that, although the Amoyá seedlings grow less in terms of height, they compensated for this difference by developing a greater number of leaflets (Fig 4B).

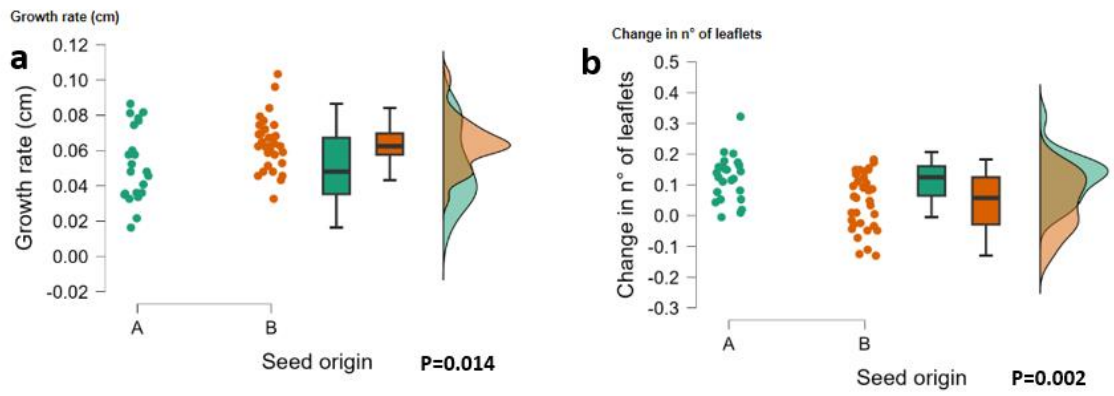

Fig.4 Comparison of growth by seed origin independently of the inoculum treatment. **a**. Height growth rate by seed origin. **b**. Rate of leaflet production by seed origin. A represents the seedlings from Amoyá and B seedlings from Boyacá

We also found significant differences between seedling's functional traits (LMR, SLA, LAR, RMR and RSR) by seed origin. Specifically, seedlings from Amoyá exhibited a significantly higher SLA, LAR and LMR than the Boyacá seedlings (p= 0.005, 0.016 and 0.005 respectively; Fig 5A, 5B and 5D). These results suggest a greater efficiency in the capture and use of sunlight and natural resources available by seedlings from Amoyá. On the other hand, seedlings from Boyacá showed a higher root mass ratio and root to shoot ratio than the Amoyá seedlings (p= 0.014 and 0.010 respectively; Fig 5C and 5E), indicating an adaptation to different soil and nutrient conditions. The effect of the inoculum on functional traits was also evaluated, however, no significant differences were observed (S1).

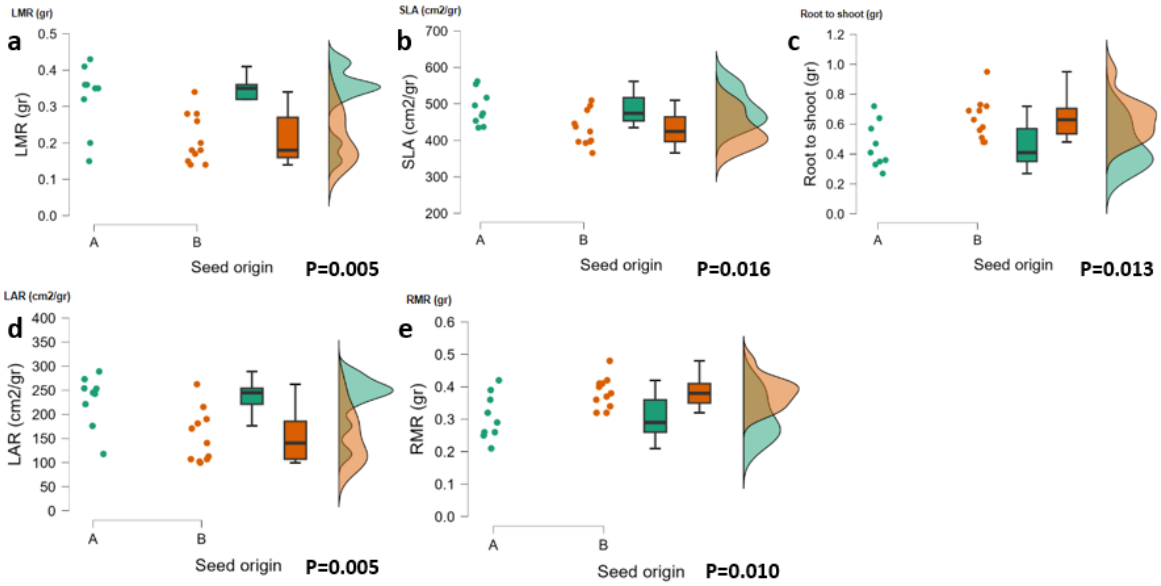

Fig.5 Comparison of seedling functional traits by seed origin. **a**. Leaf mass ratio (LMR) p=0.005, **b**. Specific leaf area (SLA) p=0.016, **c**. Root to shoot p=0.013, **d**. Leaf area ratio (LAR) p=0.005, **e**. Root mass ratio (RMR) p=0.010

Root and soil mycobiome composition

We found a total of 561 OTUs and a total of 132.921 reads in all samples, where the mean of reads by sample was 4.288, equivalent to 4.56GB of data. Also, we found that the majority of OTUs weren't identified to genus (75.36% of OTUs) or family (60.97% of OTUs), this high percentages of unidentified taxa represent an important gap knowledge that affected the resolution of our analysis given that results are usually based exclusively on the identified taxa. This implies that the percentages presented here do not reflect the total number of OTUs and individuals found, but only those that have been identified, leaving out, in some cases, a considerable part of the database.

From all identified OTUs in all samples, the 6 most abundant families/orders were Nectriaceae with 36.68% of reads, Lophiostomataceae 21.6%, Helotiales with 11.19%, and Lasiosphaeriaceae with 6.48%, Archaeorhizomycetaceae with 5.94% and lastly Bolbitiaceae with 5.79% (Fig 6). Also the most diverse families with the greatest number of OTUs were Nectriaceae with 30 identified OTUs, Glomeraceae with 15, Herpotrichiellaceae with 14, and Aspergillaceae and Clavicipitaceae with 11 identified OTUs. We observed a similar community composition between the negative control TE and the treatments AV, AN, BV, BN samples, which corresponded to seedlings initially grown in commercial sterile peat. The most representative family in these samples was Nectriacea, with this family accounting for a very high percentage, between 23- 99%, of the total number of reads (Fig 6).

The fungal community composition of the wild seedlings (N) showed a high percentage of Helotiales with more than 50% abundance in almost all samples and Lophiostomataceae with an abundance of 89.8% in only one of the wild seedlings (N_5). The analysis of native soil from Juglans adults used as inoculum (SN) revealed a high abundance of the Lasiosphaeriaceae family (between 4%-51% across all samples) and Archaeorhizomycetaceae (39.2%) and Bolbitiaceae (38.3%) in samples SN_3. Sample SN_3 was collected in a grazing area with livestock, therefore we believe this could have influenced the observed changes in fungal community composition.

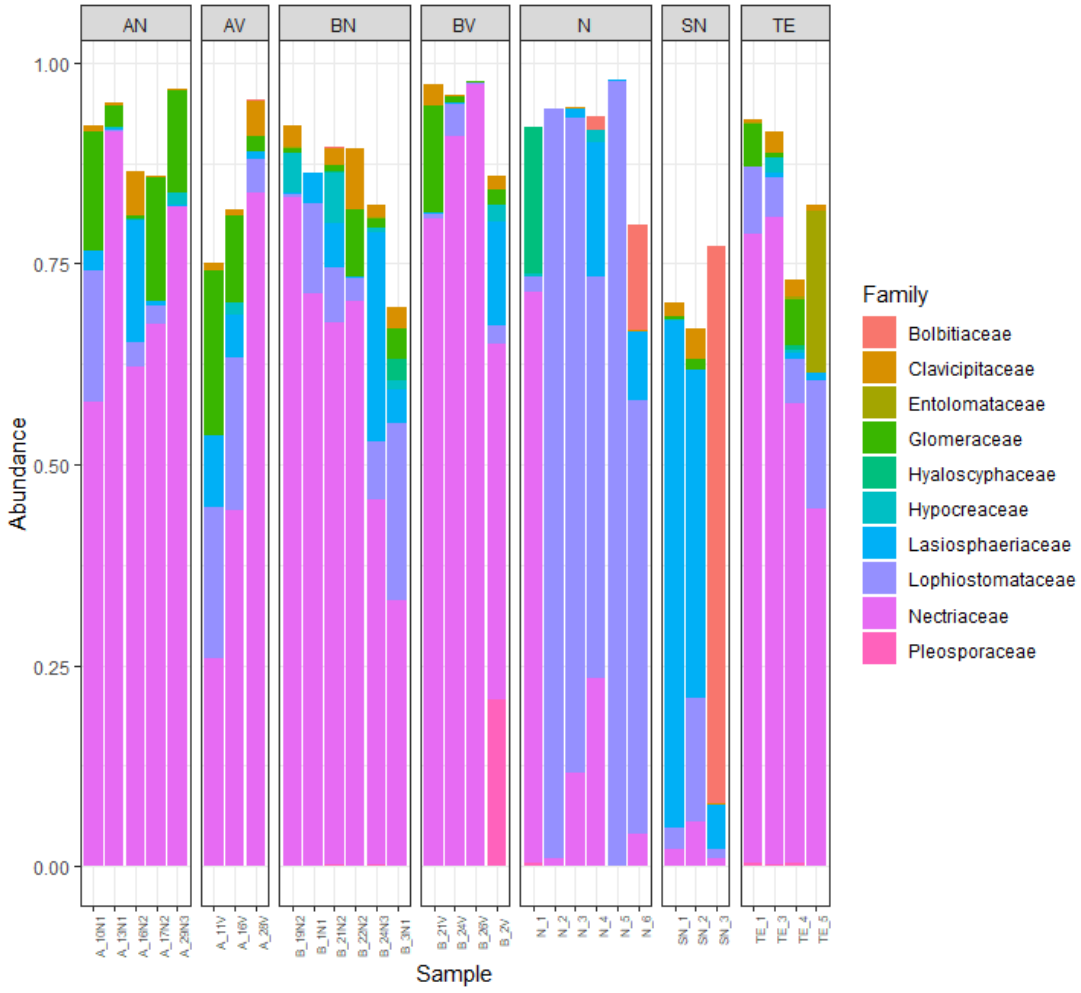

Fig.6 Relative abundance of all identified families of root-associated fungi from the treatments: Seedling germinated in sterile peat (TE), Native soil (SN), Native seedling (N), Seedling grown in nursery with seed origin Amoyá (AV), Seedling grown in nursery with native inoculum with seed origin Amoyá (AN), Seedling grown in nursery with seed origin Boyacá (BV) and Seedling grown in nursery with native inoculum with seed origin Boyacá (BN).

Regarding the distribution of the identified genera in all samples, the fungal community was mostly dominated by saprophytic fungi and plant pathogens with *Lophiostoma* (35.24%), *Fusarium* (19.08%) *Podospora* (9.75%), *Dactylonectria* (9.58%), and *Conocybe* (9.45%) being the most abundant. According to the fungal traits classification, *Lophiostoma* is identified as having a primary wood saprophytic lifestyle and *Podospora* as dung saprophytic lifestyle, while *Fusarium*, *Dactylonectria*, *Ilyonectria* and are mainly characterized as phytopathogens. For the wild seedlings the genus *Lophiostoma* (between 2-99%), and *Dactylonectria* were the most abundant.

At the genus level, we confirmed an increase in the presence of pathogenic genera in seedlings grown in commercial sterile peat, where the predominant genera were *Fusarium* and *Dactylonectria*. On the other hand, the SN and N samples exhibit a prevalence of genera with primary lifestyles as saprotrophs (*Podospora*, Fig 7). This strong differences highlights the impact of the cultivation environment on the diversity of root-associated fungal communities

Regarding AMF, we found that only 0.53% of the fungal OTUs analyzed presented this primary lifestyle. Similarly, only 1.54% of the total number of reads corresponded to AMF in the families *Rhizophagus* representing 0.70% of reads, *Glomus* 0.03%, *Funneliformis* 0.77% and *Claroideoglomus* 0.04% (Fig 8).

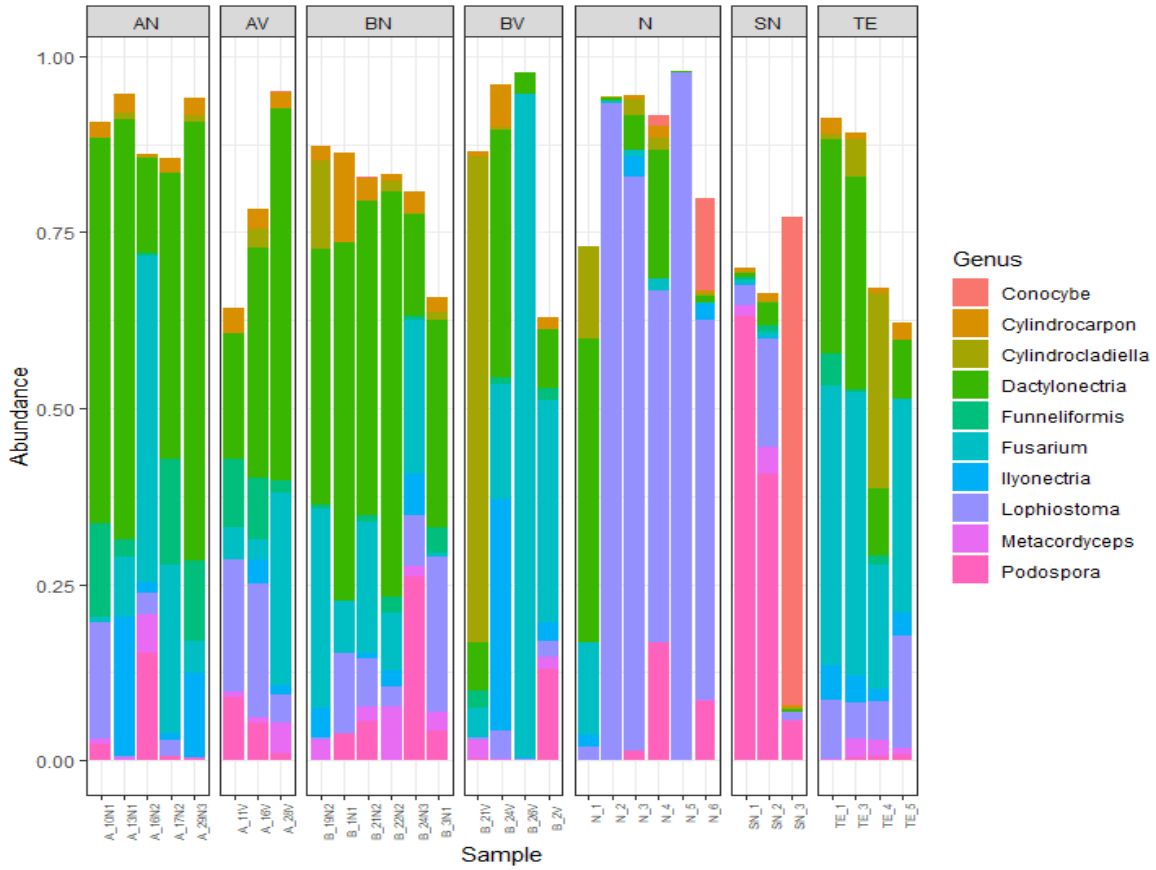

Fig.7 Abundance of top 10 genera of root-associated fungi identified under the treatments: seedling germinated in Sterile peat (TE), Native soil (SN), Native seedling (N), Seedling grown in nursery with seed origin Amoyá (AV), Seedling grown in nursery with native inoculum with seed origin Amoyá (AN), Seedling grown in nursery with seed origin Boyacá (BV) and Seedling grown in nursery with native inoculum with seed origin Boyacá (BN).

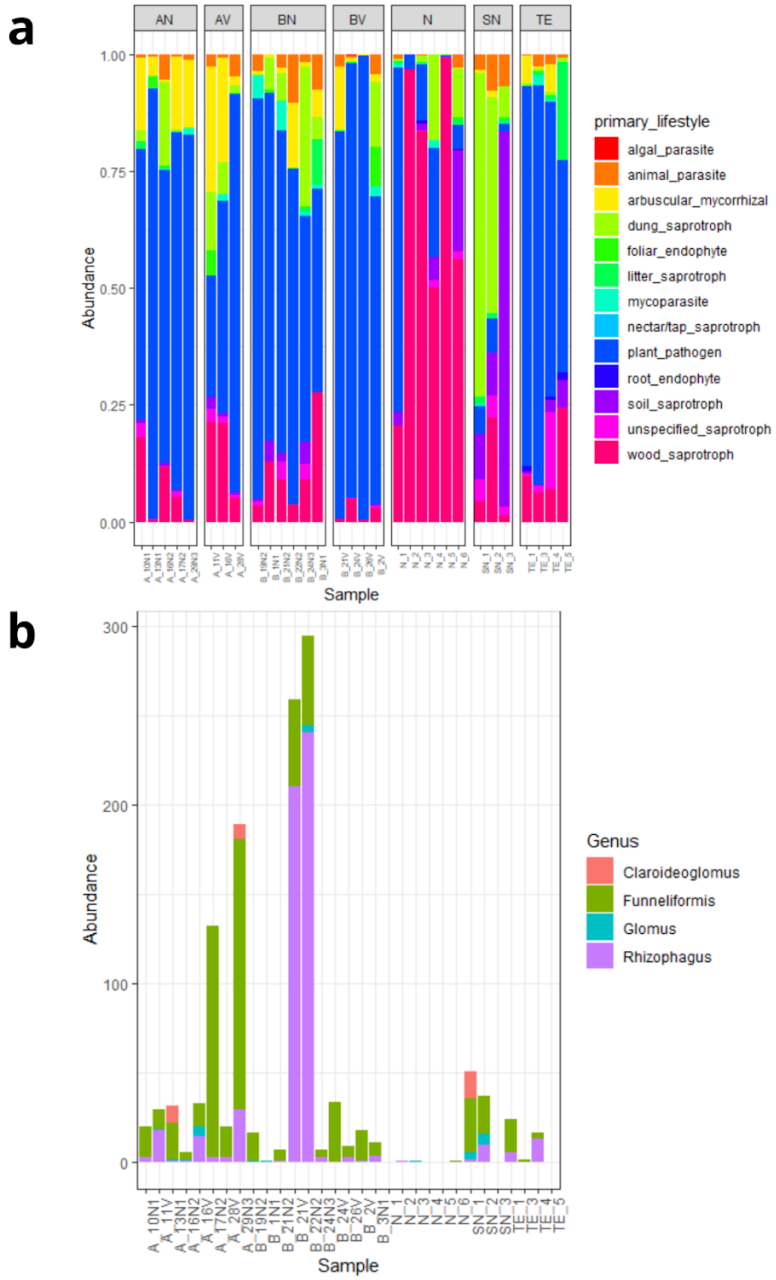

Fig.8 a. Primary lifestyle of the fungal communities for all the treatments and **b**. Abundance of genera identified for primary lifestyle of arbuscular mycorrhizae

Alpha Diversity

Overall we found a total of 561 OTUs in all the samples. The ANOVA results for the richness index Fisher indicate significant differences among the treatments (Fig 9, p= 0.0307). The Tukey test showed that the samples corresponding to TE and SN were the only treatments showing a significant difference in their diversity (p= 0.034, Fig 9).

Besides native seedling and native soil that showed the highest diversity (Fig 9), the greenhouse treatments with the greatest species richness and greatest diversity were BN and AV. Nevertheless, the diversity and richness of all treatments were very similar, and when compared using the Tukey test, no significant results were observed between the AV, BV, AN, and BN treatments. The species present in the TE treatment are intriguing given that this substrate was initially sterile and could have been environmentally colonized after seed plantation.

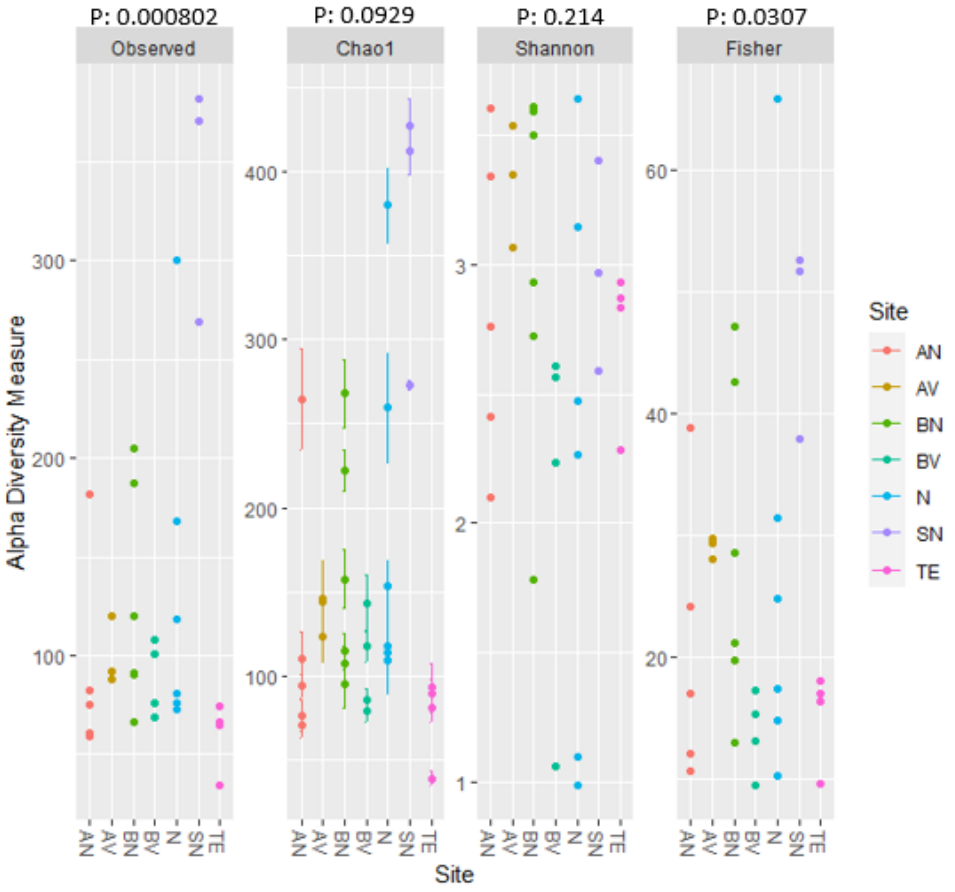

Fig.9 Alpha diversity analysis with total species richness and the Chao1, Shannon and Fisher indices for the all treatments: seedling germinated in sterile peat (TE), Native soil (SN), Native seedling (N), Seedling grown in nursery with seed origin Amoyá (AV), Seedling grown in nursery with native inoculum with seed origin Amoyá (AN), Seedling grown in nursery with seed origin Boyacá (BV) and Seedling grown in nursery with native inoculum with seed origin Boyacá (BN).

The species accumulation curves show a high number of Operational Taxonomic Units (OTUs) consistently in all the treatments, exceeding 500 OTUs (Fig 10A). It is notable to highlight that the treatments with the highest abundance of OTUs corresponded to the ones

that involve *Juglans* native soil, namely, SN, N AN and BN. In contrast, we found a lower number of OTUs in treatments that did not include *Juglans* native inoculum, such BV and AV. Finally, we found a low number of OTUs in the TE treatment that didn't include any soil inoculum (Fig 10B).

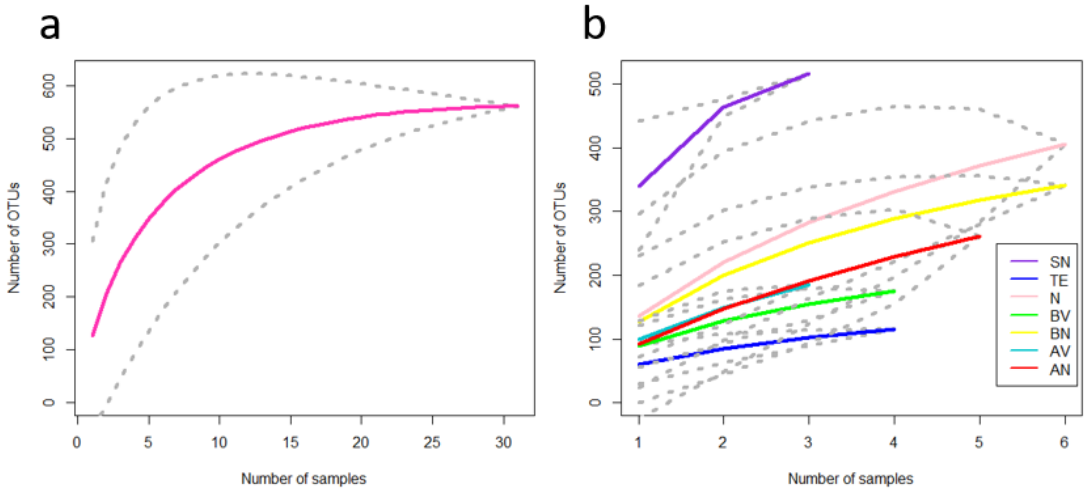

Fig.10 Species accumulation curves showing the total number of OTUs identified in all samples by treatment (SN, N, TE, AV, AN, BV, BN)

Beta Diversity

The results of the Multidimensional Scaling (NMDS) analysis for the analysis of beta diversity revealed significant differences in the fungal community composition among the evaluated treatments. As expected, we found significant differences between the TE treatment and the SN and N (SN and N clustered together, ADONIS test p=0.004975, Fig 11). All the other treatments showed intermediate species composition between TE and SN probably due to the strong effect of the TE treatment in all transplanted seedlings.

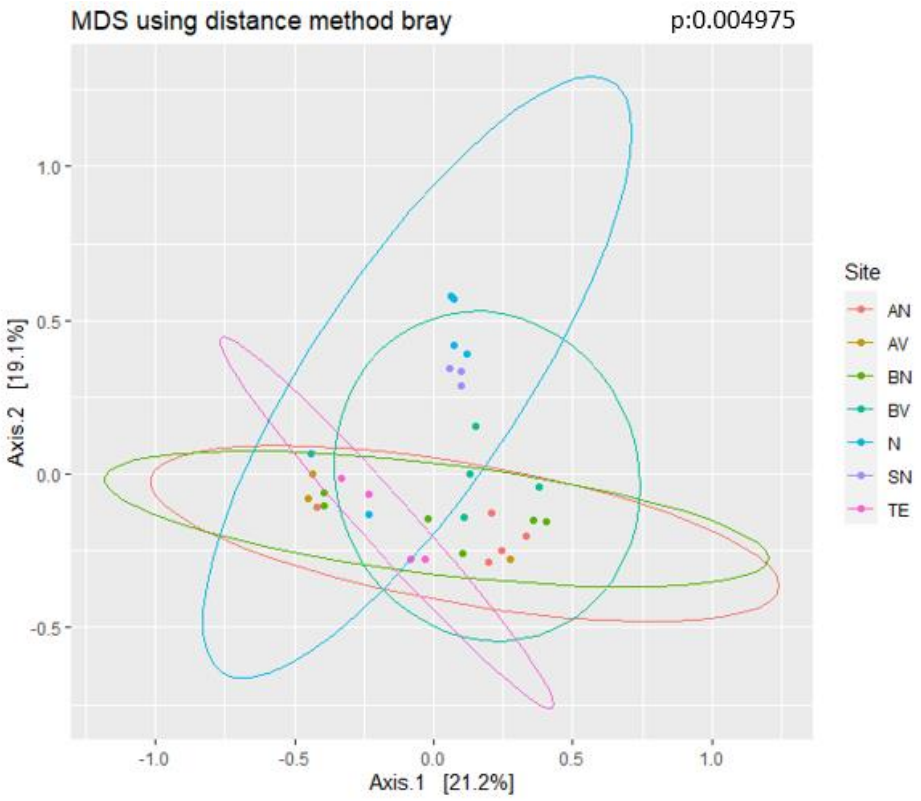

Fig.11 Ordination analysis comparing fungal communities from all samples and treatments (SN, N, TE, AV, AN, BV, BN) using Non-metric Multidimensional scaling (NMDS) using distance method bray-curtis. ADONIS test p=0.004975.

Discussion

To our knowledge this is the first study that uses eDNA to characterize the root-associated fungal communities of *Juglans neotropica*. Our findings show that *J. neotropica* seedlings are positively influenced in their growth when sown with native soil inoculum from adult conspecific trees, which might contain a microbiome that can improve their performance. However, the results of the molecular tests showed that the root-associated fungi composition did not vary significantly between the seedlings inoculated with native *Juglans* soil and those that were not, since a similar microbial composition was observed between the treatments, therefore more research is needed to completely understand the plant responses to inoculation.

Our results show that native soil and wild *Juglans* seedlings hold a very high diversity of soil and root-associated fungal species and a different species composition compared with seedlings grown in the greenhouse. In addition, our plant performance data show that in the case of *Juglans neotropica*, inoculation with live conspecific soil inoculum has a positive impact in seedlings growth. This positive impact could come from an increase in positive interactions like defense against pathogens acting as a defensive barrier, competing for

resources, and producing metabolites that inhibit the growth of harmful agents (Qu Q 2020). In addition, these microorganisms can activate defense mechanisms in plants, triggering biochemical reactions that strengthen their immunity; the inoculation with key microbiota members can improve plant traits (Williams & Ginzel 2022; Qu Q 2020; Compant 2019). More research is also needed to understand the exact mechanism.

The soil and root microbiome plays a fundamental role in restoring ecosystems and plant conservation. Research has highlighted the critical influence that microbial communities have on soil health and plant development (Berendsen et al. 2012). These microbial communities facilitate the uptake of nutrients essential for plant growth and play a crucial role in protecting against pathogens and regulating abiotic stress (Mendes et al. 2013). Microbial diversity in the soil and around roots contributes to ecosystem stability and promotes resilience against environmental perturbations (van der Heijden et al. 2015). Therefore, understanding and managing these microbial communities becomes a critical factor in ecological restoration and conservation efforts of plant species.

In our study, we found a low diversity and richness of AMF, with very low abundance in all samples; these results could be associated with our choice of primers given that it has been shown that the ITS region it's not ideal for characterizing AMF communities (Öpik et al 2014) but we can't discard that there was low initial abundance. Applying AMF in field sites to protect endangered plants is difficult thanks to the complexity of plant community structures and the large amount of fungal inoculum needed (Bothe et al. 2010). Some factors can affect the colonization of AMF. The effectiveness of these interactions, such as the colonization of roots by these mycorrhizae, can vary greatly. The threshold value for an effective symbiosis still needs to be clarified.

The effectiveness of symbiosis between plants and AMF can be influenced by the diversity and richness of AMF taxa (Bothe et al. 2010). The presence of AMF was detected in both the seedlings cultivated with sterile peat and those with native inoculum, but their prevalence was relatively low in both cases (Fig 8B). However, 4 genera of AMF could be identified, which are *Claroideoglomus, Funneliformis, Glomus and Rhizophagus*, where the genera with the highest prevalence in the samples are *Rhizophagus and Funneliformis*, standing out in samples from the BN and AN treatments (Fig 8B). These fungi mitigate soil-borne diseases and support overall plant health by strengthening nutrient absorption, regulating water balance, and promoting soil structure. Its fundamental role in acquiring nutrients and water relations helps plants cope with environmental conditions (Jeffries et al. 2003). Additionally, AMF contributes significantly to the biogeochemical cycling of nutrients within ecosystems, promoting a balance of nutrients essential for the health and resilience of plant and soil communities (Genre et al. 2020).

As shown in Fig 8A, in the analysis of the seedling germinated in sterile peat samples, a high abundance, 17.11% (37.33% of reads) of all species were identified as having a primary phytopathogen lifestyle, suggesting the presence of organisms that can negatively

affect plants in this particular environment. In this case, 26.32% of the total phytopathogens belong to the genus *Fusarium*, 15.79% to *Cylindrocladiella*, 10.53% to *Mycoleptodiscus* and the genera *Curvularia, Septoria, Phaeoacremonium, Nectria, Cylindrocarpon, Volutella, Gibberella, Ilyonectria* and *Dactylonectria* make up the remaining 47.37 %. Some well known phytopathogenic species were identified, such as *Phaeoacremonium venezuelense, Volutella ciliata, Gibberella zeae, Cylindrocladiella elegans* and *Dactylonectria anthuriicola*. Commercial sterile peat could have a high potential for spreading species that can harm seedlings. The Nectriaceae family, one of the most abundant families in our results, can have a primary lifestyle as plant pathogens as can be seen in Fig 6 and Fig 8, therefore understanding the factors leading to this pattern should be studied carefully. The most abundant genus among the phytopathogens found in the samples was *Fusarium*; this plant pathogen represents a threat to essential and vital crops and cereals for the human and animal diet because it causes diseases such root rot (RR), crown rot (CR), and *Fusarium* head blight (FHB) (Askun 2018; Booth 1971).

The findings of our study raise concern because sterile peat, widely used for planting seedlings, seems to facilitate the conditions for pathogenic proliferation in the roots during seedling germination and could have long lasting effects in the fungal community composition even after the seedlings were transplanted into live soils. This finding raises questions about the quality and safety of fundamental material in seedling germination since the significant presence of these pathogens could negatively impact the growth and development of plants from the first stages of their cultivation. Chavarro et al. (In prep) also found a large percentage of pathogenic fungi in sterile peat substrate used for propagation of *Coccoloba uvifera* seedlings in Cartagena, Colombia. Therefore, more research is needed to understand the origin of these microorganisms within commercial sterile peat and the potential flaws in sterilization of this substrate.

On the other hand, in the native soil samples, a high abundance of species with primary lifestyles of dung saprotroph and soil saprotroph was observed, indicating a community of microorganisms adapted to decompose organic matter in the soil. Furthermore, when examining samples from native seedlings, a notable abundance of species primarily associated with wood saprotrophs was evident, suggesting a relationship with the decomposition of plant materials (Fig 8A).

We also observed strong differences between the effect of individual *Juglans* inoculum sources. As we can see in Fig 2E , inoculum 2 produced higher growth rates in seedlings originating from Amoyá. Likewise, the genotype of the plant can be decisive both for the development and growth of the seedling and in the attraction and selection of beneficial microorganisms in its root environment (Busby et al, 2022; Compant 2019). This was demonstrated by the significant difference that was observed between the response of the seeds of Amoyá origin versus the Boyacá seeds, with the Amoyá seedlings, that were local, showing a more positive response to the native soil inoculum than the seedlings of Boyacá origin.

Furthermore, it was observed that Amoyá seedlings tended to focus their development on leaf growth, while Boyacá seedlings showed a greater preference for root development and growth. In this way, the dynamic interaction between forestry propagation practices and plant genotype can shape the microbial community associated with roots, influencing their diversity and functionality (Compant 2019). This comprehensive approach can improve plant health, resistance to diseases and stress to biotic and abiotic factors, as well as efficiency in nutrient absorption (Werner 1992; Vandenkoornhuyse et al. 2015).

Overall, our study underlines the crucial relevance of integrating the native microbiota into the plant propagation processes to increase the chances of survival, increase growth rate and strengthen resistance to pathogens. We observed that native soil inoculation can have diverse impacts depending on seedling genotype, underscoring the importance of recognizing that inocula do not have a uniform effect in all situations. It is essential to highlight that the root mycobiome of native *J. neotropica* individuals in our study area was characterized by a high abundance of saprotrophic fungi, which carries numerous benefits for soil nutrient cycling in the ecosystems where these plants thrive. Furthermore, it is essential to recognize that plant restoration plans often neglect microbial communities, which play a positive and indispensable role in plant health. Integrating these microbial communities in restoration plans could significantly improve the effectiveness and sustainability of these projects. However, much more research is still needed to generate better strategies for integrating the microbiome into the seedlings that will later be transferred into the ecosystems.

It is important to emphasize that this study had limitations in the available budget, which directly affected the ability to conduct comprehensive sampling. Furthermore, another important limitation was the impossibility of measuring the functional characteristic of the Specific root area (SRA) due to the limitations of the available equipment. In particular, the small size of the available scanners limited our ability to obtain accurate and representative measurements of this important parameter. These limitations should be considered when interpreting the results and considering their generalization to a broader context.

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Declarations

Financiation

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Data disponibility

- All the sequences produced in the study will be submitted to the Genebank's Sequence read archive (SRA)

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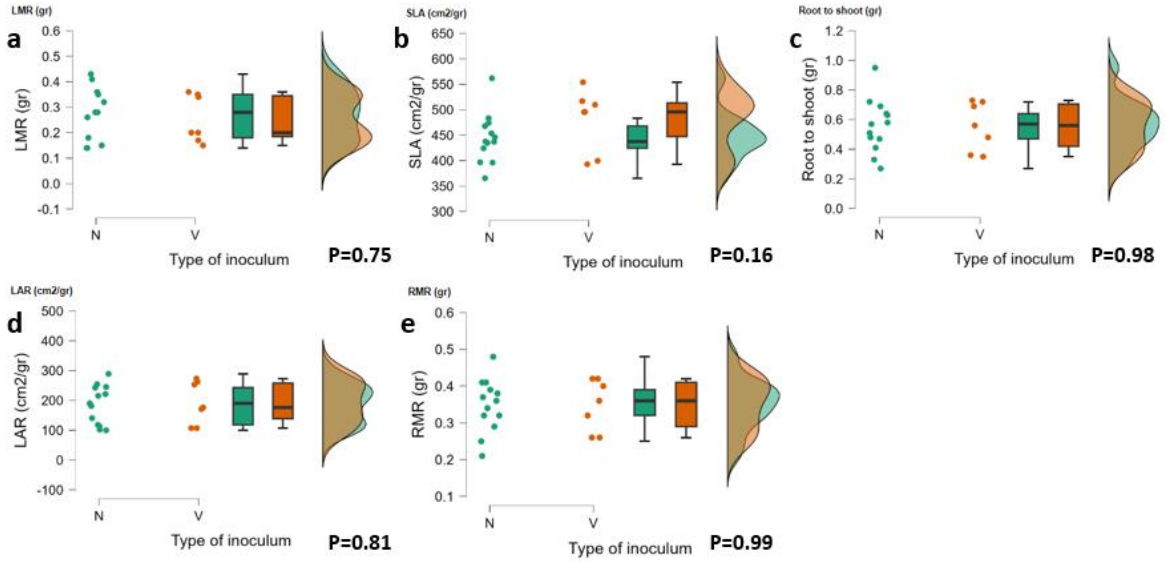
Online resources - Supplementary material



S.1 Comparison of seedling functional traits by type of inoculum. a. Leaf mass ratio (LMR) p=0.75, b. Specific leaf area (SLA) p=0.16, c. Root to shoot p=0.98, d. Leaf area ratio (LAR) p=0.81, e. Root mass ratio (RMR) p=0.99