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Links between Tropical Forest Restoration and Fungal Communities

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Abstract

Tropical forests are continuously reshaped by deforestation and natural regeneration. While the processes governing above-ground forest regeneration are relatively well understood, the role of the below-ground biome, particularly soil fungal communities, in driving forest recovery remains a major open question. This study investigates the relationships between tropical forest regeneration and soil fungal communities during the early stages of post-agricultural recovery in tropical dry and wet forests in Mexico and Australia. By integrating vegetation surveys, soil physicochemical data, and fungal community analyses, we assessed how above- and below-ground components respond to forest regeneration. Our results reveal clear regeneration signals in Mexican forests, with contrasting trajectories between forest types. Wet forests exhibited pronounced plant species turnover, whereas dry forests showed more stable community compositions. Across sites, fungal communities were dominated by saprotrophic fungi and displayed similar relative abundances of arbuscular and ectomycorrhizal fungi. Soil properties, particularly pH and nutrient availability, emerged as the primary drivers of fungal community composition. A strong coupling between plant and fungal communities was detected in Mexican wet forests. In contrast, fungal communities in Mexican dry forests became increasingly homogeneous over time, indicating convergence during early recovery, accompanied by a decline in fungal richness. Both arbuscular and ectomycorrhizal fungi were closely associated with vegetation recovery, underscoring their functional importance for plant establishment and growth. Overall, this study highlights the tight linkage between soil fungi and forest regeneration and emphasizes the need to incorporate below-ground processes into the assessment and management of tropical forest restoration.

Zusammenfassung

Tropische Wälder werden durch Abholzung und natürliche Regeneration ständig umgestaltet. Während die Prozesse, die die oberirdische Waldregeneration steuern, relativ gut verstanden sind, ist die Rolle des unterirdischen Bioms, insbesondere der Bodenpilzgemeinschaften, für die Wiederherstellung des Waldes noch nicht ausreichend erforscht. Diese Studie untersucht die Zusammenhänge zwischen der Regeneration tropischer Wälder und Bodenpilzgemeinschaften in den frühen Phasen der Sukzession nach landwirtschaftlicher Nutzung in tropischen Trocken- und Feuchtwäldern in Mexiko und Australien. Durch die Integration von Vegetationserhebungen, physikalisch-chemischen Bodeneigenschaften und Analysen der Pilzgemeinschaften haben wir untersucht, wie ober- und unterirdische Komponenten auf die Waldregeneration reagieren. Unsere Ergebnisse zeigen deutliche Regenerationssignale in mexikanischen Wäldern, wobei sich die Waldtypen in ihrer Entwicklung unterscheiden: Feuchte Wälder wiesen einen ausgeprägten Artenwechsel auf, während trockene Wälder eine stabilere Zusammensetzung der Pflanzengemeinschaft besaßen. An den meisten Standorten wurden die Pilzgemeinschaften von saprotrophen Pilzen dominiert und wiesen eine ähnliche relative Häufigkeit von arbuskulären Mykorrhiza-Pilzen und Ektomykorrhiza-Pilzen auf. Die Bodeneigenschaften, insbesondere der pH-Wert und die Nährstoffverfügbarkeit, erwiesen sich als die wichtigsten Einflussfaktoren für die Zusammensetzung der Pilzgemeinschaften. In mexikanischen Feuchtwäldern wurde eine starke Kopplung zwischen Pflanzen- und Pilzgemeinschaften festgestellt. Im Gegensatz dazu wurden die Pilzgemeinschaften in mexikanischen Trockenwäldern im Laufe der Zeit zunehmend homogener, was auf eine Konvergenz während der frühen Erholungsphase hindeutet, begleitet von einem Rückgang der Pilzvielfalt. Sowohl arbuskuläre Mykorrhiza-Pilze als auch Ektomykorrhiza-Pilze standen in engem Zusammenhang mit der Erholung der Vegetation, was ihre funktionelle Bedeutung für die Etablierung und das Wachstum von Pflanzen unterstreicht. Insgesamt weist diese Studie auf den engen Zusammenhang zwischen Bodenpilzen und der Regeneration von Wäldern hin und betont die Notwendigkeit, auch unterirdische Prozesse in die Bewertung und das Management tropischer Wälder miteinzubeziehen.

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1 General Background Introduction

1.1 Tropical Forests Under Change

Of the approximately 4 billion hectares of forest worldwide, 45 % are in the tropics (FAO, 2020). Although these forests make up less than 14% of earth's land surface (FAO, 2020), they are home to more than half of all terrestrial invertebrate species (Pillay et al., 2022), a majority of the world's already described and still undescribed tree species (Gatti et al., 2022) and probably millions of insect species, with the most part being currently unknown to science (Novotny & Miller, 2014). When looking at fungi, far less is known about their biodiversity in the tropics since most molecular fungi research takes place in non-tropical areas, with long term studies being rare (Stallman et al., 2024).

In general, we do not know a lot about the total extent of biodiversity in these forest ecosystems, and with the current rate of forest loss, we might never know: The earth loses millions of hectares of forest per year (FAO, 2020). While annual global forest loss has decreased in the last few decades (FAO, 2020), the loss of tropical forests has been rising, with an average of around 4 MHa per year (Goldman et al., 2025).

This should point out one thing: A huge part of life on earth and especially in the tropics is already under threat of extinction (IUCN, 2025) and a prerequisite for trying to change this is knowledge. Thus, in this chapter I shall briefly summarize what tropical forests are and how they are structured. It shall expand on the extent and the reasons behind land-use change in these tropical forest ecosystems, but also elaborate on how these recover, naturally or assisted.

1.1.1 Ecological Zones and Forest Structure

According to the Food and Agriculture Organization of the United Nations (FAO) a forest is considered tropical if it is frost-free throughout the year and never has a minimum temperature lower than 18 °C. As shown in **Fig. 1**, the tropics encompass different ecological zones, which are defined by their precipitation and duration of the dry[1] season. Following this, a rain forest has a dry season lasting between 0-3 months, a moist deciduous forest between 3-5 and a tropical dry forest between 5-8 months (FAO, 2012).

[1] In this framework, a month is dry if the precipitation in mm is equal or lower than double the mean temperature in °C.

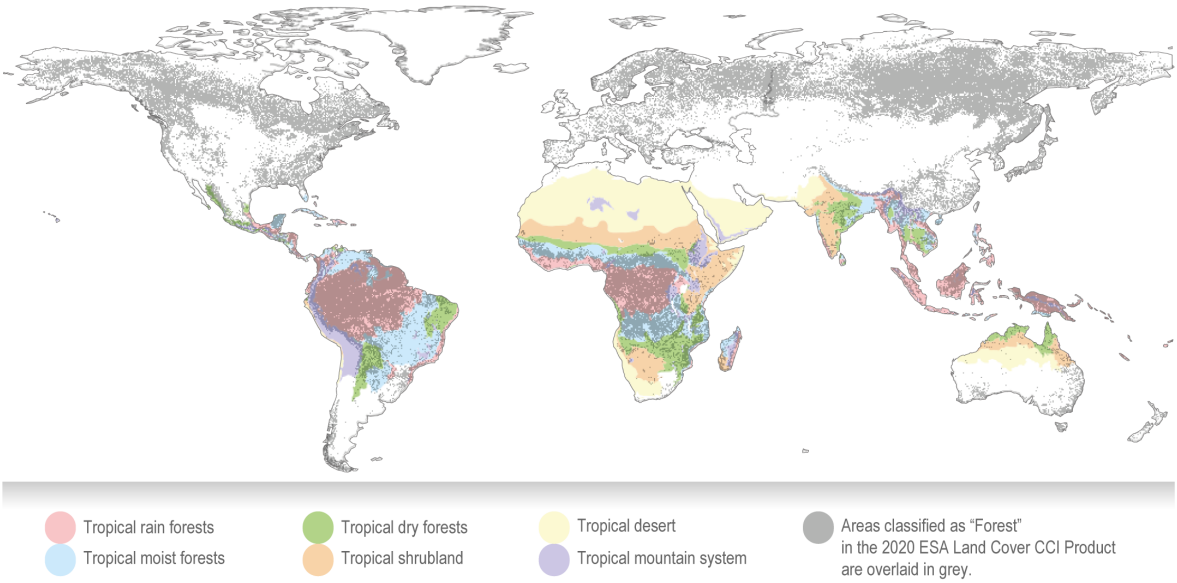

Fig. 1. Tropical ecological zones as defined by the FAO. Adapted from Ometto et al. (2022). The colored areas show where the meteorological conditions meet the definition of each zone. Tropical wet forest zones are shown in red and blue and tropical dry forest zones in green. Their intersect with the gray area shows the true forest extent within each ecological zone.

The structure of tropical dry forest can be described by vegetation strata (Osborne, 2000): After a relatively sparse understory, trees of 10-15 m with narrow crowns emerge. The competition for light is strong here due to the vast middle-story layer, which builds the roof of the rain forest. These trees are usually 25-35 m high and their crowns grow into all directions in search of light. Above that stratum, single very tall trees with heights of 45-50 m emerge. Depending on the moisture content, other plants, like epiphytes and lianas, grow on and from the trees, which can be seen in **Fig. 2**.

Fig. 2. Regenerating tropical wet (cloud) forest in Tandayapa, Ecuador. Taken by Matthias Meschik.

In contrast to wet forests, the structure of tropical dry forests strongly depends on the amount of received rain and is not as pronounced (Petruzzello, 2022). In general, they

have a lot of deciduous tree species as well as lianas and epiphytes.

Estimated by Chazdon et al. (2025), there have been around 888 MHa of undisturbed tropical wet forest and 570 MHa of undisturbed tropical dry forest worldwide in 2023. *Undisturbed* in this context means that the last disturbance has occured more than 20 to 40 years ago depending on the forest context. However, in this respective time frame an area of 225 MHa has undergone forest loss while only 202 MHa of tropical forest have been regenerating across the Americas, Africa, Asia and Oceania. Interestingly, regenerating tropical forests are relatively young, with the median years since the last disturbance being below 10 across all continents. This is a result of frequent re-clearance of forest land for land use, which shall be discussed in the following section.

1.1.2 Forest Loss and Land-Use Change

Land-use change is the most important driver of terrestrial ecosystem degradation and biodiversity loss worldwide (IPBES, 2019). More than a third of the terrestrial land surface is currently used for agricultural purposes. The most widespread form of land use change, and in turn ecosystem degradation is agricultural expansion.

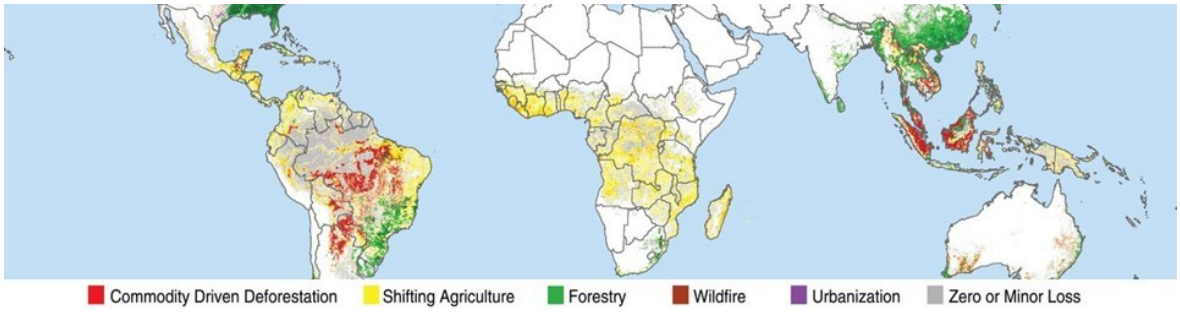

Fig. 3. Primary drivers of forest cover loss from 2001 to 2015. Adapted from Curtis et al. (2018), with the following category definitions: *Commodity-driven deforestation* refers to the permanent conversion of forests or shrublands into land used for agriculture, mining, or energy infrastructure. *Shifting agriculture* involves small- to medium-scale clearing of forest or shrubland for farming, which is later abandoned and typically followed by forest regrowth. *Forestry* describes large-scale logging or tree harvesting within managed forests or plantations, where forest regrowth is evident in subsequent years. *Wildfire* is large-scale forest loss caused by burning, without subsequent signs of human land conversion or farming activity. *Urbanization* denotes the conversion of forest or shrubland to expand or intensify urban areas.

A global analysis from Curtis et al. (2018) suggests that between 2001 and 2015 an estimated 27% of global forest loss can be attributed to long-term or permanent land conversion for commodity production, such as agriculture or mining. Another 26% was attributed to forestry and 23% to wildfires. However, as **Fig. 3** shows, in many tropical areas, shifting agriculture is predominant, causing around 24% of global tropical forest

loss. In this practice, the land is cut or burned down and used for agricultural purposes for a short period of time, hence its alternative name *slash-and-burn* agriculture (Mertz et al., 2009). Afterwards, the land is either left to itself to regenerate or planted with trees for commercial use. In terms with traditional ecological knowledge, this practice keeps the overall ecosystem intact because the burned area is only cultivated for a few years and left to regenerate for many decades (Chazdon, 2014). However, a growing population has forced farmers to shorten the regeneration period to a decade or less, making shifting agriculture unsustainable and stimulating ecosystem degradation.

Another big threat to tropical forests are fires. In seasonally dry tropical forests, fires occur naturally on a regular basis and dominant tree species are adapted to reoccurring wildfires (Chazdon, 2014). Tropical wet forests are fire resistant due to the high moisture content and the absent litter accumulation due to evergreen tree species. However, this changes during drought. Especially in the Amazon, multiple strains on the ecosystem can occur at once, such as drought enhanced by climate change, logging and wild fires (Chazdon, 2014). In these cases, a positive feedback is at work: Logging opens up the canopy and decreases the humidity in the understory. This promotes the colonization of (invasive) grasses, which serve as perfect tinder during a severe drought and decrease humidity even more. A usually very fire-resistant ecosystem is therefore transformed into a fire-prone one . The wildfires then burn through the forest, opening up the canopy even more and stimulating grass growth. In years of extreme drought, this has an especially huge impact. For instance, in 2024, tropical primary forest loss increased by 80% compared with the former year (7 Mha) and 50% of this was caused by wildfires (Goldman et al., 2025).

As this section shows, the destruction is immense and with the loss of habitat, a loss of the habitat-building species comes: In the tropics and subtropics, 30% of all tree species are threatened in lowland moist forests, 41% in montane moist forests, and 39% in dry forests (Rivers et al., 2023). The need for restoration is therefore more urgent then ever before.

1.1.3 Natural Forest Regeneration

If a tropical forest ecosystem has been degraded over many years or decades, the question of how to restore it to native or old growth conditions is of utmost importance in a time of accelerating biodiversity loss and global climate change. As explained in detail in **1.1**, there are different approaches to ecological restoration, but the most feasible on a global scale is natural regeneration. Global models suggest that 215 mHa of land could be restored through natural forest regeneration, with over half the area lying in Brazil, Indonesia, China, Mexico and Columbia (Williams et al., 2024). In this section

I shall describe mechanisms of natural forest regeneration in the tropics, timelines and how it relates to forest attributes.

The underlying process of natural forest regeneration is succession. This is the concept of how populations, communities and ecosystems change over time after part or the whole of the ecosystems has been disturbed (Poorter et al., 2023). There are numerous theories on how succession takes place, including ideas relating to individual forest patches all the way to the socio-economical context of restoration. Thus, the synthesis theory in Chazdon (2014) for wet tropical forests shall be discussed in more detail.

A forest regeneration trajectory strongly depends on its initial conditions. As **Fig. 4** shows, first short- and long-lived pioneer trees establish in the area, which has been disturbed through processes like tree falls, wild fires, landslides, or which have been used for agriculture and subsequently abandoned to regrow. The disturbance can be categorized by spatial extent, frequency, duration and severity (Waide & Lugo, 1992). Importantly, natural, moderate disturbance can enhance forest structure as well as composition and resistance, since it allows for the colonization of disturbance-tolerant *and* disturbance-sensitive species (Chazdon, 2014).

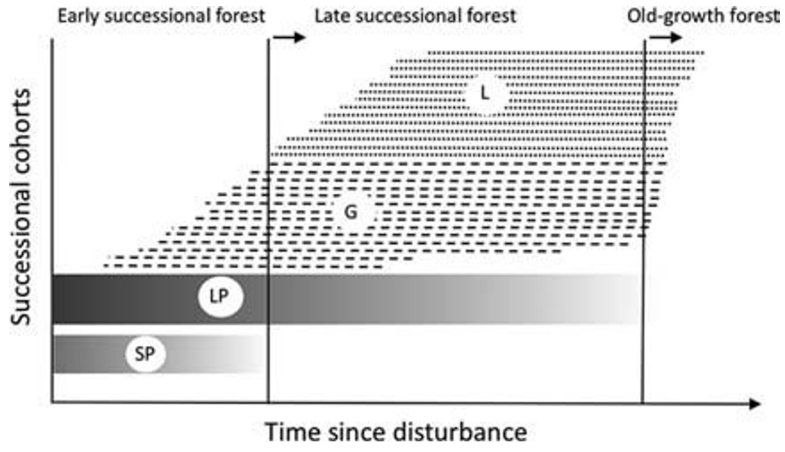

Fig. 4. Conceptual illustration of successional stages in a tropical wet forest. Taken from Chazdon (2014). A framework based on Wirth et al. (2009) for distinguishing early successional forests, late successional forests, and old-growth forests. Short-lived (SP) and long-lived (LP) pioneer trees are thought to establish quickly and almost at the same time during the start of succession. In contrast, generalists (G, mid-successional species) and late-successional specialists (L) continue to colonize throughout the process. The shift from early to late successional forest occurs when late-successional species replace the short-lived pioneers. The progression from late successional to old-growth forest happens when the long-lived pioneer cohort declines, representing the final remnant of the original disturbance.

The fast growing pioneer trees can grow up to 20 m, creating another vegetation stra-

tum below them. After short-lived pioneers die out, room is made for longer-lived generalists and late successional specialists. This creates the complex vertical structure, explained in **1.2.1**, reaching a stable but not static state after 100-200 years (Chazdon, 2014). In old growth forests, species can be categorized into three categories (Chazdon, 2014): Light demanding species depend on large canopy gaps for establishment and regeneration. Shade tolerant species can establish and recruit without the presence of gaps. Gap-requiring species, by contrast, need gaps for successful recruitment into the canopy, though not necessarily for seedling establishment.

In reality, the picture is much more complicated: Following the mosaic theory, small forest patches up to 100 m have unique dynamics and over the whole ecosystem create a complex mosaic of patches, with their own dynamics, depending also on the dynamics of adjacent patches (Chazdon, 2014). Succession can also stop at a given moment if (invasive) grasses or ferns quickly colonize the disturbed area, taking up space and making it impossible for trees to establish. This *arrested succession* can be controlled by human interference. Overall, succession is slower in tropical dry forests, but they are more resilient when it comes to disturbance due to their simple structures. (Murphy & Lugo, 2003).

Successional processes in dry tropical forests differ from those in wet forests. This results from slower canopy closure, predominantly deciduous trees, and the larger role of resprouting in tropical dry forests (Holl, 2007). Following Arroyo-Mora et al. (2005) four successional stages, based on vegetation structure and leaf-flushing patterns, could be made out in the tropical dry forests of northwestern Costa Rica. After the pasture stage, early succession is marked by scattered woody plants, shrubs, and grasses, with a single tree layer reaching 6-8 meters in height. Intermediate forests develop two vegetation layers, 10-15 meters tall, with up to 80% of the trees losing their leaves during the dry season. In late successional stages, forests have three layers and reach 15-30 meters in height. Evergreen crowns cover 50-90% of the canopy, shedding their leaves only briefly and at different times of the year.

In general, tropical forests regenerate mainly through four different modes after a disturbance following the framework developed in Chazdon (2014): Firstly, trees resprout from damaged stems or roots, which plays an important role in the tree regeneration in shifting agriculture, especially in dry forests. Secondly, they germinate and grow from the soil seed bank, in which seeds can stay dormant for decades. Here, the conditions for germination are seed specific, for instance some seeds need temperature variations others red light. Thus, disturbance events, like tree falls, play an important role because they dramatically change the topsoil conditions, promoting germination.

Especially seeds of pioneer trees can stay in the seed bank for many decades, waiting for the right conditions. Thirdly, some trees or seedlings survive the disturbance, which makes them important nuclei for local seed dispersal (*advanced regeneration*). These so-called remnant trees play a crucial role in natural regeneration after agricultural land use. These three tree sources form the ecological memory of a forest, making it resilient. Lastly, seeds often come from outside the disturbed area, like a nearby forest patch, transported through wind or animals. This is crucial not only for areas with little or no trees, but also for late-successional tree species, whose seeds are usually short-lived, germinating quickly after dispersal. After agricultural land use, the forest regeneration modes depends on the land use intensity, with fewer modes available the higher the intensity is.

There are many factors that influence natural regeneration. **Fig. 10 a)** shows positive and negative socio-ecological factors for the probability, that natural regeneration occurs, as well as for the quality of natural regeneration. In general, accessibility, soil quality and vegetation cover promote agricultural areas going into natural regeneration, whereas its quality depends on the initial vegetation, the proximity to other forests and the land use history. When it comes to local and landscape conditions, that support rapid and diverse regeneration of abandoned agricultural fields or pastures, the following are important (Chazdon, 2014):

- Preservation of fertile topsoil
- Close proximity to existing forest patches
- Ability of trees to regenerate from roots or stems
- Presence of a viable soil seed bank
- Seed availability from early- and late-successional woody plants in the seed rain
- Ongoing inflow of native species from neighboring areas
- Natural suppression of weeds through pioneer shrub or tree establishment
- High levels of animal and microbial diversity (e.g., insects, vertebrates, soil fungi)
- Safeguards against repeated fire events

If conditions are suitable, the question arises, how do forest attributes change with time. **Fig. 10 b)** gives an overview. Soil (bulk density and Carbon) recovers the fastest, followed by plant functioning (wood density and specific leaf area) (Poorter et al., 2021). Tree species diversity and forest structure (biomass and structural heterogeneity) take already multiple decades to recover to 90% of old growth values. With over 100 years, species composition takes the longest, as late successional specialists have to establish.

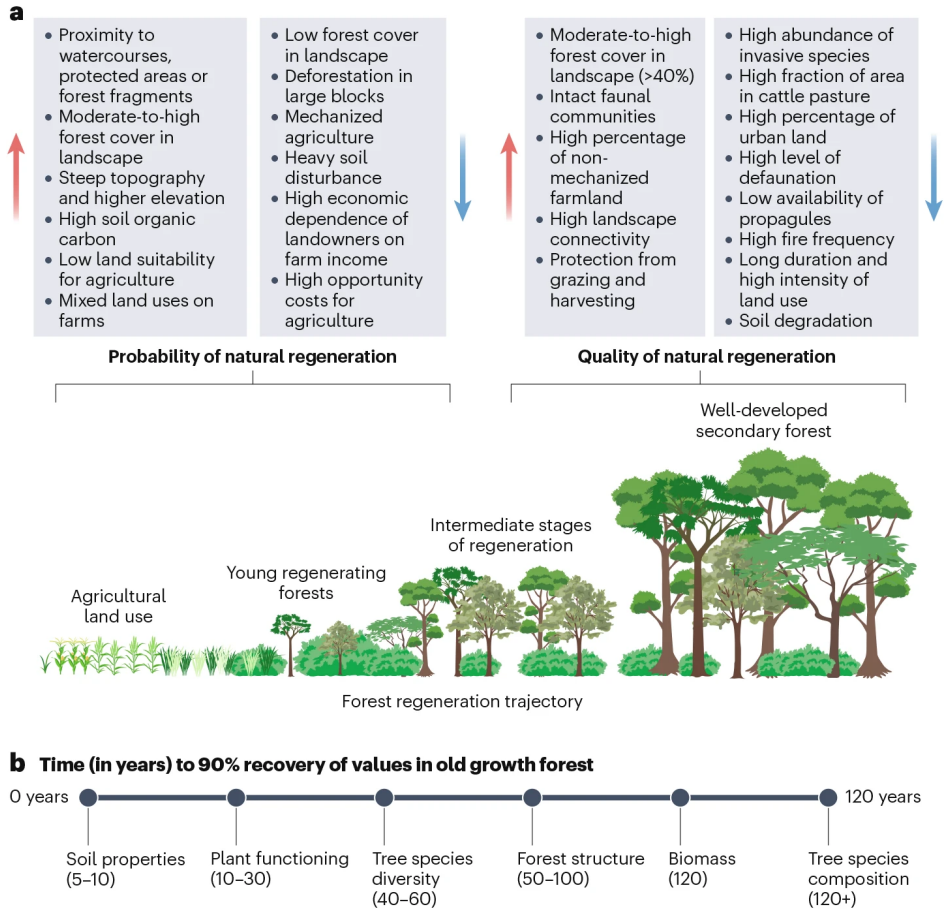

Fig. 5. Natural regeneration of tropical forests: Important factors and recovery timeline. Taken from Chazdon et al. (2025). **a)** Local and landscape biophysical as well as land-use factors influence both the likelihood of natural regeneration (left) and the quality and pathways of regeneration (right), either positively (upward arrow) or negatively (downward arrow). **b)** The pace of recovery differs among various forest attributes.

In this section I gave a broad overview of the complexity of tropical forests and their succession. However, the picture becomes more complicated when taking microbes into account. In the following section I shall focus on microbes, especially fungi, and their ecological roles, in general as well as in tropical forests.

1.2 Fungal Ecology

1.2.1 Plant-Soil Feedback

The relationship between plants and the components of the surrounding soil is a complex one. On the one hand, the physical, chemical and biological properties of the soil determine how plants and plant communities will develop. On the other hand, the soil is also deeply influenced by the plants which grow on it. At first glance, this situation looks like a chicken-or-the-egg problem, but ultimately it can be described by

the plant-soil feedback (van der Putten et al., 2013).

Plants change the soil directly through root exudates, litter inputs, and interactions with microbes, especially nitrogen-fixing bacteria or mycorrhizal fungi (see **1.3.2**) (van der Putten et al., 2013). Thus, each plant species creates a unique soil environment. This can be more or less suitable for the same plant species or others surrounding it. If it improves the performance of individuals of the same species (*conspecifics*) and enhances the chance of conspecifics monopolizing the local habitat, one speaks of a positive plant-soil feedback. However, if the habitat is made less suitable for a species by growing in its soil and the probability of being replaced increases, negative plant-soil feedback effects are at work.

Since soil-feedback effects influence the plant community over time, they have a clear impact on succession (van der Putten et al., 2013). However, research has been contradictory up to this point. On the one hand, Reynolds et al. (2003) found that early successional stages have a positive plant-soil feedback. Here, plants often associate with N-fixing bacteria and mycorrhizal fungi, exploring the soil and enriching it with nitrogen. However, after some time pathogens may accumulate, turning the soil-plant feedback negative. Now the pioneer species have a competitive disadvantage against the pathogen-resistant late-successional species. On the other hand, (Kardol et al., 2006) found that specifically in secondary succession pathogens and negative soil-plant feedbacks seem to be predominant in the early successional stages. In later stages, this trend is reversed as symbionts, especially mycorrhizal fungi, play a more important role.

1.2.2 Mycorrhizal Fungi

As seen in the last chapter, soil microbes play a crucial role in many ecosystem processes. One of the most import groups are mycorrhizal fungi, which form symbiotic associations with over 90% of terrestrial plant species (Brundrett & Tedersoo, 2018). They influence nutrient and carbon cycling (van der Heijden et al., 2015), soil structure (Rillig & Mummey, 2006) as well as plant diversity and productivity (van der Heijden et al., 1998, 2008).

Depending on the mycorrhizal type, the symbiosis can have different benefits for plants and fungi, but most commonly mycorrizhal fungi provide nutrients, such as nitrogen or phosphorus, while receiving carbon from host plants in return (Smith & Read, 2008). Mycorrhiza can also protect plants from pathogens (Jung et al., 2012) and drought (Cosme, 2023). Even though this symbiosis is for the most part on the mutualistic end of the symbiotic spectrum it can very well be or become parasitic (Smith & Read,

2008). This chapter shall give an overview on different mycorrhizal types and focus on the most important one in tropical ecosystems: arbuscular mycorrhiza (Soudzilovskaia et al., 2019).

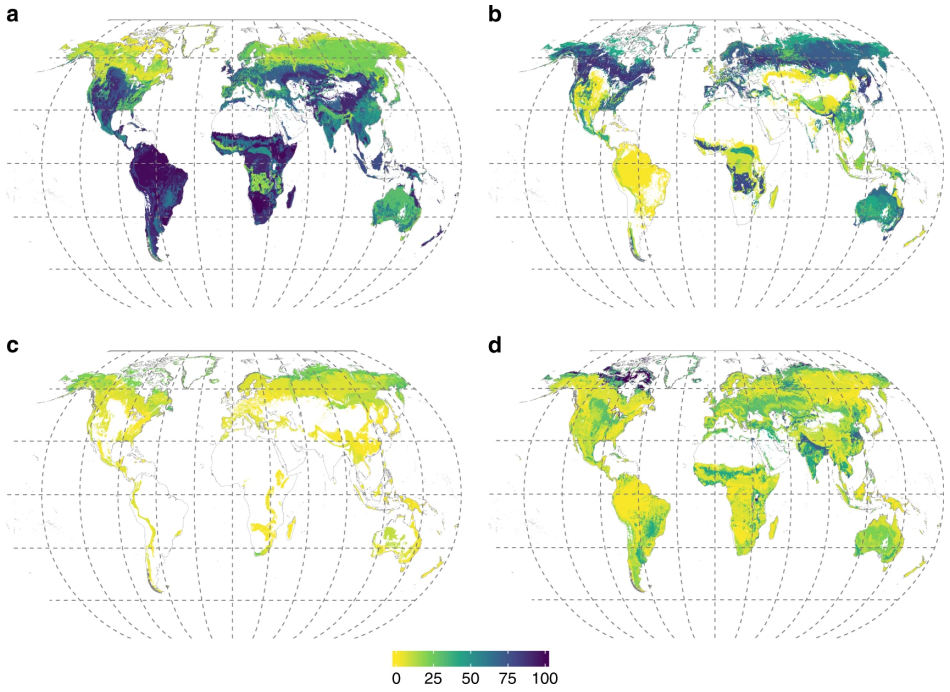

Fig. 6. Percentage of aboveground plant biomass of mycorrhizal vegetation. Adapted from Soudzilovskaia et al. (2019). **a)** Arbuscular mycorrhizal plants, **b)** ectomycorrhizal plants, **c)** ericoid mycorrhizal plants, and **d)** non-mycorrhizal plants.

Most vascular plant species form a symbiosis with one of the main groups of mycorrhiza fungi: 72% are arbuscular mycorrhizal (AM), 10% are orchid mycorrhizal (OrM), 2.0% are ectomycorrhizal (EM), 1.5% are ericoid mycorrhizal (ErM) and only 8% are completely nonmycorrhizal (NM), whereas 7% do not have consistent NM-AM associations (Brundrett & Tedersoo, 2018). A global distribution of the most common type of mycorrhiza can be seen in **Fig. 6**. Ectomycorrhizal Fungi (EMF) are most important in temperate and boreal climates. Although they only are associated with 2.0% of vascular plant species, these are mostly the tree species, that build the forest habitats in these areas (van der Heijden et al., 2015). Arbuscular mycorhizal fungi (AMF) on the other hand are dominant especially in the tropics, where they form symbioses with most plants. There are of course key differences between these types of fungi. The plant colonization ability of EMF and AMF is described in **Fig. 7**, with the main morphological difference being, that AMF grow inside the plant roots cells, while EMF grow in between them, forming a protective net around the root tip.

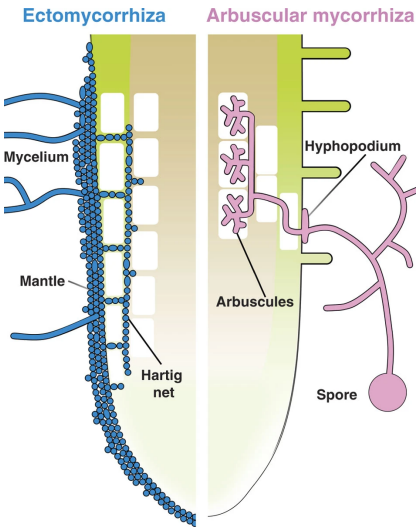

Fig. 7. Illustration of root colonization structures of ectomycorrhizal (blue) and arbuscular mycorrhizal (pink) fungi. Taken from (Bonfante & Genre, 2010). EMF form a thick mantle of tightly packed hyphae around the root tip, while a network of hyphae, known as the Hartig net, penetrates between the outer root cells. By contrast, in AMF, the root tip itself is usually not colonized. Instead, hyphae emerge from a spore, attach to the root surface by forming a hyphopodium, and then grow both between and within root cells. Colonization ultimately leads to the development of arbuscules, finely branched, tree-like fungal structures inside the inner root cells (brown), that serve as the main sites of nutrient exchange.

AMF are usually associated with Phosphorus uptake, since they lack the saprotrophic capacity to mine for Nitrogen (van der Heijden et al., 2015). This is also why they are more present in the heavily Phosphorus-depleted tropical areas. Furthermore, their growth is especially dependent on temperature (Soudzilovskaia et al., 2015). EMF are able to mobilize both organic and inorganic forms of Nitrogen from the soil and are thus crucial for the Nitrogen cycle (van der Heijden et al., 2015). Their growth is mostly determined by soil pH and they benefit from relatively constant precipitation (Soudzilovskaia et al., 2015). In reality, AMF and EMF often coexist in the same ecosystem. Plant roots are often colonized by more than one fungus and the fungi themselves are in symbiosis with more than one plant at the time. This results in the interconnection of plants by mycorrhizal mycelial networks (van der Heijden et al., 2015).

1.2.3 Fungi in Tropical Forest Restoration

Fungi are fundamental to tropical forest restoration because they drive nutrient cycling, soil carbon dynamics, and plant-soil interactions. Research in selectively logged Bornean forests shows that soil fungal communities recover more slowly than bacterial ones, and that active restoration practices such as removal of understory vegetation can even promote fungal communities that diverge from old-growth forests, as these practices may be selecting for different microbial communities (Robinson et al., 2025). These findings highlight that restoration design influences below-ground trajectories.

Successional changes in fungal composition occur even when overall fungal diversity remains constant: dry and moist tropical forests display contrasting AMF and pathogen communities, with dry forests having a higher AMF and lower pathogen diversity,

meaning strategies effective in wet forests may not translate to dry forests (Saltonstall et al., 2025). Similarly, decomposer fungal communities respond to the recovery strategy, as active restoration can increase richness and density of fungi driving leaf litter breakdown compared to passive restoration, which effects nutrient release and carbon turnover (Rivera et al., 2025).

Among fungal groups, AMF are central to seedling establishment, soil fertility, and stress tolerance (Lethielleux-Juge, 2025). They support above- and belowground biodiversity, help prevent invasion, and contribute to soil carbon stabilization. AMF inocula may help to accelerate ecological restoration (Huante et al., 2012). However, their effectiveness depends on the local context. For instance, native AMF consortia and early-successional strains outperform exotic or single-species inocula (Lethielleux-Juge, 2025). A global literature analysis confirms the rapid growth of research on AMF in restoration but notes gaps in long-term monitoring and specificity of AMF-plant relationships (Arévalo et al., 2025). However, the effect of EMF in tropical forest restoration is still unknown. There is evidence suggesting that EMF community composition, rather than EMF richness, is the primary driver of tree growth responses (Anthony, 2025), but how this translates to restoration is an important and open question (Keller et al., 2025).

Belowground processes in restoration are tightly linked to soil carbon storage, yet carbon dynamics are often overlooked in restoration management. Enhancing tree species with strong mycorrhizal associations can increase soil C sequestration, while invasive-species management may have unintended negative consequences for soil C pools (Keller et al., 2025). To properly evaluate success, restoration monitoring frameworks must integrate soils, roots and soil biota rather than relying solely on aboveground vegetation. Toro et al. (2025) suggest monitoring at least bulk density, aggregate stability, organic matter, pH, decomposition rate, macrofauna abundance before and after intervention, but interestingly, there is no concerted effort to consider fungi. Yet, even after decades, restored fungal communities may still differ from old-growth soils, indicating a need for long-term, fungi-aware restoration planning (Eaton et al., 2024).

1.3 Statistical Methods

Analysis of Variance (ANOVA) is a classical statistical method used to test whether the means of two or more groups differ significantly (Sokal & Rohlf, 2022). It works by partitioning the total variation in the response variable into variation between groups and variation within groups, and then comparing these using the F-statistic. If the

between-group variation is significantly larger than the within-group variation, ANOVA concludes that at least one group mean differs from the others. ANOVA assumes that residuals are normally distributed, that groups have homogeneous variances, and that observations are independent.

Permutational Multivariate Analysis of Variance (PERMANOVA) is a non-parametric multivariate test used to compare differences in community composition or other high-dimensional data among groups (Anderson, 2001). It was introduced as an extension of ANOVA that does not require normality, does not require homoscedasticity, and works on distance matrices rather than raw data. Instead of comparing means directly, PERMANOVA partitions variation in a distance matrix, such as Bray–Curtis, into within-group and between-group components. Significance is assessed using permutation tests, where group labels are repeatedly shuffled to generate a null distribution of pseudo-F statistics.

The Mantel test is a statistical method used to measure the correlation between two distance matrices, typically representing ecological, genetic, spatial, or environmental dissimilarities (L. Legendre & Legendre, 2012). It evaluates whether pairs of objects that are similar or different in one matrix tend to be similar or different in the other. Instead of comparing raw variables, the Mantel test uses pairwise distances, such as Bray–Curtis dissimilarity for community composition, Euclidean distances for environmental variables, or geographic distances for spatial separation. The test statistic is usually a Pearson correlation coefficient computed between the two sets of distances.

Because distance matrices contain non-independent entries (each sample contributes to multiple pairwise distances), traditional parametric tests are invalid (L. Legendre & Legendre, 2012). The Mantel test solves this by using permutation testing: one distance matrix is repeatedly permuted, the correlation is recalculated for each permutation, and the p-value is obtained by comparing the observed correlation to its null distribution. This makes the Mantel test non-parametric and free from assumptions of normality. However, it still assumes that the distances meaningfully represent underlying relationships and that the data do not include strong spatial or temporal autocorrelation that could bias permutation results.

Principal Coordinate Analysis (PCoA), a metric multidimensional scaling method, is an ordination method used to represent the relationships among samples based on a distance or dissimilarity matrix (Borcard et al., 2018). Unlike PCA, which requires raw data and Euclidean distances, PCoA can use any distance measure, including Bray-Curtis, which makes it very useful for ecological and multivariate community data.

The goal of PCoA is to place samples in a low-dimensional space such that the Euclidean distances among points in that space approximate the original dissimilarities as closely as possible (Borcard et al., 2018). It begins by converting the distance matrix into a centered matrix of scalar products and then performing an eigenvalue decomposition. Each eigenvalue corresponds to the variance explained by an axis, and each eigenvector provides the coordinates of samples along that axis.

Distance-Based Redundancy Analysis (dbRDA) is a constrained ordination method that relates multivariate community or ecological data to a set of explanatory variables (P. Legendre & Anderson, 1999). It is essentially a two-step procedure: transforming community data into a distance (dissimilarity) matrix, and performing a constrained ordination (like RDA) on that distance representation (Borcard et al., 2018). The method was introduced to bridge the gap between traditional RDA, which requires Euclidean distances and linear relationships, and ecological community data, which typically use non-Euclidean dissimilarities such as Bray–Curtis. dbRDA allows to model how much variation in multivariate community structure can be explained by environmental variables, treatments, or spatial gradients.

The method begins by computing a distance matrix among samples based on community data (Borcard et al., 2018). This matrix is then transformed via Principal Coordinates Analysis (PCoA) so that non-Euclidean dissimilarities can be represented in a Euclidean space. Only the axes that correspond to non-negative eigenvalues are retained. These PCoA axes are then used as the response matrix in a redundancy analysis (RDA), in which explanatory variables are used to constrain the ordination. This yields canonical axes that represent the strongest relationships between the community dissimilarities and environmental predictors. Significance of the model and individual terms is typically assessed through permutation tests, similar to PERMANOVA.

Nonmetric Multidimensional Scaling (NMDS) is an ordination technique used to visualize patterns in multivariate ecological data based on a matrix of pairwise dissimilarities (Borcard et al., 2018). Unlike methods that rely on Euclidean distances, NMDS is a rank-based method. This means it focuses on preserving the order of distances, not their absolute magnitudes. Because of this, NMDS is particularly well suited for ecological community data, which often use non-Euclidean dissimilarity measures such as Bray–Curtis or Jaccard, and which frequently violate assumptions of linearity, normality, and Euclidean geometry.

NMDS works by iteratively arranging samples in a low-dimensional space such that

the rank order of observed dissimilarities closely matches the rank order of distances in the ordination space (Borcard et al., 2018). A key feature of NMDS is the stress value, which quantifies the mismatch between the observed dissimilarity ranks and the ordination distances. Lower stress indicates a better fit, and common guidelines suggest that stress < 0.1 indicates a good ordination, 0.1 - 0.2 is acceptable, and > 0.3 indicates a poor representation of the data (Clarke, 1993).

Partial Least Squares Regression (PLSR) is a multivariate regression technique designed for situations where predictors are highly correlated or numerous relative to sample size (Wold et al., 2001). Instead of regressing the response on the original predictor variables, PLSR constructs a small number of latent components that capture the covariance between the predictor matrix X and the response variables Y. These components are linear combinations of the original predictors, chosen specifically to maximize their ability to predict Y, rather than merely explaining variance in X.

Variable Importance in Projection (VIP) is a commonly used measure in PLSR that quantifies the contribution of each predictor variable to the PLS components used in the model. VIP values summarize how strongly a variable helps explain both the predictor space X and the response variables Y, weighting each variable's influence by the amount of variance explained by each PLS component. Larger VIP scores indicate greater importance for predicting the response, whereas smaller values reflect weak or negligible contributions. A widely applied guideline is that variables with VIP > 1 are considered important, those between 0.8 and 1.0 moderate, and those below 0.8 unimportant for the model (Chong & Jun, 2005).

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2 Links between Tropical Forest Restoration and Fungal Communities

2.1 Introduction

Tropical forests are among the most biodiverse ecosystems worldwide (Gatti et al., 2022; Novotny & Miller, 2014; Pillay et al., 2022). Although they make up less than 14 % of earth's land surface (FAO, 2020), they are home to an immeasurable number of species. However, large areas of tropical forest have been converted to agricultural land, resulting in extensive ecosystem degradation (Curtis et al., 2018; IPBES, 2019). Following agricultural abandonment, forest regeneration offers an important pathway for ecosystem recovery (Williams et al., 2024), yet the processes governing regeneration remain incompletely understood.

Forest recovery is commonly assessed using aboveground vegetation indicators such as species richness, community composition, and biomass accumulation (Chazdon et al., 2025). In contrast, belowground components, particularly soil fungal communities, have received comparatively less attention (Stallman et al., 2024), despite their fundamental role in nutrient cycling, plant establishment, and ecosystem functioning. Soil fungi encompass diverse functional guilds, including saprotrophs, mycorrhizal fungi, endophytes, and plant pathogens, all of which can influence forest successional trajectories.

In tropical ecosystems, arbuscular mycorrhizal fungi dominate most plant-fungus associations, while ectomycorrhizal fungi, although less widespread, may strongly affect plant growth and nutrient dynamics where suitable host species occur (Soudzilovskaia et al., 2019). Agricultural land use can substantially alter fungal communities through soil disturbance and changes in nutrient availability, and the recovery of fungal communities following land abandonment may shape, or respond to, vegetation regeneration.

Plant and fungal communities are linked through reciprocal feedback (van der Putten et al., 2013). Vegetation composition influences fungal communities via litter inputs and host associations, while fungi can affect plant recruitment, growth, and competition via processes like nutrient uptake and pathogen infection. These interactions may differ between tropical dry and wet forests, which experience contrasting climatic conditions and regeneration strategies (Saltonstall et al., 2025). However, there is a significant knowledge gap regarding fungi in tropical forest restoration, with only a few studies investigating their responses to ecosystem restoration (Eaton et al., 2024; Huante et al., 2012; Rivera et al., 2025; Robinson et al., 2025).

The aim of this thesis is to investigate the links between tropical forest regeneration, soil fungal communities, and associated soil properties in regenerating tropical dry and wet forests in Mexico and Australia. Specifically, this study addresses the following questions: 1. How do vegetation communities regenerate over time following agricultural abandonment? 2. How do fungal community composition and functional guild structure differ between forest types and countries? 3. Which soil properties best explain variation in fungal communities? 4. To what extent are fungal communities linked to vegetation composition, turnover, and growth during restoration? 5. How do fungal communities change over time during early stages of forest recovery?

By integrating vegetation data, soil characteristics, and fungal community analyses, this study provides an integrated perspective on above- and below-ground interactions during tropical forest regeneration and contributes to a more comprehensive understanding of ecosystem recovery processes.

2.2 Materials and Methods

2.2.1 Site Descriptions

This study focuses on four different tropical forests, two in Southern Mexico and two in North-Eastern Australia. In each country a tropical dry as well as a tropical wet forest were sampled and analyzed. All the plots are from areas, which have been previously used for agricultural purposes and have been left to regenerate since 2020.

Nizada (16°39′ N, 95°00′ W) is located in a dry deciduous forest near the south coast of Mexico in the province of Oaxaca. It lies at an altitude of 90 - 120 m a.s.l. and has a mean annual temperature of 27.7 °C and a mean annual precipitation of 900 mm (Hordijk, Poorter, Martínez-Ramos, et al., 2024). However, the precipitation is very seasonal, as 90% of the rainfall comes down between late May and mid October.

Loma Bonita (16°05′ N, 91°00′ W) is located in a tropical rain forest in the Mexican province of Chiapas near the border of Guatemala. It lies at an altitude of 115 - 300 m a.s.l. and has a mean annual temperature of 24.4 °C and a mean annual precipitation of 3000 mm (Hordijk, Poorter, Martínez-Ramos, et al., 2024). The driest season is between February and April, with a rainfall of less than 100 mm per month.

The Australian forest sites are located in the Atherton Tableland at an elevation of 700 - 1000 m a.s.l. in Queensland (17°23′ S, 145°35′ E - 17°27′ S, 145°38′ E) ("Pantrop", 2025). The tableland experiences a rainfall gradient and has a distinct dry season

between July and October. In the northern part, around Yungaburra, lies a semi-deciduous dry forest that receives about 1400 mm of rainfall per year. In the southern part, around Tarzali, a wet evergreen forest is located, with an average annual rainfall of 2200 mm.

2.2.2 Plant Community and Soil

The vegetation was identified in 25 m x 25 m plots on recently abandoned pasture or agricultural land (Hordijk, Poorter, Meave, et al., 2024). Individuals were considered trees if their height was greater than 1.5 m, as opposed to seedlings. For each of the latter height and diameter at 30 cm height were measured. The plots were surveyed during the dry season between January and April in the Mexican forests and between July and November in the Australian forests. Typically, the Mexican forests were surveyed from 2020 to 2024 and the Australian ones from 2021 to 2023. However, some plots were were newly established and some abandoned. Appendix **B.1** shows the available forest data per year.

The Mexican forests have two types of plots, namely forest cover and biodiversity. The first type of plots were generally surveyed, while on the second type experiments took place. In this thesis all the forest cover plots and the control plots of the biodiversity plots were taken into account. In total, there are 65 plots with trees or seedlings in them in any of the years, which classifies them as relevant for further analyses. Of these, 27 are Mexican wet forest, 25 are Mexican dry forest, 11 are Australian wet forest and two are Australian dry forest plots. To summarize, 38 plots are from wet forests and 27 from dry forests and there are in total 52 Mexican and 13 Australian plots.

For each plot, three soil samples (0-15 cm) were taken, or, in the case of high stone content, five samples were taken (Hordijk, Poorter, Martínez-Ramos, et al., 2024). Per site the samples were pooled and then analyzed. Several soil parameters were measured. The ones relevant to this study are pH, available nitrogen measured with the Kjeldahl method, available phosphorus measured with the Olsen extraction method, and clay content.

2.2.3 Fungal Community

Soil samples were taken from the center of the 25 x 25 m plots during the dry season. The Mexican dry forest samples were collected in February and March, and the Mexican wet forest samples were collected in March and April. The Australian samples were taken in November. They were air dried in the field to preserve microbial DNA.

Due to the remote nature of the plots, it was not possible to preserve the samples on ice or in a freezer. After the samples were fully dried, they were shipped to the University of Vienna, where the following analyses took place. In total, 107 soil samples made their way to Vienna. The soil for 15 Mexican dry forest plots was sampled twice, once in 2020 and once in 2024.

The DNA was extracted using the E.Z.N.A.® Soil DNA Kit with the following protocol: For each sample 250 mg of soil was weighted added to a disrupter tube together with 725 μL of the SLX-Mlus buffer solution. After bead beating at 4 m/s for 40 seconds, the samples were vortexed for 5 minutes in order to lyse them. Afterwards 72 μL of DS buffer was added. The samples were vortexed, incubated for 10 minutes and then centrifuged at maximum speed for one minute. To remove inhibitors, the supernatant was mixed with 135 μL of chilled P2 buffer and 200 μL of cHTR reagent. After vortexing and centrifuging, the supernatant and an equal volume of XP1 buffer was added together, vortexed and filtered through a filter column. The last step was repeated with the lysate to bind most of the DNA. The DNA extract was then washed, first with 500 μL of 100% Isopropanol and then twice with 700 μL of DNA wash buffer, that was before diluted with ethanol. In between these steps, the filter column was centrifuged at 10,000 g for one minute and the filtrate was discarded. To remove all residual ethanol, the coloumn was centrefuged at maximum speed for 2 minutes. To elute the DNA, 100 μL of an 70°C warm elution buffer was added to the column and after 2 minutes was centrifuged at maximum speed for 1 minute. The DNA-containing filtrate was then again added to the same coloumn and centrifuged. The DNA extract was stored at -20°C.

For sequencing, at first the full length ITS primers ITS9munngs (forward, GTACACACCGCCCGTCG) and ITS4ngUni (reverse, CGCCTSCSCTTANTDATATGC) have been used. However, only a few samples showed a successful PCR result. The procedure and its results can be found in Appendix **A**. Thus, we switched to the following ITS2 primers: gITS7ngs (forward, GTGARTCATCRARTYTTTG) and ITS4ngsUni (reverse, CCTSCSCTTANTDATATGC). The sequencing was done by the Joint Microbiome Facility (JMF) of the Medical University of Vienna and the University of Vienna. They used Illumina sequencing on a MiSeq platform with the v3 reagent kit (2 x 300 bp). This is a high-output paired-end sequencing method based on sequencing-by-synthesis, where DNA bases are identified by their fluorescent labels. The run produces two reads of 300 bp, one from the forward direction and one from the reverse direction. Because both ends of the same DNA fragment are sequenced, the overlapping region can be merged into a single, representative sequence. All data was passed through the dada2 bioinformatic pipeline (*sensu* Borgmann-Winter et al. (2023)).

2.2.4 Statistical Analysis

The statistical analysis was conducted in R version 4.3.2. For all plots the package **ggplot2** was used. Anova was performed using the **aov()** function, and the post-hoc test was conducted with the **TukeyHSD()** function to obtain significance values between groups. Alternatively, to check for homogeneous variances, a prerequisite for the **aov()**, the Levene test (**leveneTest()**) from the **car** package was used. If the variances were heterogeneous, a linear regression (**lm()**) with HC3 robust standard errors was applied. For this, the heteroscedasticity-consistent covariance matrix estimation (**vcovHC()**) from the **sandwich** package was used. To perform an anova-like test, **linearHypothesis()** from the **car** package was used. To double-check the **emmeans()** function from the **emmeans** package was used. Permanova was done using the **adonis2()** function from the **vegan** package.

The plant pivot table was calculated with **aggregate()** and **reshape()**. The Mexican vegetation data had to be cleaned first, and the Australian vegetation data had to be transformed, as the formats between the countries differed. The cleanup corrected most spelling mistakes of plant names and selected only the controls of the biodiversity plots. The Shannon and Simpson index was calculated with the **diversity()** function from the **vegan** package.

The composition of vegetation classification was calculated using **aggregate()**. Here, the composition was calculated for each plot, and then the average, where each plot was weighted by its number of individuals, was calculated over all plots per forest type, year, and country. The mycorrhizal association of the tree and seedling genera was calculated using **aggregate()** and **ave()** based on the FungalRoot database (Soudzilovskaia et al., 2020). Here, all genera of all plots were added together and then the percentages were calculated for each forest type separately. Also, a NMDS on the species composition of the Mexican dry and wet forest was performed.

The community turnover was calculated by creating a distance matrix for all sites *separately*. This matrix was calculated for the plant community between the first and last available year. This yielded one dissimilarity value for each site. The annual mean growth is the mean of the yearly growth rates and was calculated for each plot as follows:

$$\text{Annual Mean Growth} = \frac{1}{k}\sum_{i=1}^{k}\frac{v_{i+1}-v_i}{y_{i+1}-y_i}, \tag{1}$$

where y_i is the year of observation i, v_i is the sum of heights in year y_i and k is the

number of years minus 1.

The taxonomic characterization of the ASVs was done in dada2 using the **assignTaxonomy** function against the UNITE database (Abarenkov et al., 2010). For the guild association of the fungi, the FUNGuild database was used (Nguyen et al., 2016). As FUNGuild suggests multiple guilds per fungus with a given likelihood, they were simplified manually. All different saprotrophs, except for wood saprotrophs, were summarized as saprotrophs and no distinction was made between different parasitic forms.

Since one fungus can belong to multiple functional guilds, these were split up proportionally. This means that the abundance of each unique fungus per plot was divided by the number of functional guilds of this fungus and each guild was assigned the given proportion. For example, if there were two ASVs, both assigned as pathogen-saprotroph, then one ASV was assigned to the pathogen guild and the other to the saprotroph guild. Ecologically, this means that each possible guild has been realized accordingly. In an abundance analysis saprotrophs were considered wood saprotrophs if they were capable of breaking down wood, even if they had other saprotrophic abilities. Here, in order to calculate the percentages of the guilds, the **aggregate()** function was used. A linear regression with HC3 robust standard errors was used to calculate the differences in fungal guilds between the two forest types. For this analyses the total ASV counts were used instead of percentages, as the latter produce non-independent predictors. Usually the forest type was the first and most important distinction. However, in this case the country effects were also looked at using the same methods. A dbRDA on the guild percentage data and soil parameters as well as one on the fungal community data and soil parameters were performed. This proportional split was only done for these abundance-related analyses. For the following analyses, when examining a specific guild, the other guilds associated with a specific ASV did not matter.

A dbRDA was also performed on the fungal community data. For this, the **capscale()** function from the **vegan** package was used. Afterwards, an anova function from the same package (**anova.cca()**) was done to receive the p value of the model and its terms. A Mantel test was performed with 9999 permutations, linking fungal community data to the tree community data from the soil sampling years for all fungi as well as for specific guilds. For this, the data was first Hellinger-transformed using the **decostand()** function, and then the Bray-Curtis dissimilarity matrix was calculated with the **vegdist()** function; both are from the **vegan** package. The latter was used in preparation for the NMDS and the mantel test, for which the functions **metaMDS()** and **mantel()** were applied, respectively. To look at the community shift, an NMDS and a betadispersal analysis were performed. The betaspersal was calculated using the

betadisper() function from the **vegan** package and an **anova()** was done afterwards to get the p value.

The community turnover was calculated in the same manner as that for the plants. The fungal community turnover was related to plant and soil parameters using linear regressions. PLSR models were used to establish a link between fungal richness, fungal PCoA-axes as well as soil parameters and the plant community turnover, plant PCoA1-axes and tree and seedling mean annual growth. For the PCoA the **pcoa()** function from the **ape** package was used. The PLSR was calculated using the **plsr()** function from the **pls** package and the associated VIP scores using the **VIP()** function from the **chillR** package.

2.3 Results

2.3.1 Forest Characterization

In total there are 65 plots which have trees or seedlings in them. From that, 13 are Australian and 53 are Mexican plots. This difference arises due to the low tree and seedling number per plot in Australia, which for some plots is even 0 (see **Fig. 9**). There are 38 tropical wet forest and 27 tropical dry forest plots across both countries. A land use intensity index has been calculated for most Mexican plots in Hordijk, Poorter, Meave, et al. (2024). It ranges from 0 - 100 % and includes extent, duration, intensity and frequency of the land use practice. The mean land use in the dry forest plots is with an index of around 36 %, higher than in the wet forest plots, that have an index of around 24 %.

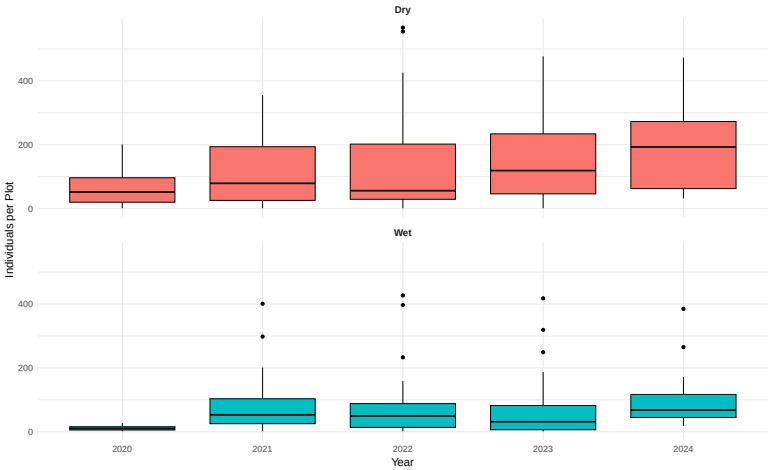

Fig. 8. Individuals per plot over time for all dry (upper) and wet (lower) forest plots. Both forest types show a significant increase of the mean from 2020 - 2024.

The forests are also regenerating a seedling community (**Fig. 8**). The number of individuals per plot is significantly different between the first and the last year for both dry ($p = 1.85 \times 10^{-3}$) and wet ($p = 1.95 \times 10^{-8}$) forests. The same is true for the sum of heights per plot for dry ($p = 8.08 \times 10^{-5}$) and wet ($p = 2.4 \times 10^{-7}$) forests. Also, the mean species richness increased in Mexico: In the dry forest plots species richness increased from 9.45 species per plot in 2020 to 13.89 in 2024. In the wet forest plots the increase is even more significant, from 3.1 in 2020 to 24.89 species per plot in 2024. For Australia, the picture is more complicated, as vegetation data is only available for the years 2021 to 2023 (see Appendix **B.1**) and there are generally few trees in these plots (see **Fig. 9**). The mean of the mean annual growth over all Mexican dry forest plots is 86.89 m per year and plot and 66.52 m per year and plot over all Mexican wet forest plots.

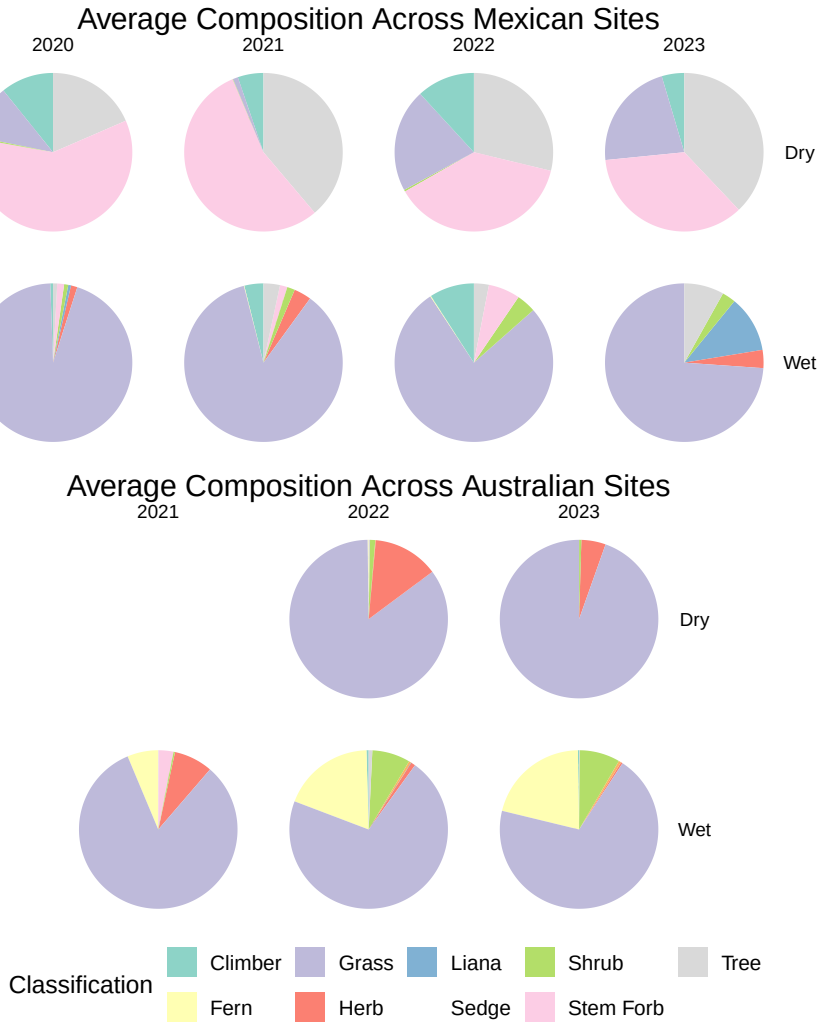

Fig. 9. Average vegetation composition for different forest types and over time. (**upper**) Vegetation data for the Mexican plots is available from 2020 - 2024. (**lower**) Australian plots were surveyed from 2021 - 2023 in the wet forest and 2022 as well as 2023 in the dry forest. Most plots show a dominance of grasses, while only the Mexican plot have a considerable amount of trees.

The vegetation groups in the Mexican dry forest plots are heterogeneous (**Fig. 9**). The dry forest plots are dominated by forbs (non-woody herbaceous plants), grasses and trees. The Mexican wet forest plots are, like all Australian plots, dominated by grasses, with an increasing number of trees over the years. However, the Australian plots show very few trees.

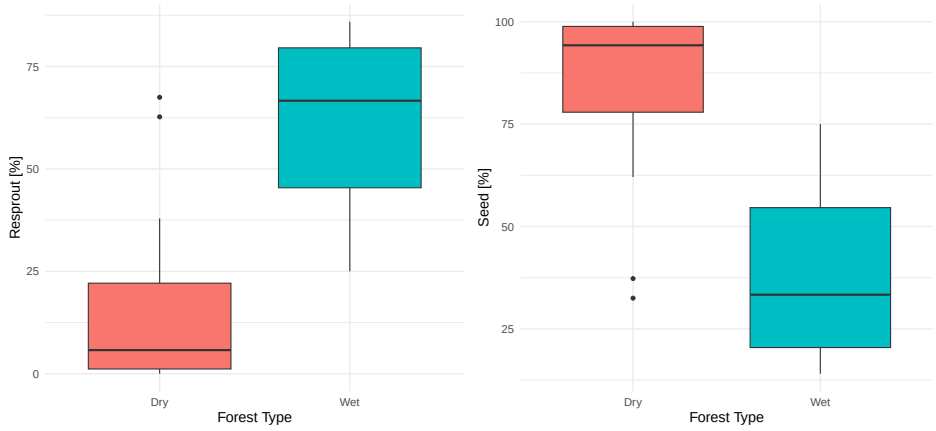

Fig. 10. Tree regeneration modes in Mexican dry and wet forest. (**left**) Trees regenerating from resprouting out of trunks or roots. (**right**) Trees regenerating from seeds.

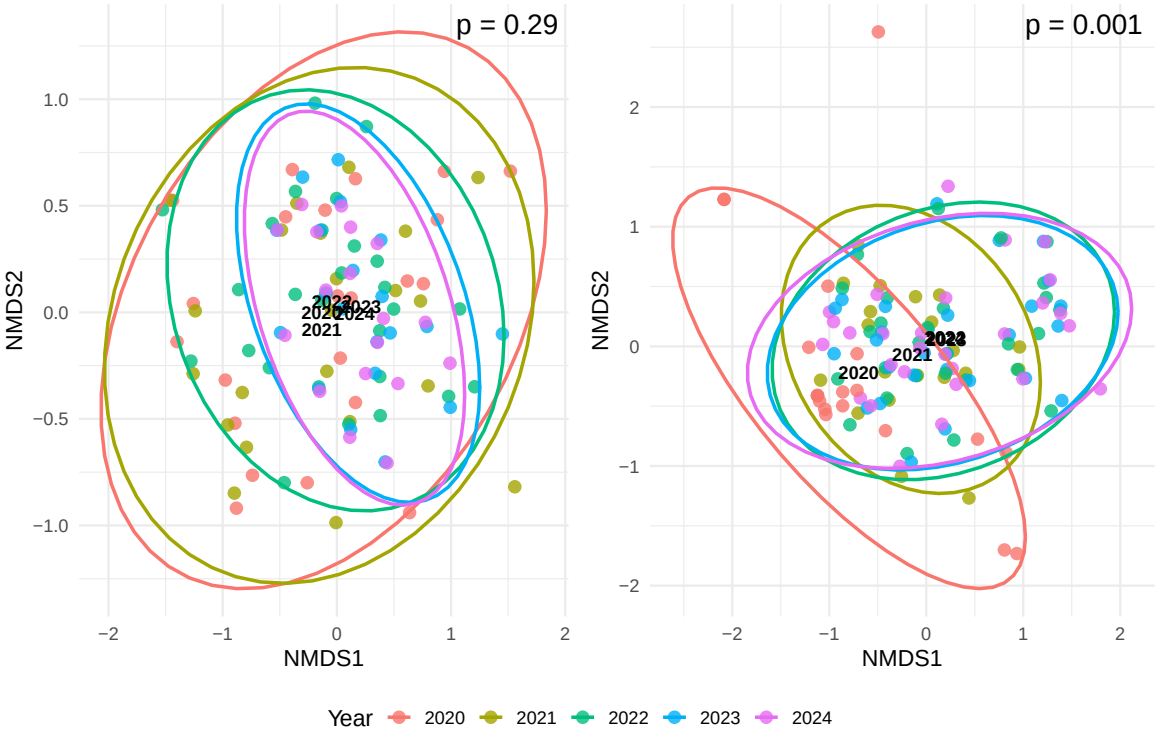

Fig. 11. Species composition (NMDS) of Mexican dry (left) and wet (right) forest over time. NMDS centroids of the respective year are depicted in bold font. The stress for the dry forest NMDS model is 0.21 and 0.17 for the wet forest model.

Two different tree regeneration modes have been surveyed in the Mexican wet and dry forests: Trees or seedlings regenerate either from resprouting or from seeds. As **Fig. 10** shows, the tropical dry forest plots regenerate significantly ($p = 3.21 \times 10^{-6}$) more from seeds compared with the wet forest plots.

Also, the species composition over time from the years 2020- 2024 has been analyzed (**Fig. 11**). A permanova test showed, that the species composition for the wet forest plots significantly ($p = 0.001$) changed over the study period, mostly due to the rather different composition in 2020. For the dry forest plots this was not the case ($p = 0.29$). **Fig. 11** also shows the centroids of the different ellipses. For the dry forest these cluster, but for the wet forest plots there is a continuous change between 2020 and 2022.

In the tropics, especially AMF play a dominant role when it comes to the association with plants (Soudzilovskaia et al., 2019). This has also been found in the Mexican dry and wet tropical forest plots. An analysis of the plant genera, shown in **Fig. 12**, found that over all plots 86.6 % of the genera in the wet and 72.9 % in dry forest associate with AMF. Interestingly, in the tropical dry forest around two percent of all plants associate with EMF and 20.3 % with both EMF and AMF.

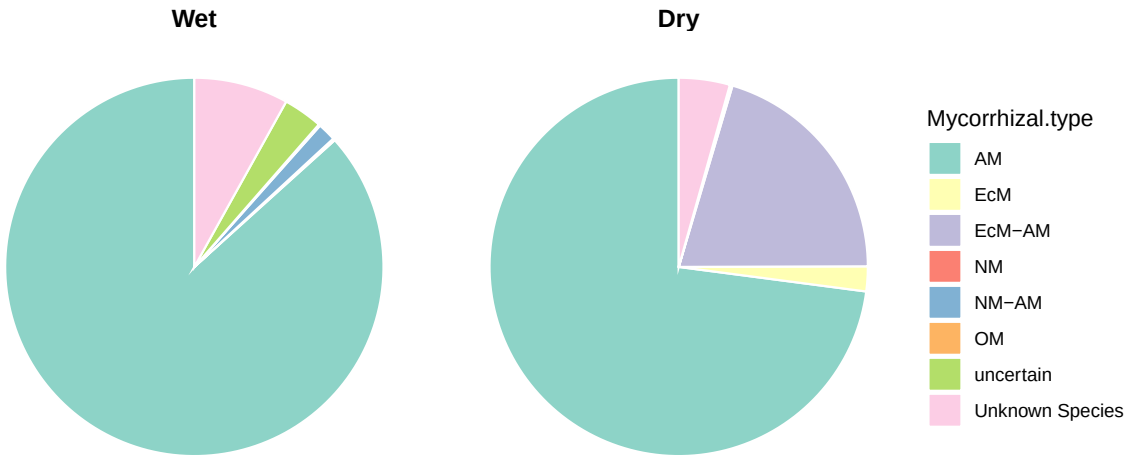

Fig. 12. Mycorrhizal assoziation of tree and seedling genera in the wet (left) and dry (right) Mexican forest in 2024. The mycorrhizal types are arbuscular mycorrhizal (AM), ectomycorrhizal (EcM), arbuscular mycorrhizal and ectomycorrhizal (EcM-AM), non-mycorrhizal (NM), non-mycorrhizal and arbuscular mycorrhizal (NM-AM) and orchid mycorrhizal (OM).

2.3.2 Fungal Guilds

The relative abundance of fungal guilds per plot was compared between forest types and countries. ASVs that could not be associated with a guild have been discarded. Several guilds differ in their relative abundance between the wet and dry tropical forest plots (**Fig. 13**). Plant pathogens, epiphytes and arbuscular mycorrhizal fungi are more abundant in the dry forest plots, whereas parasites are more abundant in the wet forest plots. The mean of the arbuscular mycorrhizal fungi over all wet forest plots is 2.3 % and 4.7 % over all dry forest plots. For ectomycorrhizal fungi it is 5.6 % and 6.1 % and for plant pathogens it is 11 % and 19 %, respectively. Overall, saprotrophs have the highest relative abundance with a mean 41.1 % in the wet and 34.5 % in the dry forest plots. Endophytes have a mean of 10.3 % in the wet and 8.6 % in the dry forest plots, establishing them as a surprisingly dominant group of fungi.

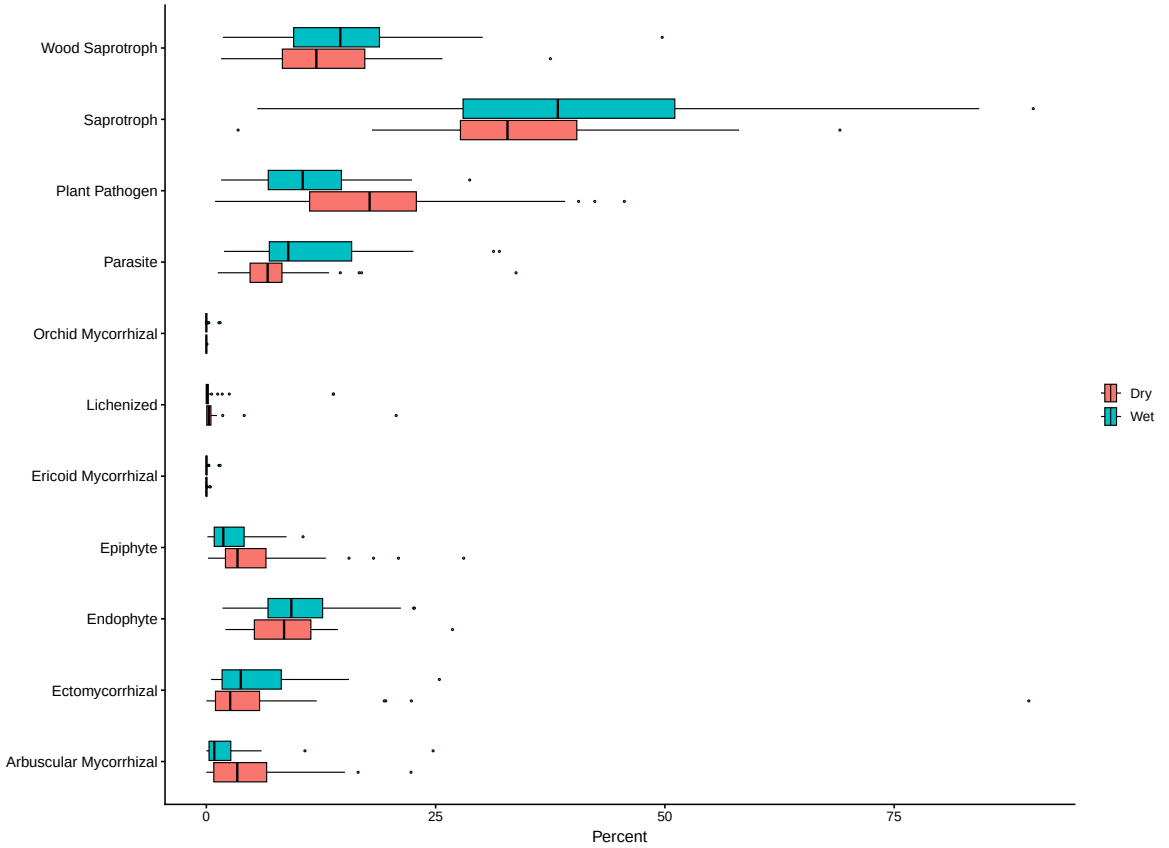

Fig. 13. Relative abundance of fungal guilds in wet and dry tropical forests in both Mexico and Australia.

Since the interaction terms were significant for some fungal guilds, they were examined first by forest type and then by country. Instead of percentages, the total number of ASVs was used for these analyses. Due to heterogeneous variances, a log transformed linear model with HC3 robust standard errors was applied. **Fig. 14** shows the to-

tal number of ASVs of arbuscular mycorrhizal fungi, plant pathogens, ectomycorrhizal fungi and saprotrophic fungi separated by country and forest type. Here, only ectomycorrhizal fungi have a significant difference in Australia ($p = 0.031$) and in Mexico ($p = 0.026$), with a higher median in the dry forest in Australia (73.47 (wet) - 217.58 (dry)) and a higher median in the wet forest in Mexico (66.50 (wet) - 44.66 (dry)).

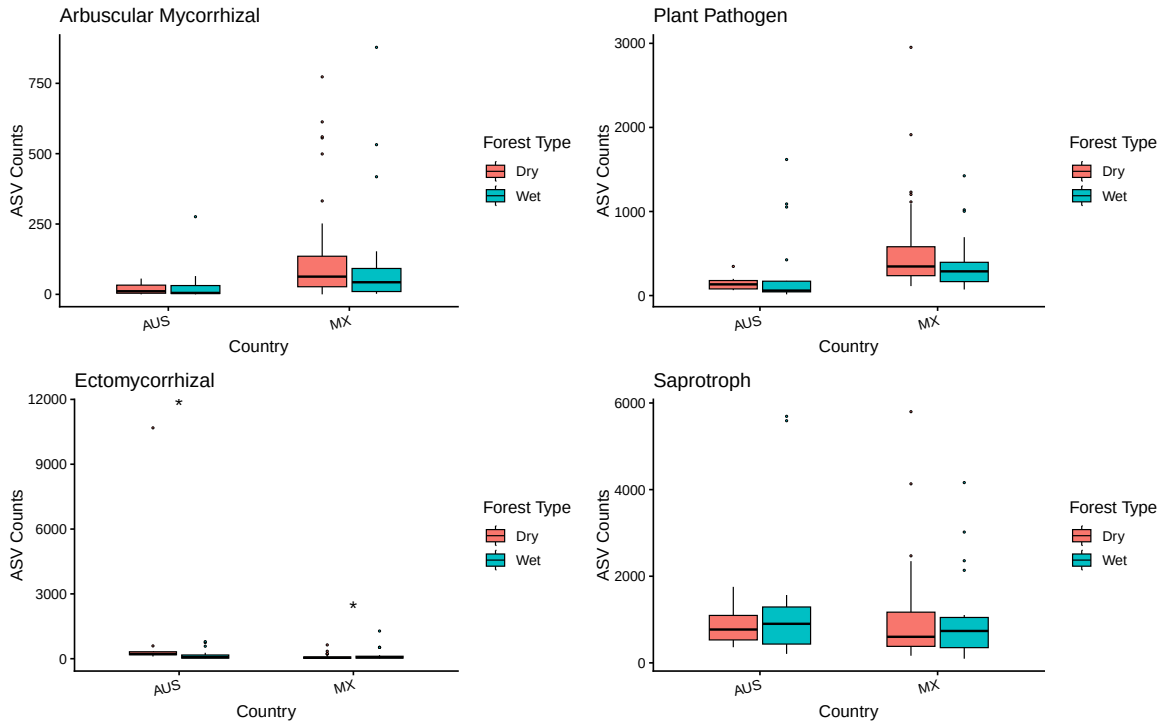

Fig. 14. Abundance of four important fungal guilds in the wet and dry forest plots separated by country. Ectomycorrhizal fungi show a significant difference between wet and dry forest in Mexico and Australia. In Australia the median is higher in the dry forest and in Mexico in the wet forest.

If looked at the countries separately, arbuscular mycorrhizal fungi are more present in the Mexican wet forest then in the Australian wet forest ($p = 0.016$) (**Fig. 15**). Ectomycorrhizal fungi are more present in the Australian dry forest compared with the Mexican dry forest ($p = 0.0003$). The abundance of plant pathogens differs between Mexico and Australia for the wet ($p = 0.009$) and dry ($p < 0.0001$) forest.

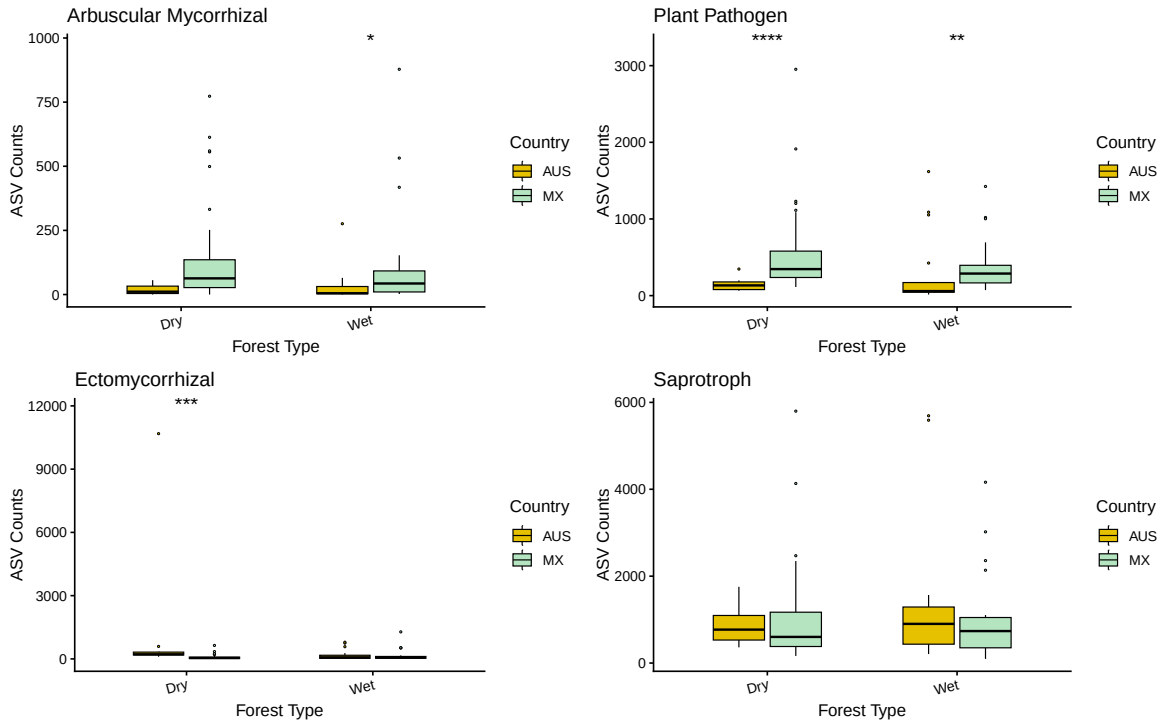

Fig. 15. Abundance of four important fungal guilds in the wet and dry forest plots separated by forest type. Arbuscular mycorrhizal fungi are more present in the Mexican wet forest. Ectomycorrhizal fungi are more present in the Australian dry forest. The abundance of plant pathogens differs between Mexico and Australia for the wet and dry forest.

A dbRDA on the fungal guild data was performed to identify predictors of variation in guild composition. Total soil nitrogen ($p = 0.001$), pH ($p = 0.014$) and the country parameter ($p = 0.025$) were significant while clay ($p = 0.084$) as well as forest type ($p = 0.071$) were marginally significant predictors of variation in guild composition. As **Fig. 16** shows, soil nutrients and clay content were associated with saprotrophs. Arbuscular mycorrhizal fungi were correlated with variation in soil pH.

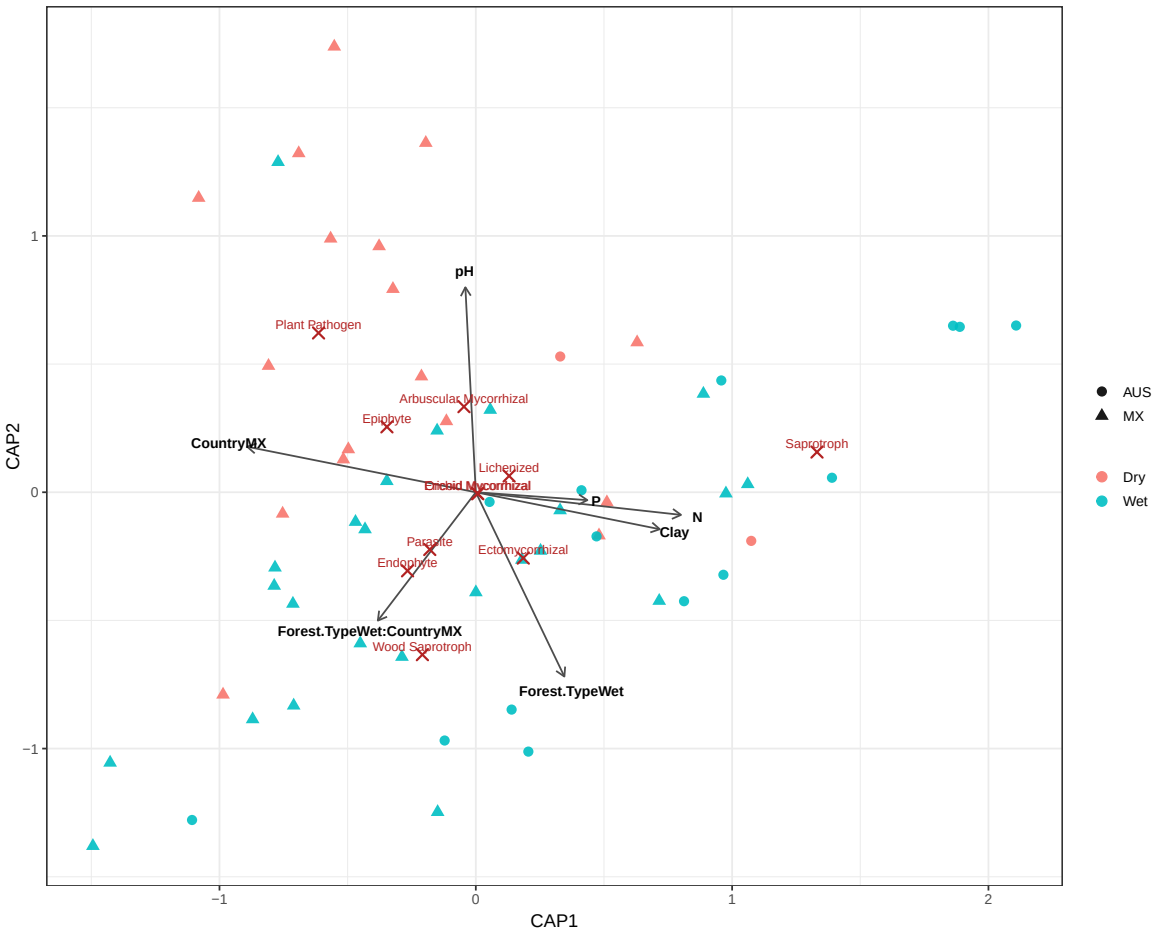

Fig. 16. DbRDA of fungal guild relative abundance data against soil parameters, country and forest type. This model is highly significant ($p = 0.001$). Nitrogen, pH and Country were significant predictors in explaining the fungal community data ($p < 0.05$).

2.3.3 Fungal Diversity, Community Composition and Vegetation Parameters

Most fungi, namely 67.4 % of the total abundance, belong to the phylum of Ascomycota, 17.7 % to the Basidiomycota and 1.8% to the Glomeromycota, the monphyletic subphylum containing the arbuscular mycorrhizal fungi (see Appendix **B.2**). 1.4 % belong to the Chytridiomycota, while the rest of the phyla include ASVs that when aggregated are below 1% of the total reads. When looking at ectomycorrhizal fungi, 35.7 % belong to the genus Russula, 33.4 % to the Chloridium, 7.3 % to Entoloma, 7.1 % to Amanita, 3.9 % to Lyophyllum and 3.3 % to Tuber (see Appendix **B.2**). For arbuscular mycorrhizal fungi, 51.6 % belong to the genus Kamienskia, 19.6 % to Septoglomus, 12.8 % to Glomus, 5.1 % to Rhizophagus and 4.9 % to Paraglomus.

A dbRDA on the fungal community data was performed to identify predictors of vari-

ation in community composition. Since the first model (see **Fig. 17**) had a significant interaction term between forest type and country, this model was split by forest type. For the wet forest model, soil pH ($p = 0.001$) and Country ($p = 0.001$) were significant and nitrogen ($p = 0.059$) was marginally significant, while for the dry forest model, only country ($p = 0.001$) was significant and pH ($p = 0.064$) as well as nitrogen ($p = 0.093$) were marginally significant. If split also by country the Mexican wet and dry forest communities showed significant models ($p < 0.05$). For the Mexican wet forest pH ($p = 0.001$) and nitrogen ($p = 0.047$) were significant and clay ($p = 0.076$) was marginally significant. For the Mexican dry forest pH ($p = 0.045$) was significant and nitrogen ($p = 0.071$) as well as phosphorus ($p = 0.085$) were marginally significant.

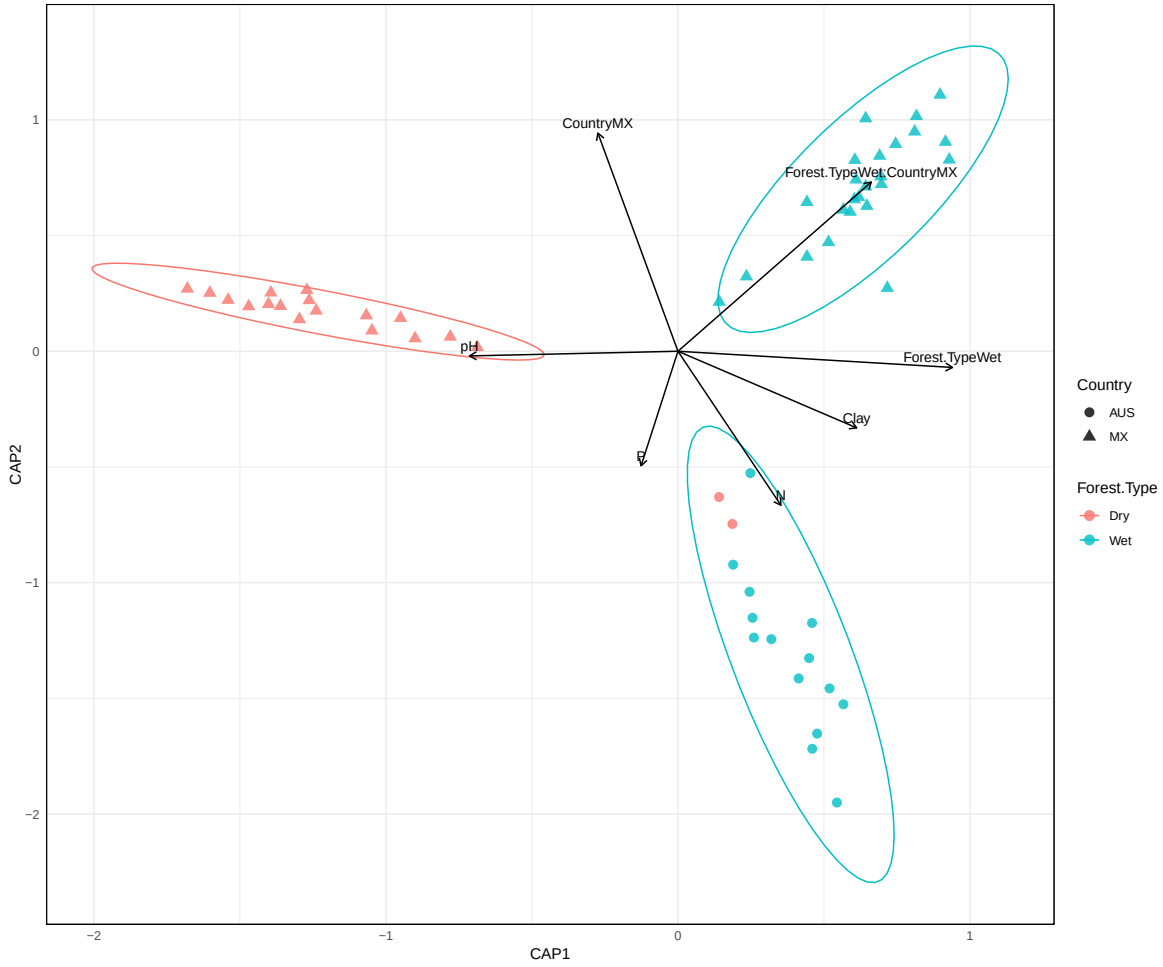

Fig. 17. DbRDA of fungal community data against soil parameters, country and forest type. This model is highly significant ($p = 0.001$) and shows a clear clustering regarding forest type and country. However, the interaction term is highly significant in this model, meaning that the model had to be split up in order to look at the effects of single variables independently.

In order to examine the links between the fungal community data and the vegetation community data, several mantel tests were performed on the Bray-Curtis dissimilarity

matrices. Since forest type and country were structuring the data, the Mexican dry and wet as well as the Australian wet forest plots were analyzed separately. Due to limited plots in the Australian dry forest, this forest type was not considered. The mantel test for all fungal data was only significant for the Mexican wet forest ($p = 0.0001$) and had a r value of 0.371. Afterwards, several mantel tests for the community data of the saprotrophic fungi, arbuscular mycorrhizal fungi, plant pathogens and ectomycorrhizal fungi were looked at. For the Mexican wet forest plots the community data of the first three guilds showed significant results: For the saprotrophic fungi ($p = 0.0002$) the r value was $r = 0.326$, for arbuscular mycorrhizal fungi ($p = 0.0002$) the r value was 0.325 and for the plant pathogens ($p = 0.0117$) the r value was 0.207.

Since the soil for 15 Mexican dry forest plots was sampled twice across a four year interval, I also examined temporal changes in the fungal community. Fungal community composition changed over time ($p = 0.003$; **Fig. 18 (left)**). It also became more homogeneous in 2024 compared to 2020 (**Fig. 18 (right)**), as tested using an analysis of dispersion in community composition ($p = 0.0458$).

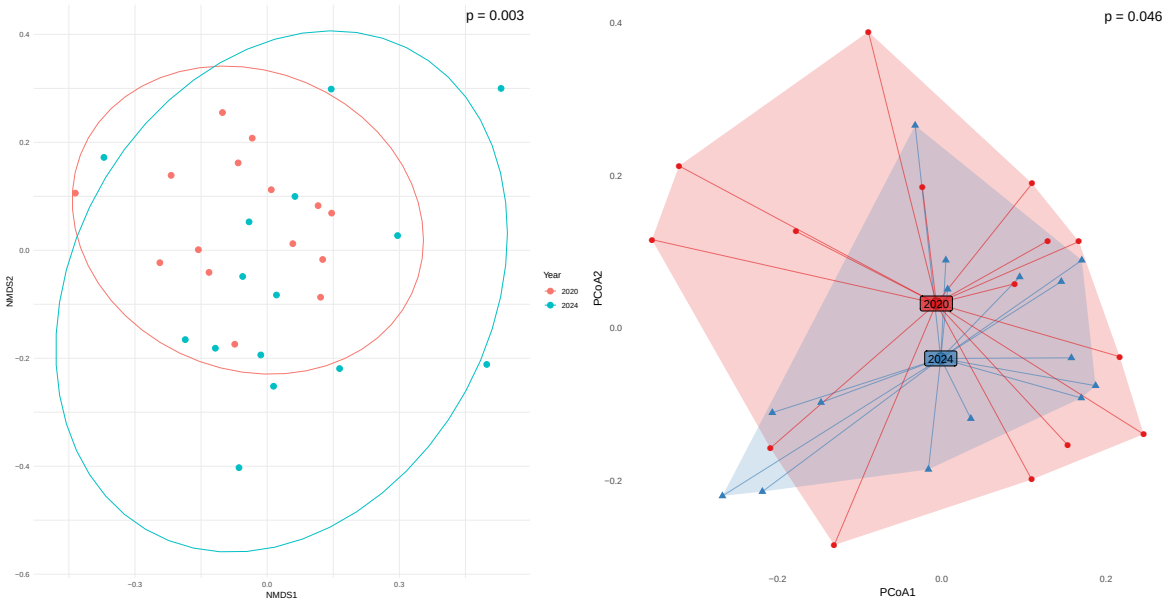

Fig. 18. Fungal communities shift from 2020 to 2024. (left) NMDS model for the fungal communities in 2020 and in 2024. The stress for the NMDS model is 0.23. **(right)** Beta dispersal for the fungal communities in 2020 and 2024. The arrows connect the points with their centroids, the multivariate average community of that year.

The fungal community turnover for the 15 sites has also been calculated, which is the Bray-Curtis dissimilarity between the community composition in 2020 and 2024. The mean Bray-Curtis dissimilarity is 0.84. None of the soil variables (pH, N, P and clay)

explained it in a linear regression. Furthermore, the fungal community turnover did not explain the plant community turnover or the annual mean plant growth.

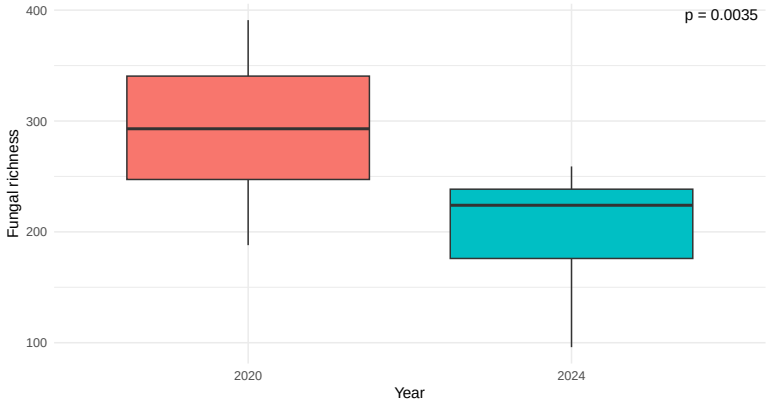

Fig. 19. Fungal richness shift from 2020 to 2024.

The alpha diversity experiences a drastic shift from 2020 to 2024, as the mean decreases by 29.2 %. As figure **Fig. 19** shows the difference in the mean is highly significant ($p = 0.0035$). However, the relationship between the differences in fungal richness and various plant parameters was not significant. The same is true when looking at saprotrophs, arbuscular mycorrhizal fungi, ectomycorrhizal fungi, and plant pathogens separately.

2.3.4 Fungal Guilds and Vegetation Parameters

In order to interlink data on the composition and diversity of fungal guilds and on physicochemical soil conditions with variation in multiple vegetation variables, I used PLSR. I separately modeled how fungal and soil variables predict plant community turnover (see **Fig. 20**), plant community composition of the final census interval (see **Fig. 21**) and the mean annual growth rate (see **Fig. 22**). The models consider only Mexican plots as the data is very unbalanced regarding the two countries. For this analysis, a total of 17 wet forest plots and 22 dry forest plots were used. In the following, the VIP values will be written in brackets after the parameters.

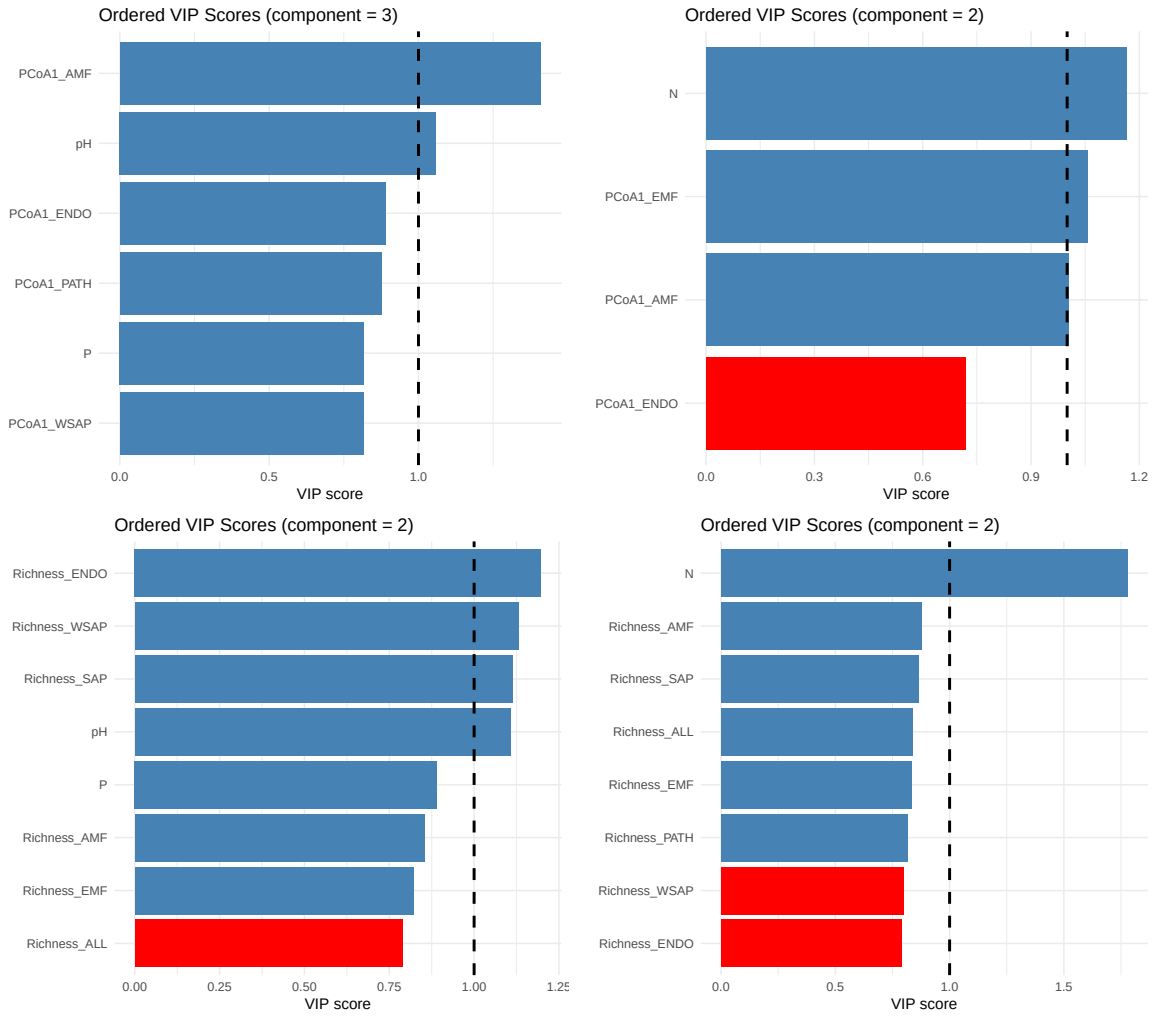

Fig. 20. VIP Scores of the fungal PCoA1 axes (upper) and fungal richness (lower) as well as soil parameters explaining the tree and seedling community turnover in the Mexican wet (left) and dry (right) forest. The correlation coefficients between the predicted and the actual plant community turnover are 0.64 **(upper left)**, 0.76 **(upper right)**, 0.44 **(lower left)** and 0.67 **(lower right)**.

The wet forest community turnover is best explained by the PCoA1 axis of arbuscular mycorrhizal fungi (1.40) followed by pH (1.06) as well as endophyte richness (1.19) and richness of saprotrophic (1.11) and wood saprotrophic (1.13) fungi. In the dry forest, soil nitrogen (1.16 / 1.77) and the PCoA1 axes of ectomycorrhizal (1.05) and arbuscular mycorrhizal (1.00) fungi have the highest effect on the community turnover.

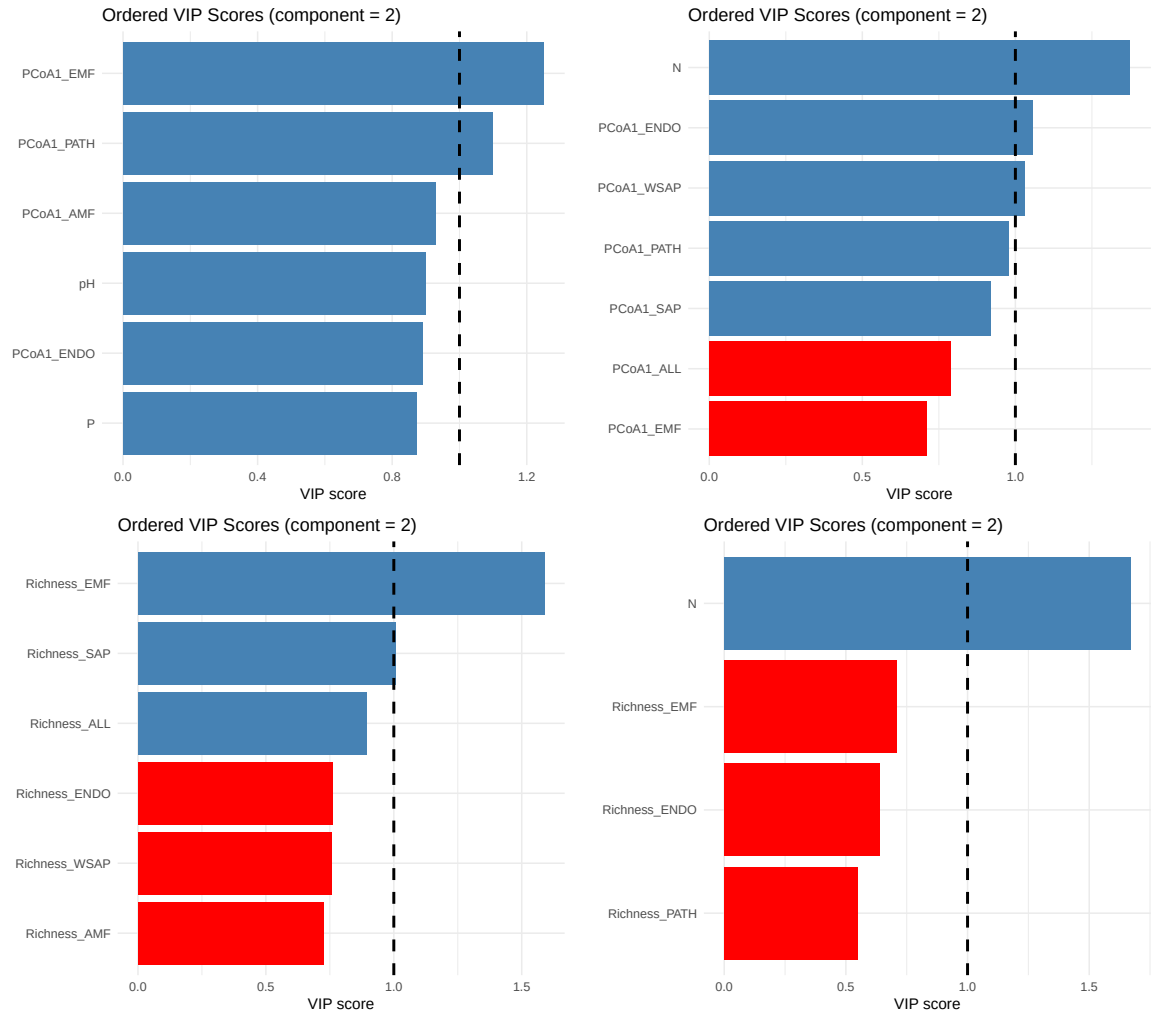

Fig. 21. VIP Scores of the fungal PCoA1 axes (upper) and fungal richness (lower) as well as soil parameters explaining the PCoA1 axis of the tree and seedling community in the Mexican wet (left) and dry (right) forest. The correlation coefficients between the predicted and the actual plant PCoA1 axis are 0.49 **(upper left)**, 0.77 **(upper right)**, 0.41 **(lower left)** and 0.61 **(lower right)**.

Plant community composition (PCoA1) in the wet forest was best explained the community composition (PCoA1) of plant pathogens (1.09), ectomycorrhizal (1.25), as well as arbuscular mycorrhizal (0.93) fungi and the richness of ectomycorrhizal (1.58) and saprotrophic fungi (1.01). In the dry forest, the best predictors were soil nitrogen content (1.37 / 1.67), the PCoA1 axis of endophytes (1.05) and wood saprotrophs (1.03).

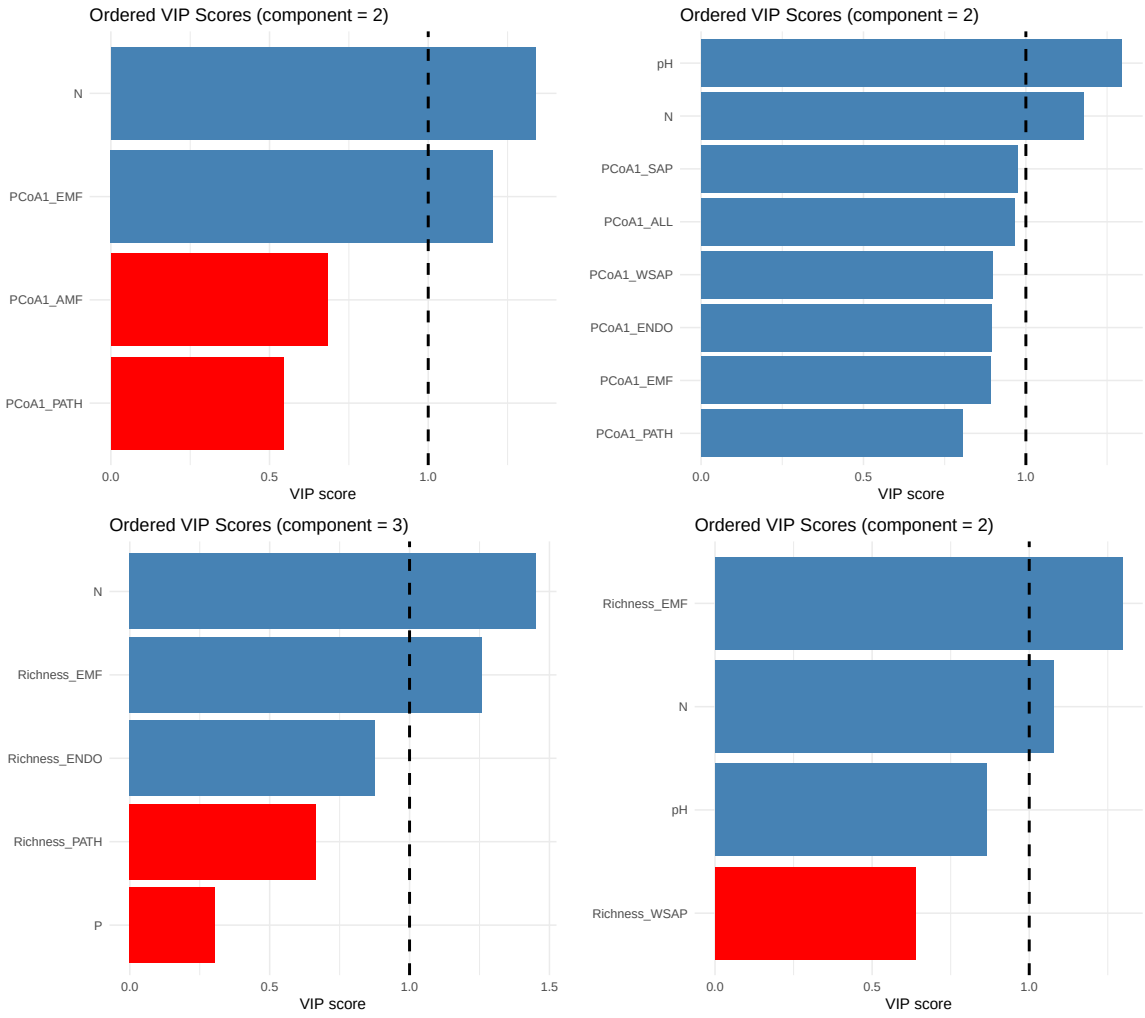

Fig. 22. VIP Scores of the fungal PCoA1 axes (upper) and fungal richness (lower) as well as soil parameters explaining the annual mean growth of trees and seedlings in the Mexican wet (left) and dry (right) forest. The correlation coefficients between the predicted and the actual annual mean growth are 0.52 **(upper left)**, 0.57 **(upper right)**, 0.54 **(lower left)** and 0.47 **(lower right)**.

Annual mean growth in the wet forest is best explained by soil nitrogen content (1.33 / 1.45), the PCoA1 axis of ectomycorrhizal fungi (1.20) and the richness of ectomycorrhizal fungi (1.25). For the dry forest it is pH (1.29), nitrogen (1.17), richness of ectomycorrhizal fungi (1.29) and nitrogen (1.07).

2.4 Discussion

Here we show that soil fungal communities, and mycorrhizal guilds in particular, are tightly coupled to vegetation recovery during tropical forest restoration. We also show that there is an especially strong link between plant and fungal community composition in the Mexican wet forest. Furthermore, during the first five years of succession in the Mexican dry forest, fungal diversity declines and fungal communities become more homogeneous. Together, these and other results demonstrate that fungal communities are highly responsive to land abandonment and early successional dynamics, and that underlying soil properties, rather than forest type alone, play a key role in mediating fungal responses and their feedbacks to plant communities. This is important because trees, particularly during establishment and early growth, are strongly dependent on fungal symbionts for nutrient acquisition and stress tolerance, meaning that belowground processes can directly shape restoration trajectories. Below, we discuss these results in greater detail and their implications for tropical forest restoration.

2.4.1 The Mexican Forests Regenerate

The Mexican forests are clearly regenerating based on their vegetation developement over time. Their species diversity, the number of individuals per plot and annual mean growth generally increased over time. Trees in the wet forest mostly regenerated through resprouting and in the dry forest via seeds. There is also a significant change in tree and seedling species composition between 2020 and 2024 in the wet forest. This suggests that the vegetation in the wet forest quickly regenerated in the first years through resprouting, indicating a higher species turnover compared with the dry forest.

In contrast to the successional dynamics in the Mexican forests, the Australian plots showed minimal recruitment of trees and seedlings during the study period. In Australia plots were dominated by grasses. The wet forest plots also have a high amount of ferns, which might indicate an arrested succession (Takeshige et al., 2023). While the lower amount of trees and seedling in the Australian forests limits direct comparability with the Mexican forests, it underscores that regeneration trajectories are strongly context-dependent and influenced by regional species pools, land-use history, and climatic constraints.

When looking at the plant genera, most of them associate with arbuscular mycorrhizal fungi. This is typical for the phosphorus depleted soils in the tropics (Soudzilovskaia et al., 2019). What might not have been captured is the fact that it might be important for seedlings to be able to switch their mycorrhizal partner depending on environmental context (Teste et al., 2020). Although this could not be confirmed in our study using

databases of known dual mycorrhizal plants (Teste et al., 2020), the dual mycorrhizal status of many plants remains unknown. There is strong evidence that, especially in the tropics, dual mycorrhizal plants may be more common than we originally imagined and that they play an important role in forest processes (Rog et al., 2025).

2.4.2 Functional Guild Structure of Tropical Soil Fungi

Saprotrophic fungi and plant pathogens dominate the fungal soil microbiome. The relative abundances of arbuscular mycorrhizal fungi and ectomycorrhizal fungi are very similar. This is partly because sequencing of the ITS region is not the most sensitive method in detecting arbuscular mycorrhizal fungi (Lekberg et al., 2018). In contrast, the primers we used are excellent at detecting Dikaryotic fungi and thus ectomycorrhizal fungi might be more present in the tropics than previously thought (Tedersoo et al., 2009, 2012).

Ectomycorrhizal fungi showed strong country-specific patterns. In Australia, ectomycorrhizal fungi were significantly more abundant in dry forests, whereas in Mexico they were more abundant in wet forests. These contrasting patterns likely reflect differences in host tree availability both within the restoration plots and in relation to the neighboring forests from which these fungi likely disperse.

Unexpectedly, endophytic fungi constituted a relatively large proportion of the fungal community in both forest types. Endophytes are increasingly recognized as important mediators of plant stress tolerance, pathogen resistance, and nutrient cycling (Bell-Dereske et al., 2017; Shankar Naik, 2019). Their prevalence suggests that endophytic associations may play a critical role in tropical forest recovery (Griffin & Carson, 2018).

2.4.3 Soil Properties Shape Fungal Community Composition

Soil physicochemical conditions can regulate microbial growth and community assembly with implications for ecosystem restoration. Soil physicochemical properties emerged as significant predictors of fungal guild structure and overall community composition. The dbRDA analyses showed that total soil nitrogen, pH, and country were significant predictors, with clay content and forest type exerting marginal effects.

Saprotrophic fungi were strongly associated with soil nitrogen and clay content, reflecting their role in organic matter decomposition and nutrient mineralization. Higher nitrogen availability may stimulate saprotrophic activity by alleviating nutrient constraints, thereby accelerating decomposition processes in regenerating forests.

Arbuscular mycorrhizal fungi were primarily associated with soil pH, which is consistent with previous studies showing strong pH sensitivity in AMF community assembly (Carrino-Kyker et al., 2016; Clark, 1997; Hazard et al., 2012; Qin et al., 2015; Van Aarle et al., 2002). This suggests that even small shifts in soil chemistry during forest regeneration can have disproportionate effects on mutualistic fungal communities.

Interestingly, forest type alone was a weak predictor when soil variables were accounted for. This indicates that differences commonly attributed to "wet" versus "dry" forests may, at least partly, be mediated through underlying soil conditions rather than climate alone.

2.4.4 Coupling between Fungal and Plant Communities

Plant-soil interactions play a central role in shaping community assembly and ecosystem processes in tropical forests. One of the most striking findings of this study is the strong coupling between fungal and plant community composition in Mexican wet forests. Mantel tests revealed significant correlations between plant community dissimilarity and overall fungal community dissimilarity, as well as for specific guilds including saprotrophs, arbuscular mycorrhizal fungi, and plant pathogens.

This coupling was absent in dry forests, suggesting that biotic interactions may be more tightly linked in wetter, more productive systems (Milici et al., 2020; Walter, 2018). In wet forests, rapid plant turnover and higher biomass production may create feedbacks that directly shape fungal community composition via litter quality, root exudates, and host specificity.

Plant pathogens also showed a significant, though weaker, association with plant community structure. This may reflect density-dependent effects, where increasing plant diversity and abundance facilitate the establishment of host-specific pathogens. Successional trajectories might be positively influenced by plant pathogens, as they have the potential to decrease competition among trees (Connell, 1971). As this study looks at early successional forests, competition might not be relevant yet, which suggests that negative plant-soil feedbacks may become more important during later stages of succession when larger trees have established, as competition becomes a more important driving factor for plant development.

2.4.5 Fungal Communities Become Less Diverse and More Homogeneous with Time

How soil fungal biodiversity changes over the course of restoration has received particularly little attention. We could show that the resampling of Mexican dry forest plots revealed pronounced temporal changes in fungal community composition over a four-year interval. Fungal communities not only shifted significantly but also became more homogeneous over time, suggesting a convergence toward a more stable community structure as succession progresses.

Despite this compositional shift, fungal community turnover was not explained by soil variables, nor did it predict plant community turnover or growth rates. This indicates that fungal succession may follow partially autonomous trajectories, influenced by biotic interactions, dispersal or unmeasured environmental factors.

The observed decline in fungal alpha diversity from 2020 to 2024 contrasts with the increasing plant diversity during the same period. This decoupling suggests that early successional stages may initially support a broad spectrum of opportunistic fungal taxa, which are later filtered as plant communities stabilize. This questions the goal of attaining high fungal biodiversity in restoration solely for the sake of biodiversity and raises the question of whether high fungal biodiversity is actually beneficial.

2.4.6 Mycorrhizal Fungi Matter in Restoration

The PLSR analyses revealed complex, forest-type-specific relationships between fungal communities, soil properties and vegetation dynamics. In wet forests, plant community turnover was best explained by the composition of arbuscular mycorrhizal fungi, soil pH, and the richness of endophytic and saprotrophic fungi. This underscores the importance of belowground mutualists and decomposers in shaping aboveground community assembly and vice versa.

In dry forests, soil nitrogen emerged as the dominant predictor of plant community turnover and composition, alongside the composition of ectomycorrhizal and arbuscular mycorrhizal fungi. This suggests that nutrient limitation may override biotic interactions under drier conditions, with fungal communities playing a secondary but still significant role.

Annual mean growth rates were best predicted by soil nitrogen and ectomycorrhizal fungal composition and richness in both forest types. This finding is particularly noteworthy, as it indicates that ectomycorrhizal fungi, despite their relatively low abun-

dance, may disproportionately influence tree growth during early forest regeneration. The link between ectomycorrhizal fungal composition and tree growth has already been shown in European forests (Anthony et al., 2022; Hupperts & Lilleskov, 2022).

2.4.7 Limitations and Implications for Tropical Forest Restoration

Although this study offers novel perspectives on fungi in tropical forest restoration, it still has some limitations. Firstly, the sample sizes are different for the Mexican and Australian forests, as there are few plots with trees in the Australian forests. Thus, only data from the Mexican forests could be used in many analyses. Secondly, the soil samples were taken during different years, ranging from 2020 to 2024. As shown in **2.3.3**, at least in the Mexican dry forest, the fungal diversity, as well as the fungal composition, changed significantly within a few years, indicating that this may also be the case for the other forests. However, most samples were taken in 2022 (see Appendix **B.1**), which means that this might be a minor issue. Thirdly, fungal functional guild assignments rely on databases that may incompletely represent tropical fungal taxa, introducing uncertainty into guild-level interpretations. Lastly, as this study relies on correlational analyses, the observed associations should not be interpreted as evidence of causal relationships or directional effects.

The results of this study highlight fungal communities as integral components of tropical forest regeneration. Restoration efforts that focus exclusively on aboveground vegetation may overlook critical below-ground processes that influence nutrient cycling, plant performance, and successional pathways.

Maintaining or restoring soil conditions that support diverse mycorrhizal and saprotrophic communities may enhance restoration outcomes. In particular, promoting systems rich in arbuscular mycorrhizal fungi and maintaining host trees of ectomycorrhizal fungi in appropriate regions could accelerate forest recovery. Furthermore, the strong coupling between plant and fungal communities in wet forests suggests that interventions targeting plant diversity may have cascading effects on soil microbial communities, potentially reinforcing positive feed-backs during restoration.

Future research should incorporate longer time series and experimental manipulations to better disentangle causal relationships. Integrating plant trait data and root colonization measurements would further strengthen inferences about plant-fungus interactions during forest regeneration.

2.5 Conclusion

This study investigated the links between tropical forest regeneration and soil fungal communities across tropical dry and wet forests in Mexico and Australia following agricultural abandonment. By integrating vegetation surveys, soil physicochemical data, and high-throughput sequencing of fungal communities, this thesis provides a comprehensive view of how aboveground and belowground components co-develop during early stages of forest recovery.

Overall, the results demonstrate that tropical forest regeneration is well underway in the Mexican sites, as evidenced by significant increases in tree and seedling abundance, species richness, and biomass over time. Regeneration pathways differed between forest types: wet forests were characterized by strong compositional turnover and resprouting-driven recovery, while dry forests showed more stable species composition and a predominance of seed-based regeneration. These contrasting trajectories highlight that successional dynamics are strongly shaped by environmental context and disturbance history.

Fungal communities differed markedly between forest types and countries, with saprotrophic fungi and plant pathogens dominating overall and ectomycorrhizal fungi showing strong biogeographic patterns. Endophytic fungi emerged as a surprisingly dominant guild, suggesting an important but often overlooked role in forest regeneration. Soil properties, particularly available soil nitrogen and soil pH, were major drivers of fungal guild composition and community structure.

A central finding of this thesis is the tight coupling between plant and fungal communities in Mexican wet forests, where changes in vegetation composition were strongly correlated with shifts in fungal community composition, including arbuscular mycorrhizal fungi, saprotrophic fungi and plant pathogens. This coupling was absent in dry forests, indicating that plant-fungal feedback is more pronounced in wetter, more productive systems. Temporal analyses further revealed that fungal communities undergo substantial compositional change during regeneration, becoming more homogeneous over time, even as plant diversity continues to increase.

PLSR modeling showed that fungal community composition and richness, especially of mycorrhizal and saprotrophic guilds, together with soil nutrients, significantly contributed to explaining plant community turnover, composition, and growth rates. Notably, ectomycorrhizal fungi, despite their relatively low abundance, were consistently associated with tree growth, underscoring their functional importance during early suc-

cessional stages.

Taken together, these findings highlight soil fungal communities as integral components of tropical forest regeneration. Forest recovery is best understood as a coupled above- and below-ground process shaped by interactions among plants, fungi, and soil properties. From a restoration perspective, this implies that successful tropical forest restoration should consider not only plant species composition but also the recovery and maintenance of functional soil fungal communities.

Future research should extend these findings by incorporating long term monitoring, experimental manipulations and direct measures of fungal functioning and plant-fungus interactions. Such approaches will be essential to fully understand the mechanisms linking soil biodiversity to ecosystem recovery and to improve the effectiveness of restoration strategies in tropical forests.

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Appendices

A Full Length ITS Sequencing

Introduction

Mycorrhizal fungi play a crucial role in tropical forests worldwide (Brundrett & Tedersoo, 2018). However, they are often overlooked when it comes to restoring these forests (Chazdon, 2014). More research on the intricate effects of these fungi on the restoring vegetation is needed. It has been shown that mycorrhizal fungi do have a crucial effect on plant biodiversity and growth (van der Heijden et al., 1998, 2008). However, in early succession mycorrhizal interaction and their role in the plant-soil feedback are contradictory (Kardol et al., 2006; Reynolds et al., 2003).

This research project aims to provide the basis for filling these knowledge gaps by supplying the fungal DNA data to link mycorrhizal communities to forest growth in regenerating abandoned pastures in Mexico and Australia. The question of how different fungal parameter affect forest regeneration, tree community turnover and tree diversity could then be addressed.

Vegetation and soil data from forest regeneration plots could be linked with the full length ITS fungal sequencing data. The fungal DNA data could then be split into different fungal guilds. From there, the effects of especially arbuscular mycorrhizal fungi, as well as saprotrophic and pathogenic fungi, could be examined. It is expected that arbuscular mycorrhizal fungi and pathogenic fungi act in an antagonistic way, the former improving plant growth parameters, while the latter might have a mixed effect on these. They usually decrease tree growth by infecting plants, but it has been shown that they can increase tree diversity as they decrease competition among trees (Connell, 1971). This study could also show how fungal communities shift over time in these regenerating forest plots as soil was sampled twice, once in 2020 and a second time in 2024.

However, the full length ITS sequencing failed due to various reasons. This report shall give an overview of the methods used and discuss why they failed in this case. It shall also give an outlook to another sequencing method, that is more likely to work. This method shall provide the basis to fill part of the existing knowledge gap and provide a better understanding of how fungal communities and trees in regenerating tropical forests interact.

Materials and Methods

The soil has been sampled in the center of the 25 m x 25 m plots in both Mexico and Australia. It has been air-dried on site and shipped to Vienna. There, 250 mg of soil has been weighted in and its DNA content has been extracted using the E.Z.N.A.® Soil DNA Kit.

PCR

For the PCR, the full length ITS primers ITS9munngs (forward, GTACACACCGCCCGTCG) and ITS4ngUni (reverse, CGCCTSCSCTTANTDATATGC) have been used. The primers were barcoded which allows for multiplexing many samples.

26 μL of PCR-grade water were mixed with 20 μL of PCR master mix in an 1.5 mL mini-centrifuge tube. Then, 1 μL of the barcoded forward primers and 1 μL of the barcoded reverse primers were added to the tube. Lastly, 2 μL of the DNA extract itself was added. The tube was then vortexed for 5 seconds and centrifuged for 30 seconds at 5,000 rpm. The 25 μL of the sample was then transferred to two dome-capped PCR strip tubes, to create two replicates. After that, the samples were spun down in a portable mini centrifuge for 20 seconds.

The ITS thermocycler conditions were the following: 95 °C for 15 minutes followed by 35 cycles: 30 seconds at 95 °C, followed by 30 seconds at 57 °C and 72 °C for 60 seconds. After the cycles the temperature was set to 72 °C for 20 minutes, followed by a hold at 4 °C.

Gel Electrophoresis

For the gel 1 g of agarose was added with 100 mL of TAE (1x) into a 250 μL microwavable flask. To create a 1 % agarose solution the flask was micorwaved until the agarose has been fully melted and the mixture was boiling. After that, 1 μL of gel red was poured into the solution. The cast was set up using an appropriate comb and the liquid was poured into the cast. It usually took around 30 minutes until the solution cooled down and the agarose solidified. The replicates of the PCR products were combined again.

After the gel has solidified and the comb has been removed, the gel was loaded with 1 μL of the 1kb ladder and 5 μL of each sample. Then the gel was run for 30 minutes at 90 V. Lastly the gel was visualized and a picture was saved.

Pooling

To pool the barcoded PCR products at equal DNA concentration, the DNA concentration was measured with the Qubit. For the Qubit two standards had to be prepared. Each standard received 190 μL of working reagent and 10 μL of the standard solution. To prepare the samples, only 2 μL of sample and 198 μL of working reagent were mixed together. After vortexing, the standards were loaded into the Qubit first, followed by the samples.

To save time, the gels had been analyzed before and the dimmest as well as the brightest sample were selected for each gel run. Only these samples were analyzed in the Qubit. The DNA concentration of the rest of the samples was estimated by the intensity of the bands compared with the intensity of the two measured samples.

Sequencing and Bioinformatics

The PCR products were sequenced using the Oxford Nanopore sequencing technique. It reads DNA by measuring electrical current changes as single molecules pass through nanopores. The samples were multiplexed with unique base pair combinations ("barcodes"), which allows multiple samples to be sequenced in one run. The bioinformatics, in particular the filtering and demultiplexing of the DNA sequencing data, was performed at the LiSC server.

Results

Gels

The gels showed mostly very weak bands, as can be seen in **Fig. 23**. This was expected and is typical for the given full length ITS primers. Nonetheless, it made it very difficult to properly distinguish between samples with a successful polymerase chain reaction and samples without one. To be sure, many of the samples had to be redone. Even after several retries, a handful of samples did not seem to work. To solve this, we first performed an inhibitor removal on the DNA extracts. As this was unsuccessful too and the DNA concentration was low in those samples, we doubled the amount of DNA extract that was used in the PCR. This seemed to do the trick. However, overall, the bands were still very weak, and it was often very hard to determine if the PCR was successful.

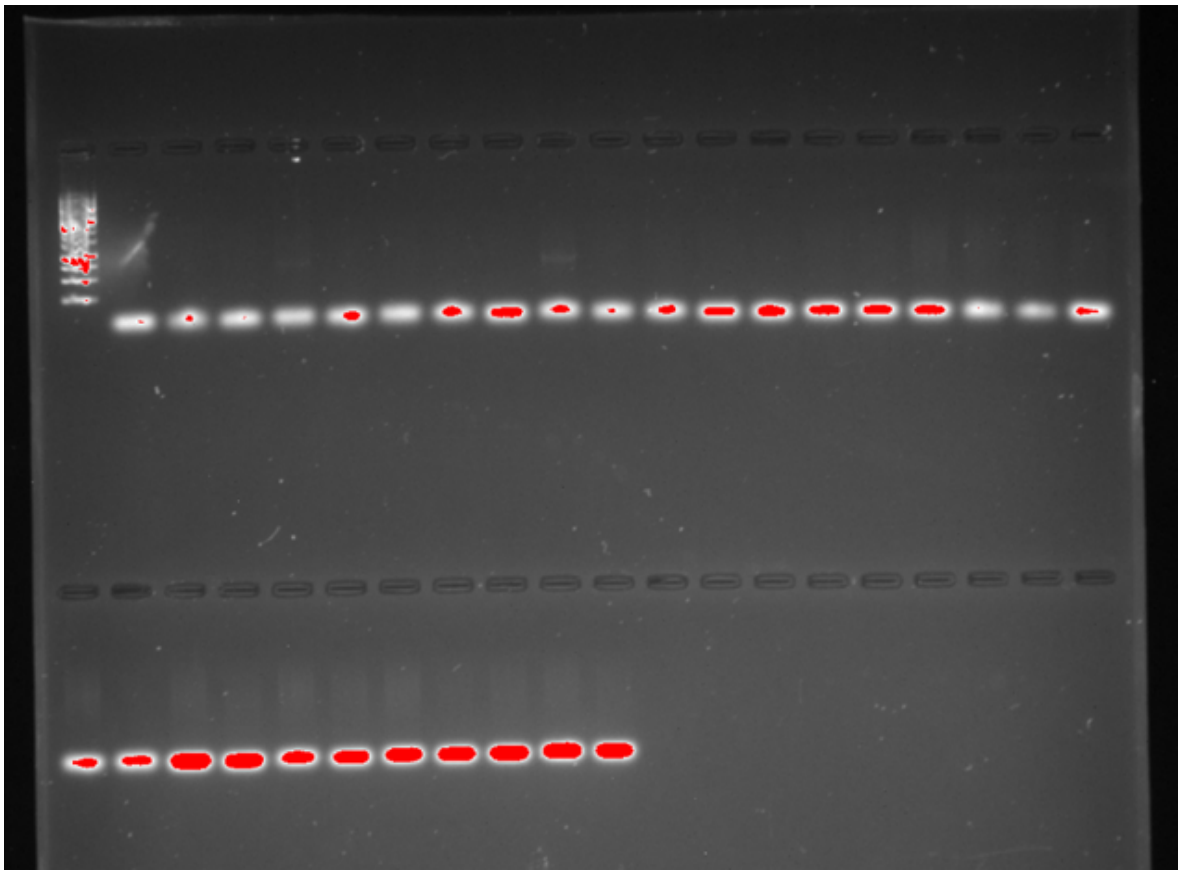

Fig. 23. Typical Gel. The first band shows the ladder. The rest of the bands show the PCR products, including a blank. While the upper row shows mixed results, most of the samples in the lower row were successful.

Sequencing Results

The sequencing was not successful. We expected the resulting DNA data to have at least multiple GB for the 107 samples. Our filtered and demultiplexed DNA data had only 10 MB, indicating that something went seriously wrong. Only two PCR products showed decent results.

Discussion

The sequencing results are paradoxical. Although many samples showed a clear successful PCR signal on the gel and many others showed a very faint one, the sequencing did not succeed. One could criticize that the decision of whether a PCR run was successful or not, by looking at the gel, was very subjective, especially for the weak bands. However, for many bands, it was very clear that the reaction was indeed successful.

Inhibitors as a reason behind this are very unlikely, as the PCR did work and we performed an inhibitor removal on some DNA extracts. The most probable cause is DNA fragmentation. Most of the samples are several years old and have undergone

multiple freeze-thaw cycles. Crystallization and enzymatic activity could have seriously fragmented the fungal DNA, and as the whole ITS1 region was looked at during sequencing, there might not have been long enough fragments present.

Conclusion

This project set out to generate full-length ITS fungal sequencing data from tropical forest restoration plots in Mexico and Australia in order to link mycorrhizal community composition with forest recovery dynamics. While DNA extraction and PCR amplification produced visible, though often weak, bands on agarose gels, the subsequent Oxford Nanopore sequencing run yielded almost no usable data. Despite multiple troubleshooting steps, including inhibitor removal and increased DNA input, the sequencing output remained extremely low, suggesting that the underlying issue occurred before or during sequencing rather than during PCR evaluation.

The most plausible explanation is that the fungal DNA in the soil samples was heavily fragmented. As highlighted in the discussion, the samples were several years old and had undergone repeated freeze–thaw cycles, which likely accelerated enzymatic degradation and crystallization damage. Because full-length ITS sequencing requires long, intact DNA fragments, the degraded state of the DNA would have prevented successful sequencing even when PCR fragments appeared faintly on gels. Consequently, the attempt to obtain full-length ITS reads was unsuccessful, and only two samples produced meaningful sequencing output.

Despite this setback, the project demonstrated the limitations of working with older, low-quality soil DNA when attempting long-read sequencing. The next step will be using ITS2 primers which look a shorter fraction of the ITS region, as such data remains essential for examining how mycorrhizal guilds contribute to forest regeneration and for advancing our understanding of plant–soil–microbe interactions in tropical restoration systems.

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B Supplement

B.1 Data Structure

Country	Forest	Soil Fungi Samples Type	Plot	Year	Vegetation Data 2020 (LT)	2021 (LT)	2022 (LT)	2023 (LT)	2024 (LT)	2020 (S)	2021 (S)	2022 (S)	2023 (S)	2024 (S)	Land Use History 2020 (T)	2021 (T)	2022 (T)	2023 (T)	2024 (T)	LUI	Soil Parameter Year	P	N	pH
MX	Dry	FC	1	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	2	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	3	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	4	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	5	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	6	2020	x	x	x			x	x	x	x			x	x	x		x			x	x
MX	Dry	FC	7	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	8	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	9	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	10	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	11	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	12	2020	x	x	x			x	x	x												
MX	Dry	FC	13	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	14	2020	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x			x	x
MX	Dry	FC	1	2024																				
MX	Dry	FC	2	2024																				
MX	Dry	FC	3	2024																				
MX	Dry	FC	4	2024																				
MX	Dry	FC	5	2024																				
MX	Dry	FC	7	2024																				
MX	Dry	FC	8	2024																				
MX	Dry	FC	9	2024																				
MX	Dry	FC	10	2024																				
MX	Dry	FC	11	2024																				
MX	Dry	FC	13	2024																				
MX	Dry	FC	14	2024																				
MX	Dry	FC	16	2022			x	x				x	x	x	x	x	x	x	x	x	x	x	x	x
MX	Dry	FC	17	2022			x	x				x	x	x	x	x	x	x	x	x	x	x	x	x
MX	Dry	FC	18	2022			x	x				x	x	x	x	x	x	x	x	x	x	x	x	x
MX	Dry	FC	19	2022			x	x				x	x	x	x	x	x	x	x	x	x	x	x	x
MX	Dry	FC	21	2022			x	x				x	x	x	x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	1	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	2	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	3	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	4	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	5	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	6	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	7	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
MX	Dry	Bio	1	2024																				
MX	Dry	Bio	2	2024																				
MX	Dry	Bio	3	2024																				
MX	Dry	Bio	4	2024																				
MX	Dry	Bio	5	2024																				
MX	Dry	Bio	6	2024																				
MX	Dry	Bio	7	2024																				
MX	Dry	Bio-Org	1	2020						x	x	x	x		x	x	x	x	x	x	x	x	x	x
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MX	Wet	FC	2	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	3	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	4	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	5	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	6	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	7	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	8	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	9	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	10	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	11	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	12	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	13	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	14	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	15	2022		x	x	x				x	x	x	x	x	x	x	x	x			x	x
MX	Wet	FC	16	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	17	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	18	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	19	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	FC	20	2022		x	x	x				x	x	x	x	x	x	x	x	x		x	x	x
MX	Wet	Bio	1	2022													x	x	x				x	x
MX	Wet	Bio	2	2022													x	x	x				x	x
MX	Wet	Bio	3	2022													x	x	x				x	x
MX	Wet	Bio	4	2022													x	x	x				x	x

MX	Wet	Bio	5	2022							x	x	x			x	x	x
MX	Wet	Bio	6	2022							x	x	x			x	x	x
MX	Wet	Bio	7	2022							x	x	x			x	x	x
MX	Wet	Bio	8	2022							x	x	x			x	x	x
AUS	Wet	FC	1	2022	x	x	x	x	x	x				x	2022	x	x	x
AUS	Wet	FC	2	2022	x	x	x	x	x					x	2022	x	x	x
AUS	Wet	FC	3	2022	x	x	x	x						x	2022	x	x	x
AUS	Wet	FC	4	2022	x	x	x			x					2022	x	x	x
AUS	Wet	FC	5	2022	x	x	x		x	x				x	2022	x	x	x
AUS	Wet	FC	7	2022	x	x	x								2022	x	x	x
AUS	Wet	FC	8	2022	x	x	x							x	2022	x	x	x
AUS	Wet	FC	9	2022		x	x		x	x				x	2022	x	x	x
AUS	Wet	FC	10	2022		x	x		x	x				x	2022	x	x	x
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AUS	Wet	FC	14	2022		x	x		x						2022	x	x	x
AUS	Wet	FC	15	2022		x	x		x	x					2022	x	x	x
AUS	Wet	FC	16	2022		x	x		x						2022	x	x	x
AUS	Wet	FC	17	2022		x	x							x	2022	x	x	x
AUS	Wet	FC	18	2022		x	x							x	2022	x	x	x
AUS	Wet	FC	19	2022		x	x							x	2022	x	x	x
AUS	Wet	FC	20	2022		x	x		x					x	2022	x	x	x
AUS	Wet	FC	21	2022		x	x							x	2022	x	x	x
AUS	Dry	FC	22	2022		x	x							x	2022	x	x	x
AUS	Dry	FC	23	2022		x	x							x	2022	x	x	x
AUS	Dry	FC	30	2022		x	x							x	2022	x	x	x
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AUS	Dry	FC	32	2022		x	x		x					x	2022	x	x	x
AUS	Dry	FC	33	2023			x			x								
AUS	Dry	FC	34	2023			x											
AUS	Dry	FC	35	2023			x											
AUS	Dry	FC	36	2023			x											
AUS	Dry	FC	46	2024					no veg data									

B.2 Figures

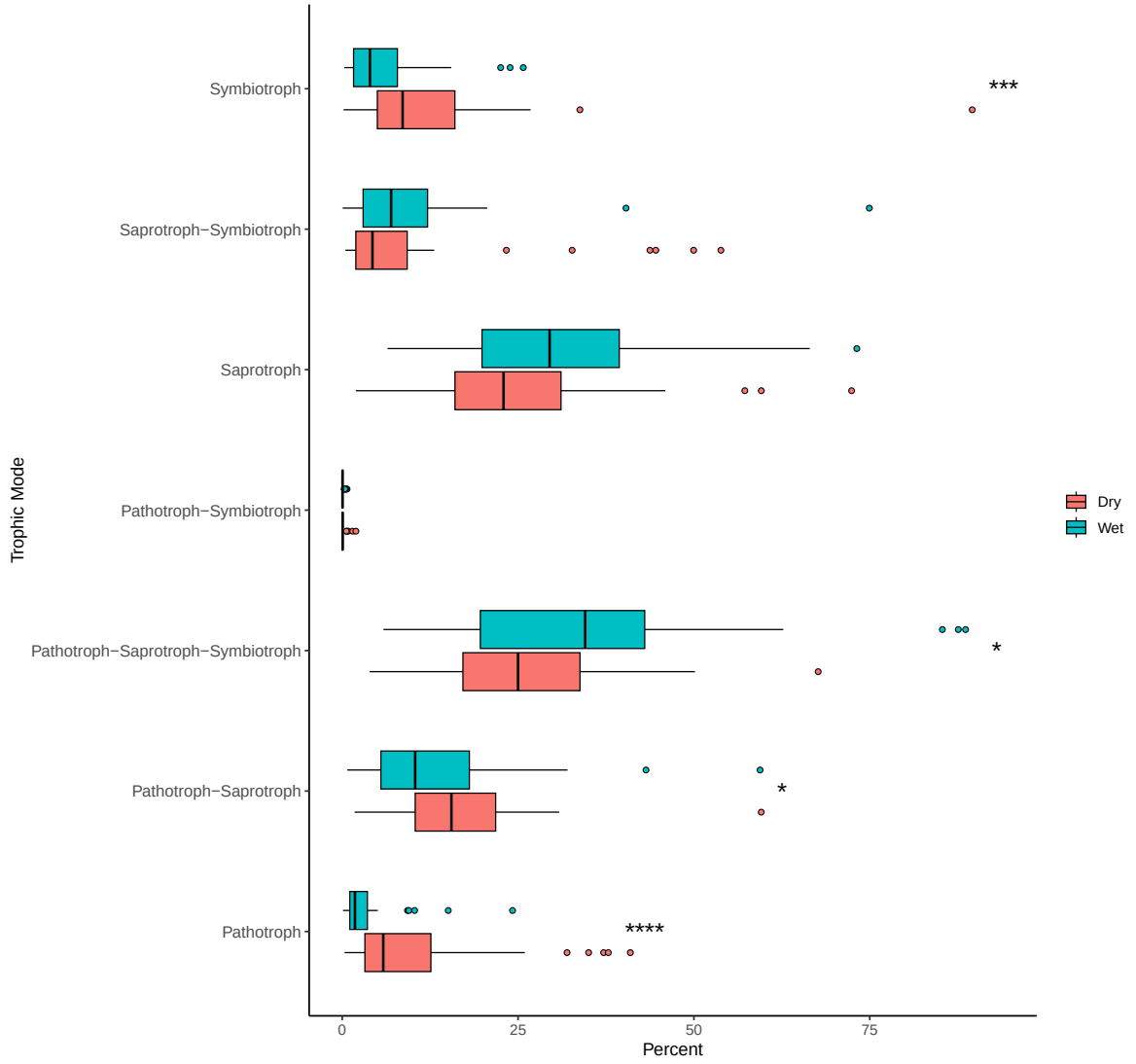

Fig. 24. Abundance of trophic modes in wet and dry tropical forests in both Mexico and Australia. Symbiotrophs, Pathotrophs, Pathotroph-Symbiotrophs and Pathotroph-Saprotroph-Symbiotrophs have a significantly different abundance between wet and dry tropical forests.

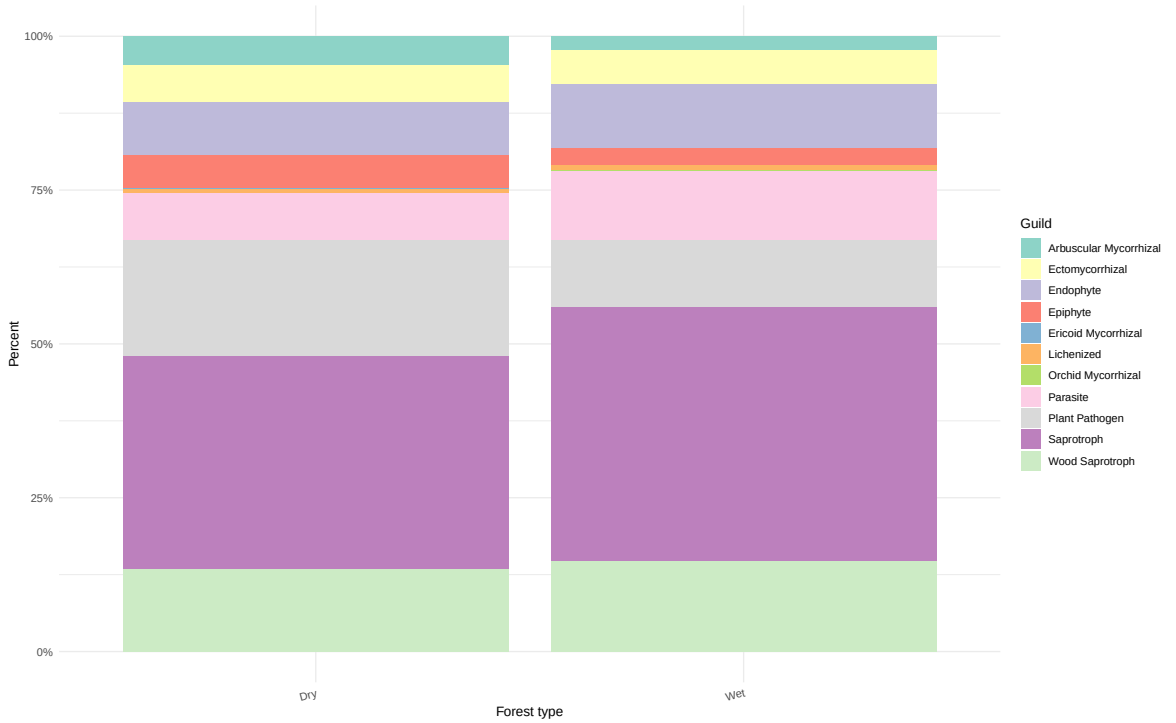

Fig. 25. Histogram of the mean fungal guild abundances in Australian and Mexican dry (left) and wet (right) forests.

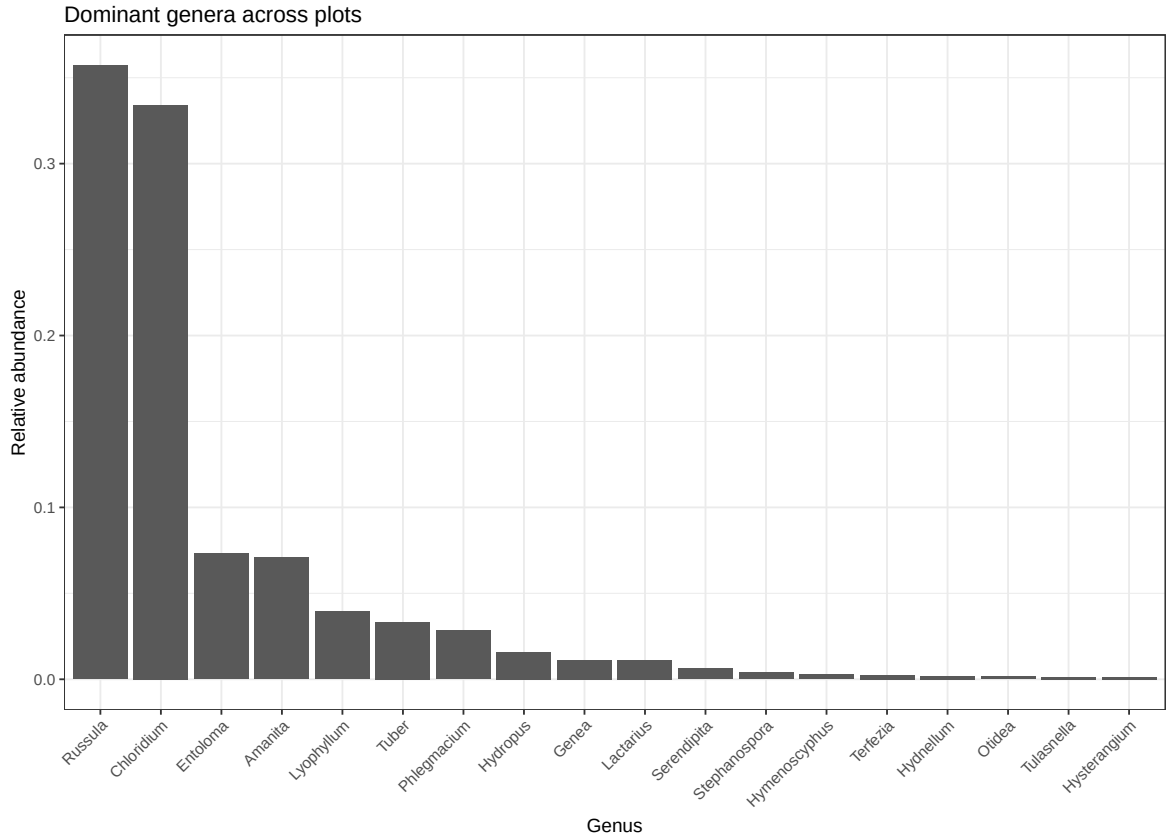

Fig. 26. Taxonomic overview of the relative abundance of ectomycorrhizal genera above 1%.

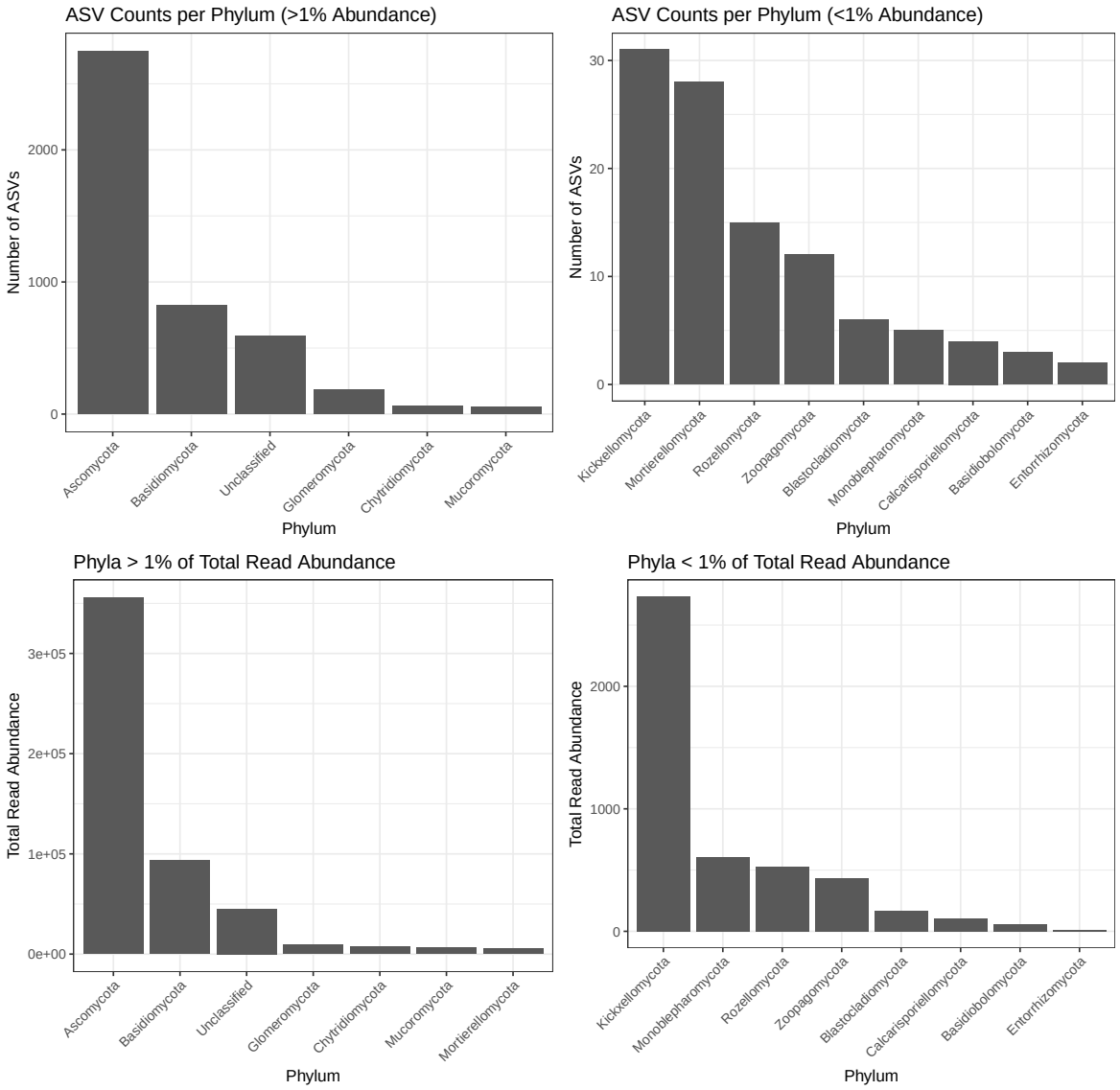

Fig. 27. Taxonomic overview split into phyla counts and abundances above 1% (left) and below 1% (right). (upper) ASV Counts per Phylum. **(lower)** Total Abundance per Phylum.