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Abstract

Most terrestrial plants worldwide make associations with mycorrhizal fungi, yet the fungal diversity of Madagascar is vastly unknown. This research project is a preliminary investigation into the mycorrhizal status of 19 tree species in and near Ranomafana National Park (RNP), a lowland tropical forest on the southeast coast of Madagascar. Arbuscular mycorrhizal (AM) colonization in these species was studied in root samples under the microscope in the laboratory of Centre ValBio (CVB). The degree of AM infection was assessed for three study areas: one directly adjacent to a protected area and two reforestation sites supported by CVB. For the 19 samples examined, 16 (84%) were determined AM colonized to varying degrees. Three species were inconclusive and require further analysis. Results also suggested that the degree of AM infection was reduced at the reforestation sites, with only 9% of samples being heavily infected compared to 25% of samples adjacent to the protected area. Additionally, above-ground fruiting bodies (mushrooms) were photographed and some later identified to describe the ectomycorrhizal (ECM) fungi abundance in the study areas. These macroscopic observations simply suggested the presence of ECM associations with certain species, and mushrooms were much more abundance in the protected area of RNP. However, no real conclusions could be made about the ECM status of trees, and only a few fungal species could be confidently identified. To conclude, this study found that most native tropical trees in RNP make AM associations to varying degrees, including the more abundant species. Land degradation from previous cultivation may influence mycorrhizal abundance as well, though more research is necessary.

La plupart de plantes terrestres mondialement font des associations avec les champignons mycorhiziens, pourtant la diversité fongique de Madagascar est grandement inconnue. Cette recherche est une investigation préliminaire sur l'état mycorhizien de 19 espèces d'arbres dans et environs le Parc National de Ranomafana (PNR), une forêt de basse terre tropicale sur la sud-est côte de Madagascar. La colonisation de mycorhizes arbusculaires (MA) dans ces espèces était étudiée dans l'échantillonnage de racines sous une microscope dans le laboratoire de Centre ValBio (CVB). Le degré d'infection MA était analysé pour trois zones d'étude : un adjacent à l'aire protégée de PNR et deux sites de reboisement (établis par CVB). Sur les 19 échantillons examinés, 16 (84%) étaient déterminés colonisés par MA des degrés divers, et l'évidence n'était

pas suffisant pour trois espèces. Ils ont besoin une analyse plus approfondie. Les résultats suggèrent que le degré d'infection MA était plus diminué aux sites de reboisement : seulement 9% d'échantillons étaient infectés lourdement par rapport à 25% à l'autre site près de l'entrée du parc. De plus, les fruits de champignons mycorhiziens (champignons) au-dessus du sol étaient photographiés et identifier plus tard pour décrire l'abondance de ectomycorhizes (ECM). Ces observations macroscopiques suggèrent simplement l'existence d'associations ECM avec des espèces spécifiques, et les fruits de champignons étaient moins abondantes aux sites de reboisement. Cependant, il n'y avait pas les conclusions fiables sur le degré de colonisation ou d'infection ECM d'arbres dans l'étude, et il y avait des espèces dont quelques étaient identifiés. Pour conclure, cette étude a trouvé que la plupart d'espèces originaires de Ranomafana font des associations MA, notamment des espèces les plus abondantes. La dégradation de terre à cause d'agriculture peut influencer l'abondance de champignons mycorhiziens aussi, mais plus de recherche est nécessaire.

Acknowledgements

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Key Terms

Arbuscule: a tree-like structure of AM fungi that infects plant root cells and functions as the site of nutrient exchange between the mycorrhizal fungus and host plant (Sessoms, 2020).

Arbuscular mycorrhiza (AM): soil microorganisms that form mutualistic symbioses with most terrestrial plants; they penetrate root cells directly to conduct nutrient exchange (Sessoms, 2020).

Centre ValBio (CVB): a research station located near Ranomafana National Park founded by Stony Brook University Institute for the Conservation of Tropical Environments' (ICTE).

Clearing: The KOH solution removes the host cell's cytoplasm and nuclei so that the stain can more readily penetrate and highlight the fungal tissues (Phillips & Hayman, 1970).

Ectomycorrhizal fungi (ECM): one of four subsets of mycorrhizal fungi that make associations with mostly temperate trees (Pinaceae and Angiosperms, some liverworts); their hyphae cannot penetrate the cell wall of host plant root cells (van der Heijden et al., 2015).

Endomycorrhizal fungi: mycorrhizal fungi that can penetrate the cell wall of host plant root cells to deliver essential nutrients; the largest subset of this group is arbuscular mycorrhizal fungi in the phyla Glomeromycota (van der Heijden et al., 2015).

Extraradical mycelium (ERM): the "roots" (hyphae) of mycorrhizal fungi that extend into the soil, forming a network that increases AM colonized plants' ability to absorb nutrients (Sessoms, 2020).

Hartig Net: fungal hyphae found in the intercellular spaces between cortical root cells of host plants; in some cases, the fungus may cover the plant cell to the extent that it has no contact with other root cells and is thus completely dependent on the fungus for nutrient acquisition (*The New York Botanical Garden*).

Hyphae: long, branching filaments of mycorrhizal fungi that grow around/inside plant roots, extending into the soil to access water in soil pores that cannot be reached by fine root hairs (Sessoms, 2020).

Mantle: an ectomycorrhizal structure that envelops host plant roots and conducts nutrient exchange (Anderson & Cairney, 2007).

Mycelium: the root-like structure of a fungus that collects nutrients in the soil; a mycelium may form fruiting bodies (mushrooms) above ground (Sessoms, 2020).

Mycology: the study of fungi

Mycorrhiza: a fungus that grows in association with plants in a symbiotic relationship (Sessoms, 2020).

Relative abundance: the number of individuals in a species relative to the total number of individuals in an ecosystem or habitat

Shannon-Wiener Diversity Index: a way to quantify the diversity of species in a community, given by $H = -\sum p_i * ln(p_i)$ where p_i is relative abundance; values typically range from 1.5 (low diversity) to 3.5 (high diversity) (Ortiz-Burgos, Selene, 2015)

Simpson's Diversity Index: a way to quantify species dominance in a community, given by $D = 1 - \frac{\sum n(n-1)}{N(N-1)}$ where n is each species' count and N is the total count of all species; values range from 0 (high dominance) to 1 (low dominance) (Ecology Center, 2023)

Species evenness: the extent to which species are equally represented within a community (Ecology Center, 2023)

Species richness: the number of different species represented in a community, landscape, or region.

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1. INTRODUCTION

1.1 Madagascar as a Biodiversity Hotspot

It is estimated that trillions of miles of fungal networks lie beneath Earth's surface, extending to a length equivalent to the radius of the Milky Way (Schueman, 2021). Despite their pervasiveness, mycorrhizal fungi remain largely undescribed around the world, and this is especially true in Madagascar (Rivas-Ferreiro et al., 2022). The island of Madagascar is known for its incredible floral and faunal diversity, but little is known about its fungal diversity (Rivas-Ferreiro et al., 2022). The first mycological explorations were made in the late 19th century but were halted by World War II (Rivas-Ferreiro et al., 2022). Only recently have mycologists taken interest in Madagascar's fungi again.

It is estimated that 50,000 mycorrhizal fungi species exist globally, making associations with roughly 250,000 plants (van der Heijden et al., 2015). Yet, Madagascar's National Report of the Convention on Biological Diversity in 2014 inventoried a mere 292 fungal species on the island (Madagascar Ministry of Environment and Forests, 2014); some authors report up to 500 species collected, with 90% of the waiting to be described to science (Ralaiveloarisoa et al., 2020). Among a staggering 50,000 fungal species, one thing is clear: Madagascar's mycological diversity is vastly unknown.

Madagascar's unique geologic history influenced its ecological and biological diversity, encouraging 80% floral and faunal endemicity (Madagascar Ministry of Environment and Forests, 2014). Madagascar drifted from the supercontinent of Gondwana about 100 mya, forming a landmass with India, then underwent a final separation around 88 mya (Rivas-Ferreiro et al., 2022). Scientists have hypothesized that its high endemism is attributed to this ancient geographic isolation (Rivas-Ferreiro et al., 2022). The island now hosts over 12,000-13,000 vascular plants, over 83% of which are endemic (*Sixième rapport national sur la diversité biologique de Madagascar*, 2019). Madagascar has the highest concentration of endemic plant families in the world: eight families are represented by 17 genera of 90 species (Ducousso et al., 2008). In particular, the humid rainforests of the east coast hold an incredible floral biodiversity and cover a total of 47,747 km^2, with 39% in protected areas (Madagascar Ministry of

Environment and Forests, 2014). This study chose to evaluate mycorrhizal fungal diversity in a lowland, tropical ecosystem due to its high level of floral diversity and endemicity.

1.2 Mycorrhizal Fungi

Forests around the world rely on a mycelial web to collect essential nutrients, like phosphorus and nitrogen, and in return, fungi use and sequester the carbon produced by trees (Cowan et al., 2017). This symbiotic relationship between fungi and plants, known as a "mycorrhiza," is a key component of nutrient/mineral transport in forest ecosystems (Ducousso et al., 2008). Almost all land plants rely on fungi for nutrient/mineral assimilation, as up to 80% of nitrogen and phosphorus is provided by mycorrhiza (van der Heijden et al., 2015). Consequently, mycorrhizal symbioses greatly influence plant productivity and diversity as well as carbon cycling (van der Heijden et al., 2015). The fungi-plant relationship is supported by a fungal mycelium that threads through the soil with hyphae that live inside and around the root cortex and epidermal cells (van der Heijden et al., 2015). These fungi extend root surface area, thereby making more nutrients accessible to the plant.

Mycorrhizal fungi are divided into four major groups based on their arrangement with host plant roots: endomycorrhizas (of which arbuscular mycorrhizas are the majority), ectomycorrhizas (ECM), orchid mycorrhiza and ericoid mycorrhiza (van der Heijden et al., 2015). Arbuscular mycorrhizas (AM) and ectomycorrhizas (ECM) are abundant in forests, as they associate with woody plants (van der Heijden et al., 2015). AM constitute the phylum Glomeromycota, whereas ECM constitute Basidiomycota and some Ascomycota; the phylum Mucoromycota contains some of both endo- and ectomycorrhizas (Naranjo-Ortiz & Gabaldón, 2019).

1.2.1 Ectomycorrhizal (ECM) Fungi

The difference between ECM and AM lies in their morphology and connection with host plants. ECM operate outside root cells and sometimes in the intercellular space between root epidermal and cortical cells, forming a "mycelial mantle" around lateral roots and/or a microscopic Hartig Net between cells (Anderson & Cairney, 2007). The interface formed by these structures is essential to the fungus-plant mutualism (Anderson & Cairney, 2007). Most

ECM fungi are in part saprotrophic, meaning they do not always need living host plants and can gain nutrients from dead organic material (van der Heijden et al., 2015). Many ECM produce fruiting bodies, otherwise known as "mushrooms," and they can be used to indicate the presence of mycelium in the soil (Anderson & Cairney, 2007). However, not all ECM reproduce with above-ground fruit, and/or their fruit may not be visible all the time (Anderson & Cairney, 2007). Thus, mushrooms are not always a reliable indicator of ECM abundance, and it is often necessary to analyze ECM fungi macro- and microscopically (Anderson & Cairney, 2007).

Globally, ECM fungi seem to be much less abundant than AM fungi, as 20,000 species are estimated to exist and associate with 6,000 plants (van der Heijden et al., 2015), a smaller figure than that of AM fungi. In Madagascar, ECM not only occur among native tree species but introduced ones as well, including *Pinus* and *Eucalyptus* (Rivas-Ferreiro et al., 2022). It is estimated that only 19% of plants in the Paleotropics make ECM associations, suggesting that there is a larger abundance of AM fungi in tropical forests (Corrales et al., 2018). There are several hypotheses about this phenomenon: perhaps there is a lower abundance and diversity of host plants or too high of soil temperatures causing rapid decomposition rates (Corrales et al., 2018). However, several reports have produced evidence that conflicts this trend. One study found that most tropical monodominant trees make ECM associations, and specific tropical locales have shown high densities of host tree species (Corrales et al., 2018). These discontinuities and the general lack of ECM research in tropical environments provoke many questions about fungal diversity in places like Madagascar. Thus, this study hopes to contribute to the mycological knowledge of one of Madagascar's eastern lowland rainforests.

1.2.2 Arbuscular Mycorrhizal (AM) Fungi

Endomycorrhizal, or arbuscular mycorrhizal, fungi have important functional differences from ectomycorrhizal fungi. AM are intracellular and can penetrate root cells directly to provide nutrients to their host plants (Ducousso et al., 2008). In addition to extraradical mycelium, AM fungi form microscopic intercellular and intracellular hyphae, arbuscules, and vesicles (Ducousso et al., 2008). These structures are visible under the microscope in the root cortical cells of AM plants. Unlike ECM, a large majority, if not all, AM fungi do not reproduce with

above-ground fruit and instead eject spores with hyphae (Naranjo-Ortiz & Gabaldón, 2019). Thus, microscopic analysis is always necessary for their identification.

As of 2014, between 341 and 1600 species of Glomeromycota are estimated to exist globally, making associations with roughly 200,000 species (van der Heijden et al., 2015), far more than ECM plants. Moreover, there has not been convincing evidence for AM fungi host-specificity, though many cases show host preferences (van der Heijden et al., 2015). Around 74% of all plants are estimated to make AM associations in the Glomeromycota clade in contrast to only 2% of plants making ECM associations (van der Heijden et al., 2015). This diversity can be extrapolated to tropical forests, as they are assumed to be dominated by AM trees, with 19% plants in the Paleotropics making ECM associations (Corrales et al., 2018). However, the mycological diversity of tropical ecosystems is poorly studied, and several studies have suggested exceptions to this trend, as previously mentioned (Corrales et al., 2018).

1.3 Reforestation in Madagascar

Since 1953, nearly half of Madagascar's forests have been clear cut for agriculture (NHER, 2022), leaving less than 10% of primary forest cover (Barraclough & Olsson, 2018). The traditional form of agriculture in Madagascar is slash-and-burn, or *tavy*, and the use of fire contributes to soil degradation and long-term nutrient loss (Barraclough & Olsson, 2018). With a rapidly growing human population (at a rate of 2.8% in 2004 and increasing) and a widespread dependence on charcoal and fuelwood, deforestation continues to be a complex issue across Madagascar (Harper et al., 2007). Therefore, NGOs, governmental organizations, and local communities have taken up reforestation efforts on degraded lands. It is probable that slash-and-burn agriculture heavily influences the mycorrhizal community, and for this reason, AM fungi abundance may be used as a proxy for assessing the effects of deforestation and disturbance (Barraclough & Olsson, 2018). This research project briefly explores the relative abundance of mycorrhizal fungi in two reforestation sites near Ranomafana National Park (RNP), using samples directly adjacent to the protected area for comparison.

1.4 Research Objective

The main goal of this work was to describe the mycorrhizal status, particularly the AM status, of different tree species in the humid forest of Ranomafana, Madagascar. 19 tree species of variable abundance were sampled and analyzed in a laboratory for the degree of AM colonization. Mushrooms were photographed and later identified in the study areas to contribute to the mycological inventory of Ranomafana. Additionally, this study hoped to conduct a brief comparison of mycorrhizal abundance between a protected area and reforestation site to ascertain whether different environmental conditions influenced mycorrhizal colonization. It was hypothesized that trees inhabiting reforestation sites with poorer soil conditions (from recent cultivation) would have lower AM colonization rates.

2. STUDY AREAS

2.1 Ranomafana National Park (RNP)

Ranomafana National Park, founded in 1991, is a dense, low-altitude rainforest in eastern Madagascar (*Centre ValBio*). It is in the region of Vatovavy-Fitovivany in Fianarantsoa Province with a protected surface area of 41,601 ha (see Figure 1 below) (*Madagascar National Parks*). The park was named after the hot springs discovered in the valley: *rano* meaning "water" and *mafana* meaning "hot" (Ranomafana National Park, *Centre ValBio*). It sits on the edge of Madagascar's central plateau and is thus very mountainous, with elevations ranging from 500 meters to 1,500 meters (Ranomafana National Park, *Centre ValBio*). Due to its size and altitude, RNP encompasses a diverse range of forest types, from lowland rainforest to cloud forest (Ranomafana National Park, *Centre ValBio*). Around the protected area, there are more than 100 villages with over 25,000 residents, most subsistence farmers (Ranomafana National Park, *Centre ValBio*). The dominant floral families are Apocynaceae, Euphorbiaceae, Rubiaceae, Palmae, Aspleniaceae, Mytraceae, and Orchidaceae (*Madagascar National Parks*). Ranomafana boasts 938 floral species, 705 of which are endemic to Madagascar (Ranomafana, *FAPBM*). The chosen study area for the vegetation anaylsis was 0.05 ha at an elevation of 973 meters (GPS coordinates: S21˚ 15.6581' E047˚ 25.1914') and accessed by the tourist trail system. A Centre ValBio guide located the area off the main trail where the forest was less disturbed by tourists. Although the land did not belong to a primary forest, it had been protected since the establishment of the national park in 1991 and was almost entirely composed of native species

(*Centre ValBio*). Root samples were collected just outside the national park, across the RN45 road, where the forest still received little disturbance.
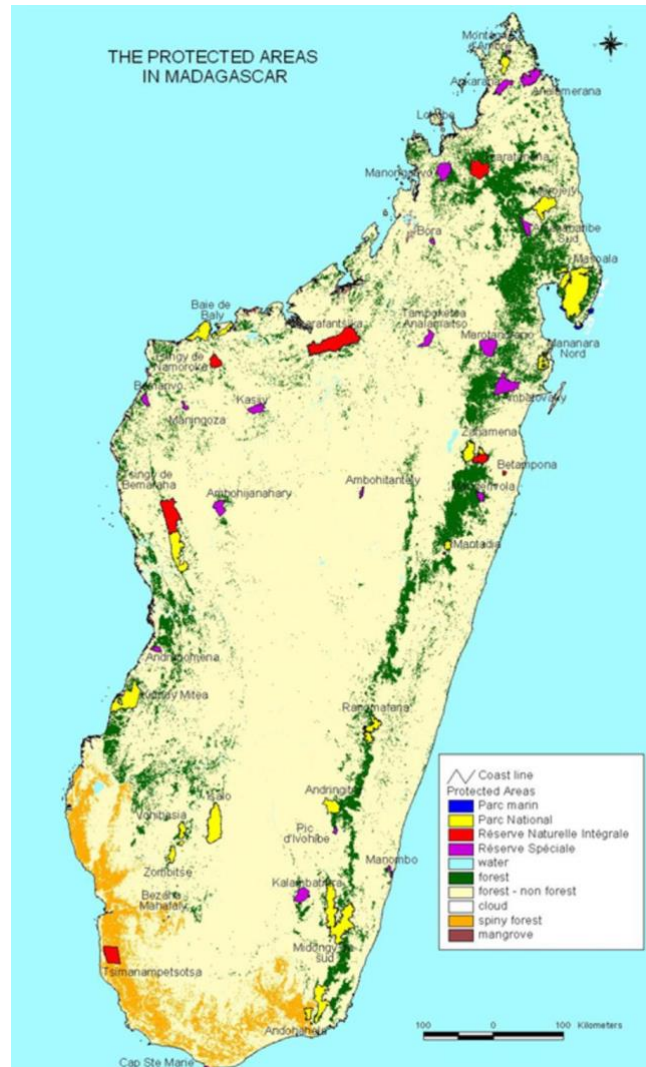
Figure 1: Map of Madagascar's protected areas and different ecosystems; the red circle points out Ranomafana, a lowland humid forest on the east coast (*Maps Madagascar*).

2.2 Centre ValBio Reforestation Sites: Akopa and Tanambao-Kelilalina

CVB is a research station founded by Stony Brook University Institute for the Conservation of Tropical Environments' (ICTE) (Centre Valbio, Main Campus, *Centre* ValBio). Centre ValBio (CVB) has partnered with local farmers to reforest land on the periphery of RNP (Reforestation, *Centre ValBio*). CVB operates twelve reforestation sites with nurseries equipped to germinate native seedlings for planting (Reforestation, *Centre ValBio*). Centre ValBio partners

with local farmers to conduct reforestation on land degraded by slash-and-burn agriculture (Reforestation, *Centre ValBio*).

The reforestation site of Akopa lies approximately one mile from the Centre ValBio facility (see Figure 3 for a map). The land at Akopa was recently cultivated to grow crops like cassava but was transformed into a reforestation site in 2017 with the help of the landowner. It now nourishes the growth of native trees. Six species were prioritized for planting: *Protorhus-Abrahamia thouvenotii* (sandramy), *Crytocarya spp.* (tavolo), *Bridelia tulasneana* (harina), *Canarium madagascariensis* (ramy), *Ocotea sp.* (varongy), and *Dilobeia thouarsli* (ramandriona). All but the latter have thrived in the past years; the soil conditions proved uninhabitable for *Dilobeia thouarsli*. Within five years, the plantation of these six species has encouraged the natural regeneration of *Harungana madagascariensis*, *Ficus politoria* (famakilela), *Dodonaea madagascariensis* (dingandahy), and *Vernonia sp.* (tavilona), to name a few. Like the other CVB reforestation sites, the land is now less-intensively cultivated, producing crops like pineapple and vanilla. This agroforestry strategy does not seem to discourage reforestation success, apart from *Dilobeia thouarsli*.

Figure 2: Image of Tanambao-Kelilalina reforestation site adjacent to rice fields

It is a similar story for the reforestation site of Tanambao-Kelilalina, which lies off RN25 west Ranomafana village (see Figure 2 for an image and Figure 3 for a map) (Fröling, 2014). The same six native species were planted here in 2017. The soil and climate are drier at this site, as it is farther from the rainforest and proximal to the cropland of Kelilalina village. In contrast to Akopa, *Dilobeia thouarsli* has thrived in these conditions. The species *Tambourissa sp.* (ambora), *Dodonaea madagascariensis* (dingandahy), *Harungana madagascariensis*, *Streblus*

dimepate (mahanoro), and *Gaertnera phyllostachya* (bararata) were observed to have naturally regenerated. Both reforestation sites, Akopa and Tanambao-Kelilalina, represented areas of secondary succession, with grasses and ferns composing much of the understory and a scattering of young trees.
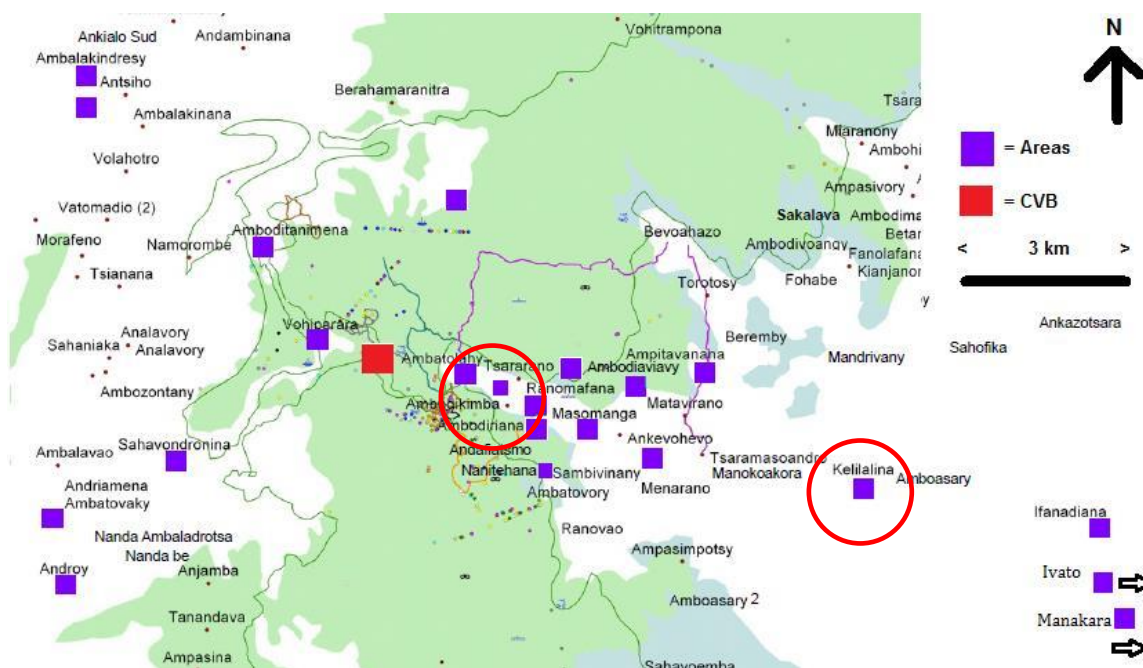
Figure 3: Map of CVB reforestation sites on the periphery of Ranomafana National Park; the red circles show the location of Akopa and Tanambao-Kelilalina sites (Fröling, 2014).

3. METHODOLOGY

3.1 Preliminary Vegetation Survey

Before conducting the mycorrhizal survey, it was necessary to ascertain the diversity of Ranomafana National Park (whether there appeared to be dominant species, which species were rarer, etc.). This information served to potentially deepen the implications of the mycorrhizal status of the plants observed. To survey the existing vegetation in the protected area of RNP, a variation of the Braun-Blanquet method was used (Westhoff & Van Der Maarel, 1978). Due to time constraints, a rectangular area of 50 m x 10 m (0.05 ha) was surveyed, as opposed to the traditional 0.1 ha (Westhoff & Van Der Maarel, 1978). This area was determined large enough to reliably represent a stand of protected native trees. The guide located the area by walking down one of the main trails for 15 minutes then diverting into the undisturbed brush and continuing for

five more minutes. The chosen area was less disturbed by tourists and researchers. A measuring tape and brightly colored string were used to delineate the borders of each sub-parcel (10 m^2). With the help of a local guide, each tree/shrub individual was identified, and its diameter at breast height (Dbh), its bole height (Hfut), and its maximum height (Hmax) were estimated. Each species' vernacular and scientific name were verified later using Centre ValBio's plant inventory.

A vegetation survey was not conducted in the reforestation sites of Akopa and Tanambao-Kelilalina. Since a specific study area was not established, observed individuals were simply documented with their vernacular and scientific name. Samples from both these sites were combined and treated as from the "reforestation site study area" when compared to the area adjacent to RNP.

3.2 Field Collection

To assess the level of ECM and/or AM fungal colonization, it is necessary to collect root samples from the field for microscopic analysis. It is important to note that fieldwork was done between the dates of April 12 and 19, 2023. These dates fall at the end of the rainy season, which begins in November and ends in April (*Duke Lemur Center*). Madagascar's east coast, and thus Ranomafana National Park, receives the most rainfall due to the rain-shadow effect brought by moist winds from the Indian Ocean (*Duke Lemur Center*). Seasonality may have a great influence on mycological abundance, thus its necessary to recognize that results may differ depending on time of year.

3.2.1 ECM Methods

Mushrooms, the fruiting bodies of mycorrhizal fungi, were photographed in the Braun-Blanquet parcel in RNP and at the two reforestation sites. The native tree species growing within a 50 cm vicinity of each mushroom were noted. However, no ectomycorrhizal mycelia were followed to their host plants, and thus, the objective of this method was more concerned with fungi identification than mycorrhizal association. Any notable characteristics of the mushroom were written down (e.g., whether it was growing on dead or live organic material). Afterwards, identification of the mushroom was attempted by referring to a mycological study conducted in

Andasibe in 2006 with Mitsinjo Association (Pirot, 2006). The guidebook *Champignons d'Andasibe (Madagascar)*, written by Paul Pirot, with pictures and descriptions was used to verify the identity of the mushrooms (see Figures 20 through 42 in the Appendix).

3.2.2 AM Methods

To assess arbuscular mycorrhizal abundance, it was necessary to collect root samples from native species. Samples were collected just outside the limits of the national park. The area of collection was secondary growth forest and not recently cultivated, thus accurately representing protected stands of native trees. First, the local guide identified a young individual of a particular species, as its root system was expected to be closest to the surface and more easily accessible. Adjacent to the protected area, an individual from 12 different species was chosen for root collection (11 native species, one non-native species, *Psidium cattleianum* (Chinese guava)). This choice was partly based on the relative abundance of these species as determined by the vegetation survey: some individuals were of a highly abundant species (like *Psychotra sp.*), while others were of a less abundant species (like *Ficus politoria*). Samples were collected by gently unearthing the topmost lateral roots (at least 2), then following these to their root tips. Between three and five 5-6 cm segments of the finest root tissue were collected from a representative individual of each species. These segments were stored in a labeled plastic bag for further microscopic analysis.

3.3 Staining Methods

To visualize AM fungi under the microscope, root staining was performed using an ink and vinegar method (Vierheilig et al., 1998). This method was necessary because traditional staining solutions, like Trypan Blue, were unavailable. Multiple trials for each sample were conducted to attain the best procedure for each sample. Only pigmented roots required clearing before staining and thus were submerged in 10% KOH solution at 90˚C for approximately 2 minutes, depending on the level of pigmentation and tissue thickness (Vierheilig et al., 1998). Clearing methods varied between samples, so several trials were conducted for each species to assure that root tissue was not damaged. Thinner roots were cleared in room temperature for two minutes longer to avoid burning the fragile tissue. Afterwards, the roots were rinsed thoroughly with tap water.

A 5% ink-vinegar solution was made using Chromia black printer ink and white household vinegar. Roots were submerged in this solution and boiled for three minutes (Vierheilig et al., 1998). Lastly, the samples were rinsed in a low concentration vinegar-water solution for 20 minutes before viewed under the microscope.

3.4 Microscopy

AM status of the tree species was determined by the abundance of mycorrhizal structures in each root sample. A Nikon Eclipse E200 microscope provided by the Centre ValBio laboratory was used. The time constraint of the study prevented the evaluation of all 83 observed plant species, thus only 19 root samples were stained and examined under the microscope: 12 samples from adjacent to the protected area, and 11 samples from the reforestation sites. Four species were compared between the two sites: *Cryptocarya spp.*, *Ocotea sp.*, *Tambourissa sp.*, and *Ficus politoria*. For each sample, five of the finest root segments were chosen. Small scissors were used to cut 1 cm segments, which were placed on glass slides for visualization. Each segment was observed for the presence of arbuscules, intracellular hyphae, intercellular hyphae, and/or fungal vesicles. Moreover, the level of colonization was estimated based on whether none, some or all the segments had these structures. A score of 0 (absent in all segments), 1- (present in one or two segments), 1 (present in three or four segments), or 1+ (present in all five segments). Finally, the species were determined to be AM- (lightly infected), AM (infected), or AM+ (heavily infected). If no AM infection was observed, samples were determined "inconclusive" (IC). This ranking was based on the 0 to 1+ score. For instance, a sample was AM+ when it possessed arbuscules, intracellular hyphae, and vesicles. However, a sample was AM- when it possessed only one of the three structures.

3.5 Statistical Analysis

Microsoft Excel was used for all statistical analyses. First, floral diversity was quantified using several methods. The relative abundance of each species was found by dividing each species' individual count by the total count of all individuals (2307). Second, the genus and species richness of each family was found. Diversity was quantified using the Simpson's Diversity Index, $D = 1 - \frac{\sum n(n-1)}{N(N-1)}$ (Ecology Center, 2023). The Shannon-Wiener Index was used

to find species evenness, $H = -\sum p_i * ln(p_i)$. The $H_{max} = ln(S)$, with S representing species richness (Ecology Center, 2023). The Shannon Equitability Index was used to determine species evenness: $E_H = H/ln(S)$, with a max value of $E_H = 1$ (Ecology Center, 2023). These vegetation statistics are helpful for context on the mycorrhizal study and further validate the immense biodiversity of the protected area of RNP.

To assess the relative levels of AM colonization between species, a series of pie charts were made. The degree of colonization by the microscopic fungal structures (ranked 0 to 1+ as previously mentioned) differed for each sample, and their relative proportions in samples from each study area and across all sites were analyzed in pie charts as well.

4. RESULTS

4.1 Floral Biodiversity and Characteristics

The preliminary vegetation survey of a protected stand of forest in RNP resulted in several conclusions about floral species density, richness, and diversity. In total, 2307 individuals of 83 species were counted and identified in the 0.05 ha study area drawn by the Braun-Blanquet method (Westhoff & Van Der Maarel, 1978). Table 1 shows the eight most abundant species along with their count and percent abundance, and Figure 4 graphically represents abundance for 80% of the most abundant species. *Psychotria sp.* (fohananasity) was the most abundant (22.5%), followed by *Dypsis linearis* (fanikara) (308). Most of these individuals were small saplings (under 1 m) and composed patches of the understory. There were 22 species in the surveyed area with only one individual observed.

Scientific Name	Count	Relative Abundance (%)
Psychotria sp.	518	22.5%
Dypsis linearis	308	13.4%
Cryptocarya spp.	172	7.46%
Vernonia sp.	131	5.68%
Cathariostachys madagascariensis	84	3.64%
Chassalia ternifolia	70	3.03%
Streblus dimepate	63	2.73%
Oncostemum nerviosium	63	2.73%

Table 1: Count and relative abundance of top eight most abundant species (comprising ~61% of all individuals).
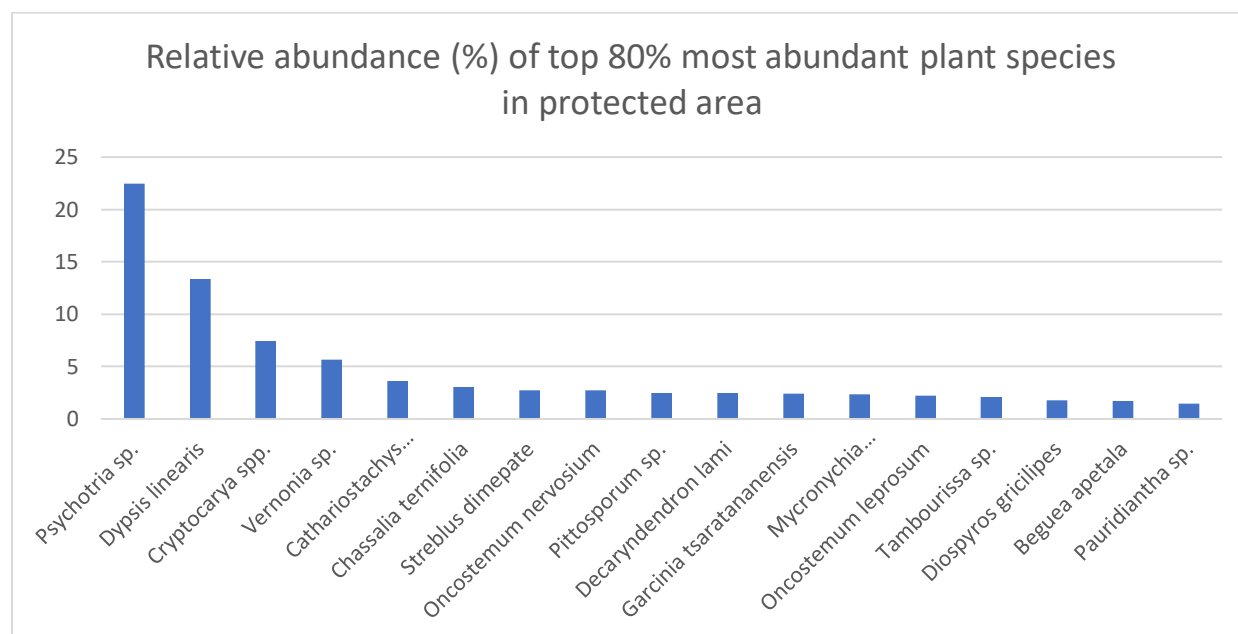
Figure 4: A bar graph showing the relative abundance of 80% of the most abundant species in the RNP protected area.

43 floral families were represented by a species richness of 83 species per 0.05 ha. The most represented families were Rubiaceae (655 individuals), Arecaceae (309 individuals), Lauraceae (195 individuals), Asteraceae (132 individuals), Primulaceae (114 individuals), and

Monimiaceae (108 individuals). Table 2 gives a breakdown of the genus and species richness of the ten most diverse families. Rubiaceae was the most diverse family, represented by 9 genera and 9 species. Approximately 28% of all individuals were in the Rubiaceae family, including the most abundant species, *Psychotria sp.* (fohananasity).

Family	Genus Count	Species Count	Individuals Count
Rubiaceae	9	9	655
Malvaceae	3	5	49
Clusiaceae	2	4	92
Sapindaceae	4	4	56
Myrtaceae	3	3	43
Monimiaceae	3	3	108
Lauraceae	2	3	195
Fabaceae	3	3	13
Euphorbiaceae	3	3	20
Bignoniaceae	2	3	3

Table 2: Shows the genus and species richness of the ten most diverse families, as well as their individual count in the protected area.

The Simpson's Diversity Index value was $D = 0.914$ (out of 1), showing a very high level of diversity. The Shannon-Wiener Index resulted in $H = 3.162$ out of an $H_{max} = ln(83) = 4.419$. Moreover, the Shannon Equitability Index gave $E_H = 0.715$, showing a high level of species evenness. These results confirm previous conclusions about RNP's notably high biodiversity as a tropical humid forest.

4.2 Arbuscular Mycorrhizal Status

Arbuscular mycorrhizal colonization was assessed based on the observation of four mycorrhizal structures: arbuscules, intracellular hyphae, intercellular hyphae, and vesicles. As described in the methodology, a degree of AM infection was assigned to each of the 19 examined species based on their 0 to 1+ score for each fungal structure. Table 3 and Table 4

outline these findings for the samples adjacent to the protected area and in the reforestation sites, respectively:

| Adjacent to Protected Area of RNP						
Plant Species	**Relative Abundance (%)**	**Arbuscules**	**Intracellular Hyphae**	**Intercellular Hyphae**	**Vesicles**	**AM Status**
Psychotria sp.	22.5%	1	1	0	1-	AM+
Cryptocarya spp.	7.46%	0	1-	0	1	AM
Chassalia ternifolia	3.03%	0	0	0	1-	AM-
Garcinia tsaratananensis	2.34%	0	1	1	1+	IC
Tambourissa sp.	2.12%	0	1-	0	1-	AM
Dombeya sp.	1.43%	0	1-	0	1-	AM
Psidium cattleianum	1.13%	0	0	1-	0	IC
Ocotea sp.	0.954%	0	0	0	1	AM-
Allophylus cobe	0.520%	1	1+	1-	1	AM+
Polyscias spp.	0.433%	1	1	0	1	AM+
Pittosporum verticillatum	0.347%	0	1-	0	1+	AM
Ficus politoria	0.260%	0	0	0	1+	AM-

Table 3: Outlines the level of colonization by a particular fungal structure (0 to 1+) assigned to each of the 12 analyzed species in the protected study area and their degree of AM infection (AM- to AM+, or IC).

Reforestation Sites: Akopa and Tanambao-Kelilalina					
Plant Species	**Arbuscules**	**Intracellular Hyphae**	**Intercellular Hyphae**	**Vesicles**	**AM Status**
Cryptocarya spp.	0	0	0	1-	AM-
Ocotea sp.	0	0	0	0	IC
Tambourissa sp.	0	1	1	1+	AM
Ficus politoria	0	0	0	1+	AM-
Ilex mitis	0	0	0	1-	AM-
Dodonaea madagascariensis	0	1	1	1+	AM
Protorhus-Abrahamia thouvenotii	0	1+	0	1-	AM
Canarium madagascariensis	0	0	1	0	IC
Streblus dimepate	0	1+	0	1+	AM
Harungana madagascariensis	0	1	0	1+	AM
Bridelia tulasneana	1	1	0	1-	AM+

Table 4: Outlines the level of colonization by a particular fungal structure (0 to 1+) assigned to each of the 12 analyzed species in the reforestation sites and their degree of AM infection (AM- to AM+, or IC).

Figure 5 shows the proportion of all samples that are heavily infected (AM+), infected (AM), lightly infected (AM-), or determined inconclusive (IC). For the four species samples common to both study areas, the samples from adjacent to RNP were used in the following calculation. The results suggest that most species (84%) were colonized by AM fungi, and almost half the species (42%) possessed an average degree of infection (AM). Of the colonized samples, only 21% appeared to be highly infected, meaning that they contained a higher abundance of fungal structures. It was determined that 16% of the total species (3 out of the 19 samples) examined had insufficient evidence of AM colonization, and further analysis would be necessary.
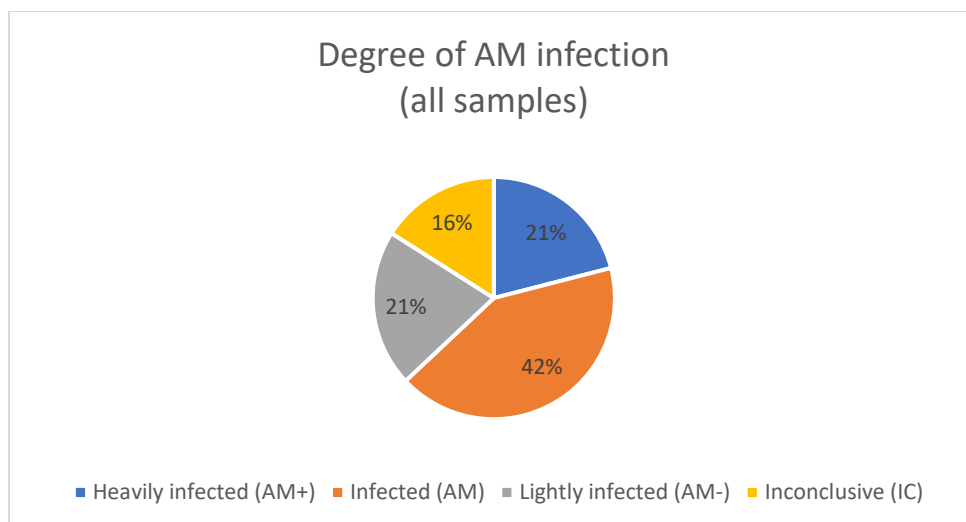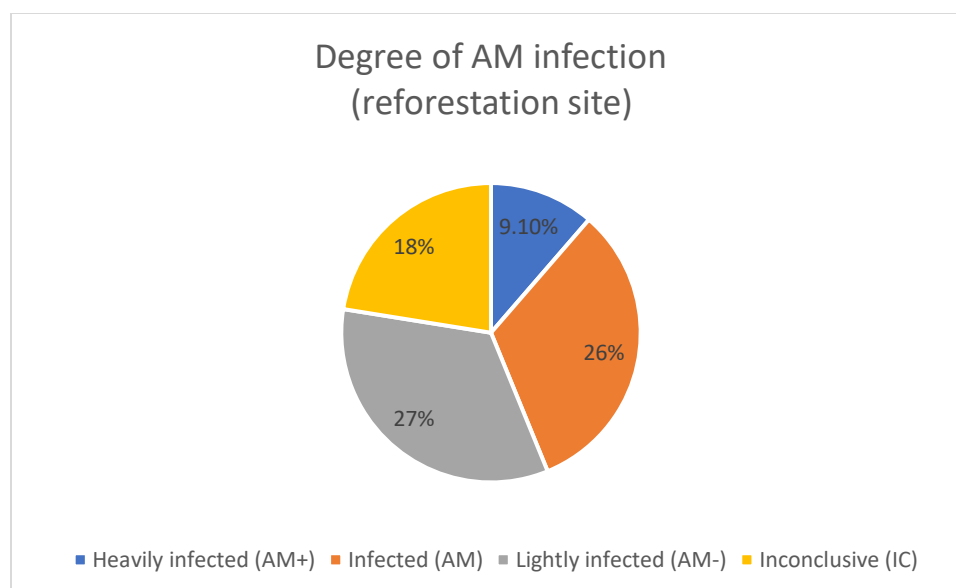
Figure 5: Depicts relative proportion of all 19 samples across the two study areas as AM+, AM, AM-, or IC.

Figure 6 demonstrates the degree of AM infection for the protected study area alone. Only 25% of plants appeared heavily infected (AM+), with the largest proportion of species ranked as "infected" (AM). Nevertheless, it is evident that most species make AM associations.
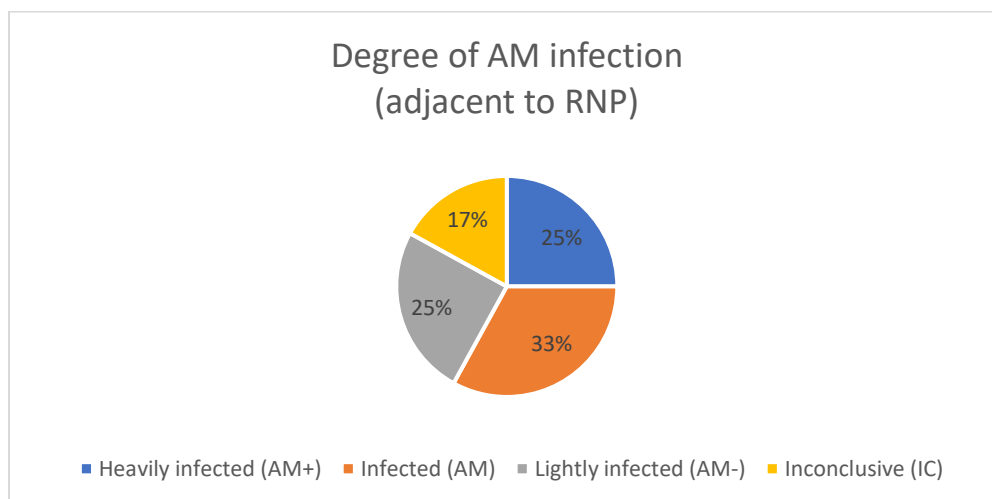
Figure 6: Depicts relative proportion of samples from adjacent to RNP as AM+, AM, AM-, or IC

The same procedure was conducted for the reforestation sites (the two sites combined), shown in Figure 7 below. A larger proportion of samples had inconclusive evidence of AM infection (18%), although the majority remained AM colonized, and fewer samples were heavily infected (AM+), only 9.1%.

Figure 7: Depicts relative proportion of samples from the reforestation sites as AM+, AM, AM-, or IC

Separate samples from four tree species, *Cryptocarya spp., Ocotea sp., Tambourissa sp.,* and *Ficus politoria* were collected from both the area adjacent to RNP and the reforestation sites. A direct comparison of AM status was made for these four species to see whether the different environmental conditions may play a role in the degree of AM colonization. Table 5 makes this comparison. AM status for *Tambourissa sp.* and *Ficus politoria* was the same for the two study areas, suggesting no significant influence of environmental conditions for these two species. The *Cryptocarya spp.* sample from the reforestation site appeared less infected than that from the protected area, and the observations of *Ocotea sp.* were inconclusive at the reforestation site. More research is needed across more species to conclude whether the soil conditions at the reforestation site truly affected AM colonization, but this brief study suggested that there may be an influence for certain native trees. It's important to note that AM colonization may not be easily generalized across species; it is possible that mycorrhizal associations are more/less resistant to environmental disturbance depending on the host plant/fungus.

	Adjacent to RNP	Reforestation sites
Species	**Degree of AM infection**	
Cryptocarya spp.	AM	AM-
Ocotea sp.	AM-	IC
Tambourissa sp.	AM	AM
Ficus politoria	AM-	AM-

Table 5: Compares the degree of AM infection between adjacent to RNP and reforestation sites for four native tree species.

For each of the four fungal structures observed (vesicles, inter- and intracellular hyphae, and arbuscules), a pie chart shows the proportion of samples containing each structure for the area adjacent to RNP, reforestation sites, and across all samples. From these data, it can be concluded that most colonized species contained fungal vesicles, with only 11% of samples without them (see Figure 8 in the Appendix). Vesicles were often very visible from the black stain, and they were determined to be fungal when they were connected to a hypha, present in intercellular space or at the edges of cells. Intercellular hyphae were much less common, as only 27% of all samples contained the structures (see Figure 11 in the Appendix). The trend was consistent across all sites (see Figures 12 and 13 in the Appendix). There may be several reasons for less intercellular hyphae observations, including inadequate staining methods. In contrast, intracellular were more commonly observed, with 68% of all samples containing these structures (see Figure 14 in the Appendix). These structures were the most apparent indicator of AM colonization; hyphal filaments were stained clearly and easily visible in many samples passing through the cell wall. Lastly, arbuscules were observed in only 21% of all samples and only 9% of the samples from the reforestation sites (see Figure 17 in the Appendix). This does not suggest a high level of AM colonization in general, although there are many limitations to proper visualization of these minute structures.

4.3 Ectomycorrhizal Status

ECM colonization was evaluated macroscopically with the observation of fruiting bodies, or mushrooms. This was an informal method, and it must be restated that no direct conclusion can be drawn about a tree's ECM status from the simple observance of mushrooms without following the fungal mycelia to its host plant. Nevertheless, this study attempted to identify and describe the observed fungi to contribute to the mycological inventory of Ranomafana.

23 mushroom species were photographed in total (see Figures 20 through 42 in the Appendix), and only two species were found in the reforestation sites, the rest in the protected study area. With the help of Paul Pirot's 2006 issue of *Champignons d'Andasibe (Madagascar)*, eight mushrooms were confidently identified. This was seen as a reliable source since Andasibe is also an east coast humid forest like Ranomafana and probably contains much of the same fungal diversity. Since no mycological guidebook exists for Ranomafana (or Madagascar in

general), it was impossible to identify all the species. However, there were several notable observations. Saprotrophic mushrooms of the genus *Marasmius* (family Marasmiaceae) were seen frequently in the protected area and always growing on dead bamboo leaves unattached to the soil (see Figures 20 and 27 in the Appendix) (Pirot, 2006). Additionally, a mushroom of genus *Hygrocybe* (family Hygrophoraceae) was spotted in the protected area (see Figures 33, 36 and 39 in the Appendix), and Pirot's 2006 study in Andasibe found that this genus is never found on recently burnt land, meaning that it may not present at reforestation sites (Pirot, 2006). In the reforestation area of Akopa, an unidentified mushroom was growing directly on the young roots of *Cryptocarya spp.* (tavolo), suggesting an ECM relationship (see Figure 42 in the Appendix).

In general, mushrooms were seen growing near *Dypsis linearis* (fanikara), *Albizia gummifera* (volomborona), *Tambourissa sp.* (ambora), *Psychotria sp.* (fohananasity), *Aphloia theiformis* (fandramanana), *Garcinia tsaratananensis* (kimbaletaka), *Chassalia ternifolia* (voananananala), and *Xylopia* buxifolia (ramiavona) (see Figures in Appendix). These simple observations don't confirm that these tree species are ectomycorrhizal, but they should encourage more investigations of the ECM status of these species. Broadly, it was evident that there were more fungal fruiting bodies in the protected study area compared to the reforestation sites.

5. DISCUSSION

The protected study area (0.05 ha) in RNP exhibited very high floral diversity and high species evenness, as indicated by the calculated Simpson's Index and Shannon-Wiener Index values. The area experiences little disturbance from human activities, allowing native species to thrive. Therefore, it is speculated that a healthy fungal community exists as well.

This study conducted a very preliminary investigation of arbuscular mycorrhizal abundance in the tropical forest of Ranomafana. The research project succeeded in determining whether the collected species were colonized by AM fungi or not, and this information is relevant to the general ecological knowledge of Ranomafana's tropical forest. A degree of AM infection was assigned for the 18 native tree species and one non-native species, *Psidium cattleianum* (Chinese guava). The results showed that 15 of the 19 tree species were AM infected to some degree, and four samples had inconclusive evidence.

This project followed a similar method to the study of Marc Ducousso and associates in 2008, although the methods differed in some ways (Ducousso et al., 2008). The 2008 study assigned a mycorrhizal status to 157 tree species from five different coastal humid forests in Madagascar, thus their findings were very relevant (Ducousso et al., 2008). They described six of the same species, *Canarium madagascariensis* (ramy), *Dombeya spp.* (hafidahy), *Harungana madagascariensis*, *Ocotea sp.* (varongy), *Polyscias spp.* (vatsilana ravimboanjo), and *Tambourissa sp.* (ambora). *Canarium madagascariensis* was sampled from Mahatsara forest 56 km north of Tamatave, *Ocotea sp.* was sampled from Tampolo forest 111 km north of Tamatave, and the other four species were sampled from Mandena forest 8 km north of Fort Dauphin (Ducousso et al., 2008). Although the 2008 study sampled from different areas, the mycorrhizal status of each species should be the same across environments, and each of these six species were determined AM colonized to some degree. Similarly, this study in Ranomafana forest confirmed that these six species were AM. However, one sample of *Ocotea sp.* from the reforestation sites was inconclusive. This could have occurred for several reasons; the amount of root tissue could have been insufficient, the stain may not have adhered to the fungus correctly, or simply the tree individual may not have had an association. Regardless, further evidence of AM associations in these native species is a relevant contribution to the mycological knowledge of Madagascar's eastern tropical forests. Importantly, Ducousso and associates' 2008 study suggested that "many dominant trees and shrubs of the eastern coast forests of Madagascar are ectomycorrhizal" (Ducousso et al., 2008); however, the most dominant species *Psychotria sp.* (22.5% abundance) was determined AM colonized and heavily infected (AM+). This is inconsistent with the 2008 study, but more research is needed on the species ECM associations.

Few conclusions can be drawn from the brief comparison was made between the reforestation sites of Akopa and Tanambao-Kelilalina and adjacent to the protected area. Firstly, two of the analyzed species had the same degree of AM colonization (*Tambourissa sp.* and *Ficus politoria*), suggesting that the previous soil degradation from cultivation may not have a considerable effect on AM colonization for these species. On the other hand, the degree of AM infection for *Cryptocarya spp.* and *Ocotea sp.* was less for the reforestation site samples. A 2018 study by Alícia Barraclough and Pål Olsson that evaluated the effect of slash-and-burn practices on AM fungi abundance in a dry tropical forest of Madagascar (Barraclough & Olsson, 2018). The study found that the predominant effects of deforestation on AM fungi were indirect; their

results suggested a reduction in the hyphal network (Barraclough & Olsson, 2018). The authors pointed out that a direct response to deforestation is still unclear, although other studies have found a decrease in fungal mycelium after prescribed fires (D'Ascoli et al., 2005). It seems that the effects of intense slash-and-burn agriculture vary "depending on the time elapsed since the disturbance" (Barraclough & Olsson, 2018). Thus, this study's small comparison of four species is insufficient for a strong conclusion about the effect of agriculture on mycorrhizal fungi abundance and diversity. Long-term research during all successional stages is recommended.

Because of the difficulty in identifying mushrooms and ECM associations, this study found little conclusive evidence of ECM associations, and AM surveys were prioritized. However, the observation of a fruiting body connected directly to the young roots of *Cryptocarya spp.* (tavolo) is promising. More investigations are needed, but this suggests that one of the more dominant species in Ranomafana may be ectomycorrhizal (*Cryptocarya spp*. had a 7.46% relative abundance in the protected study area). In addition, the presence of *Marasmius* fungus on the dead organic material of *Cathariostachys madagascariensis* (giant bamboo) suggests a potential relationship between the two species; however, it's not clear whether it would be a mycorrhizal symbiosis, since the mushrooms weren't growing on live bamboo.

5.1 Limitations and Recommendations

This study had many limitations that must be acknowledged. Firstly, the staining methods were not ideal, and standard solutions, like Trypan Blue, were unavailable. The methods had to be altered to accommodate this, so ink and vinegar were used. The staining procedure and lab work in general required many rounds of trial and error, and some roots were unable to be properly stained. This certainly affected the visibility of fungal structures under the microscope. Future studies would benefit greatly from following a clear staining procedure, like the Phillips and Hayman method (Phillips & Hayman, 1970). The time constraint of the study was also limiting, because many samples were unable to be analyzed. If this project were to be extended, it would be helpful to conduct DNA sequencing of the AM fungi to identify their species. So little is known of Madagascar's mycorrhizal diversity and fungal diversity in general, and the vast majority of fungi have yet to be described.

Concerning the reforestation site comparison, the study would have benefitted from performing the vegetation analysis on both types of study areas. The reforestation sites had a very different floral composition, so it was difficult to compare many species across the two sites. If future research was to focus on this specific exploration, it would be advisable to compare the two sites using a same group of species. This study did not completely control for the species variable (aside from four) when comparing the two study areas, and the conclusion of their difference in AM colonization is less reliable. Lastly, collecting root samples was often time consuming and difficult for older individuals, thus some species simply could not be sampled.

6. CONCLUSION

Mycorrhizal fungi, both arbuscular mycorrhizal and ectomycorrhizal, play a significant role in tropical forest ecosystems, yet very few species have been described in Madagascar. With such widespread floral diversity and endemicity, it is very possible that the island's fungal community has similar characteristics. Therefore, this research project hoped to contribute a preliminary investigation of mycorrhizal diversity in Ranomafana. Most species across the study areas appeared AM colonized, with one fifth classified as heavily infected. This has important implications for the mycological knowledge of Madagascar and supports the conceived phenomenon that most tropical plants make AM associations (Corrales et al., 2018). The protected area of Ranomafana National Park possessed high species evenness, but the most abundant species of *Psychotria sp.* was determined heavily AM infected. More research into ectomycorrhizal status is needed to determine if more dominant species like *Psychotria sp.* associate with ECM fungi as Ducousso and associates' 2008 study suggests (Ducousso et al., 2008). This is especially true considering the numerous recent studies that have indicated emerging patterns of ECM abundance in monodominant tropical ecosystems due to its resilience in nutrient-poor soils (Corrales et al., 2018).

While this study did not delve deeply into the effect of agricultural disturbance on mycorrhizal networks, the preliminary comparison between reforestation sites and areas adjacent to the national park suggested that slash-and-burn agriculture may decrease mycorrhizal fungi abundance and colonization rates. This could have significant implications for future

reforestation methods. For instance, fungal inoculation could become a more effective and widespread method of ensuring the longevity of planted saplings. Moreover, studies like this one are important for understanding which fungal species would be most beneficial to reforestation efforts, whether those species are AM or ECM.

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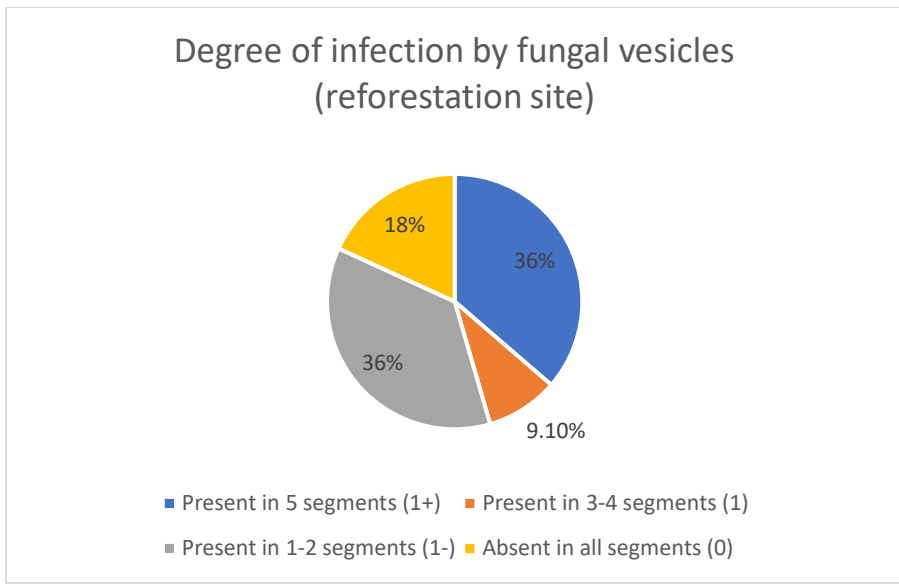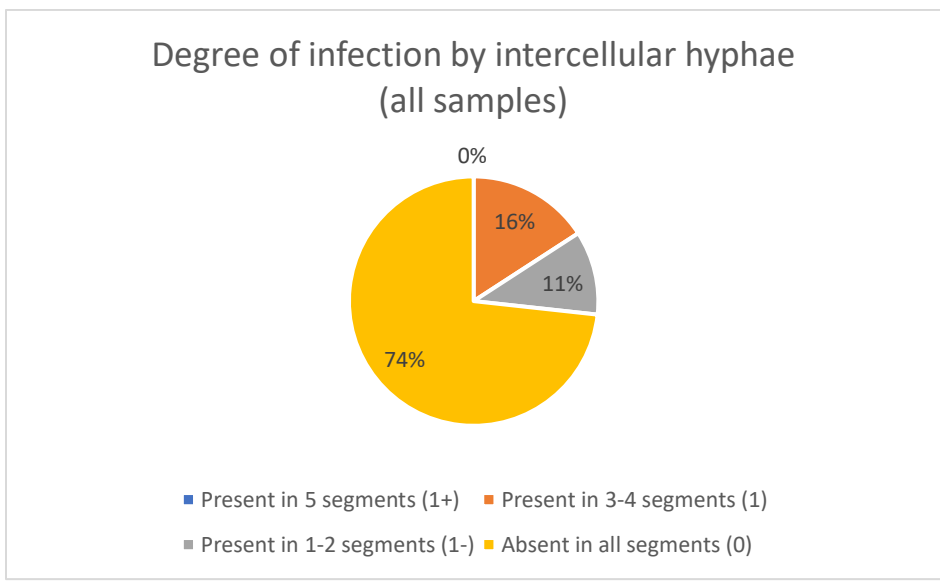
8. APPENDIX
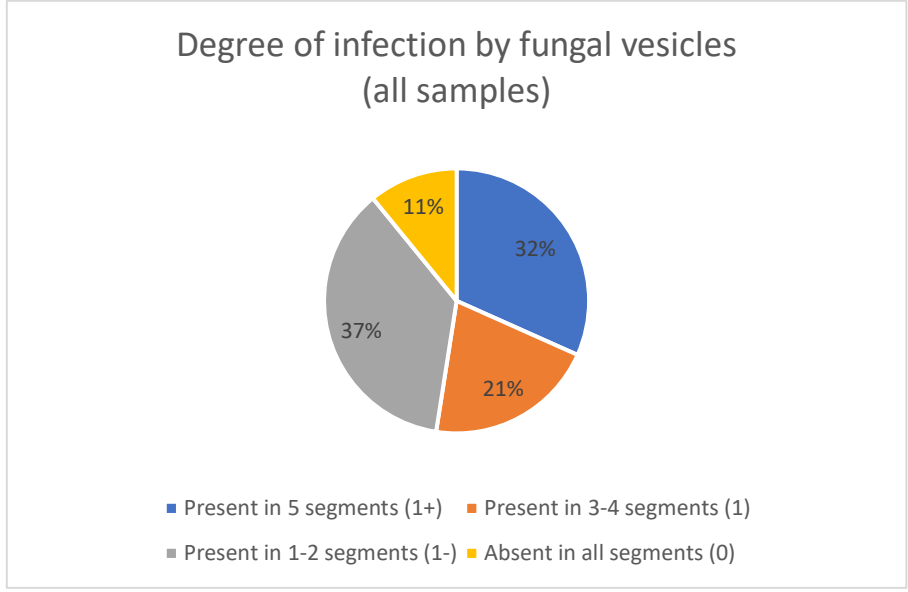
Fig. 8: Depicts the proportion of AM infection in the analyzed tree species by observation of fungal vesicles in all samples across the three sites; shows that most samples contained fungal vesicles, and roughly a third contained a high quantity.
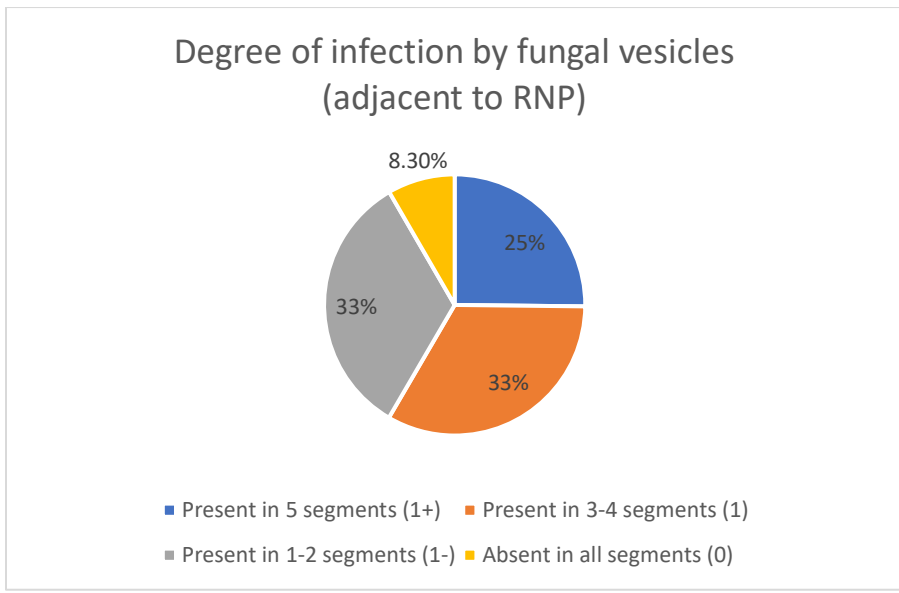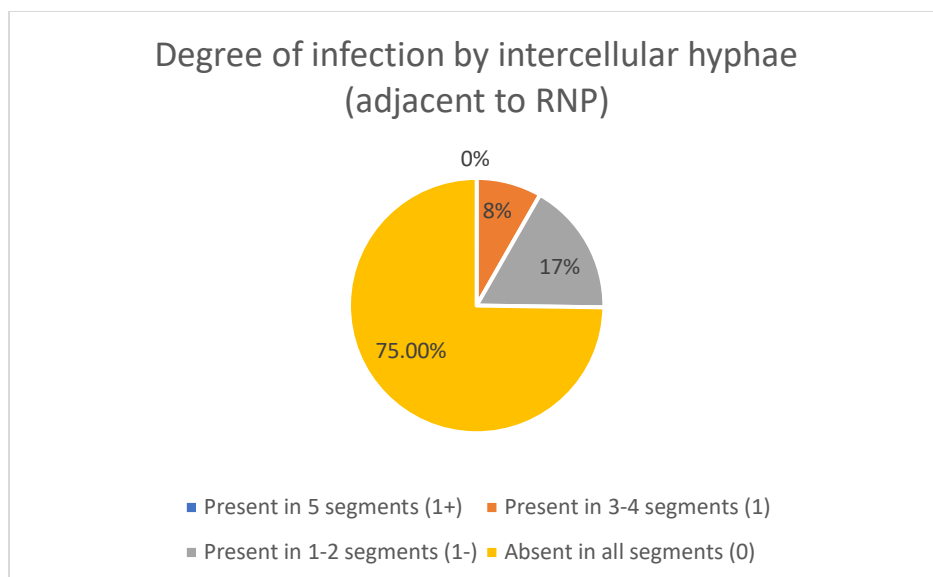

Fig. 9: Depicts the proportion of AM infection in the analyzed tree species by observation of fungal vesicles in the protected study area; shows that over 90% of colonized species had fungal vesicles.
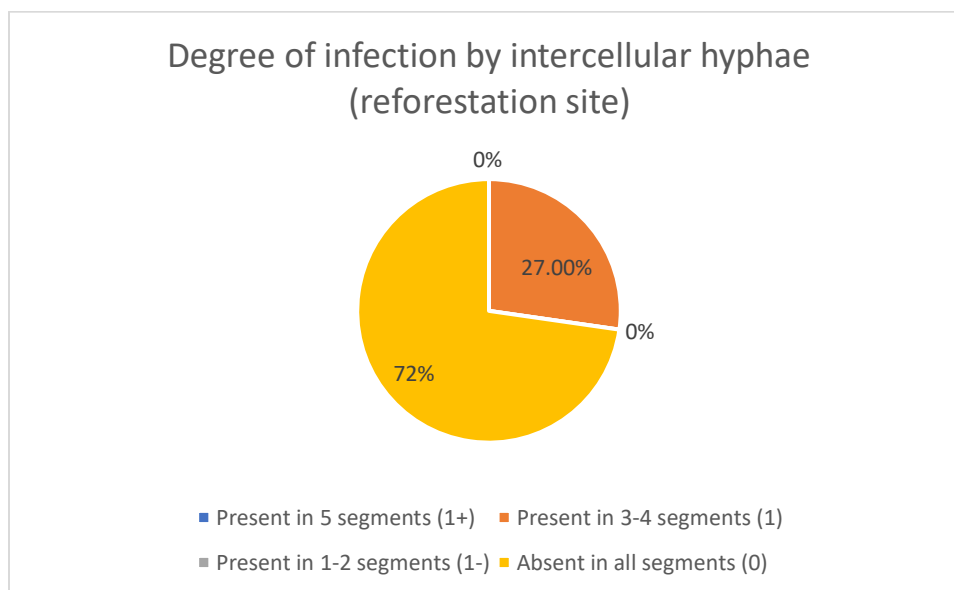
Fig. 10: Depicts the proportion of AM infection in the analyzed tree species by observation of fungal vesicles in the two reforestation sites; shows that most species contained fungal vesicles.
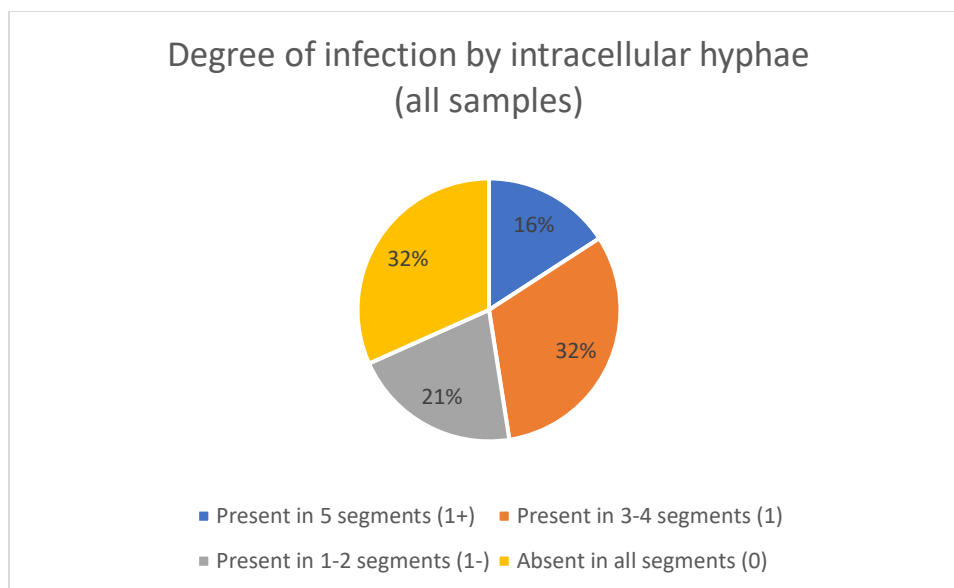

Fig. 11: Depicts the proportion of AM infection in the analyzed tree species by observation of intercellular hyphae in all samples across the three sites; shows that intercellular hyphae were absent in most samples.
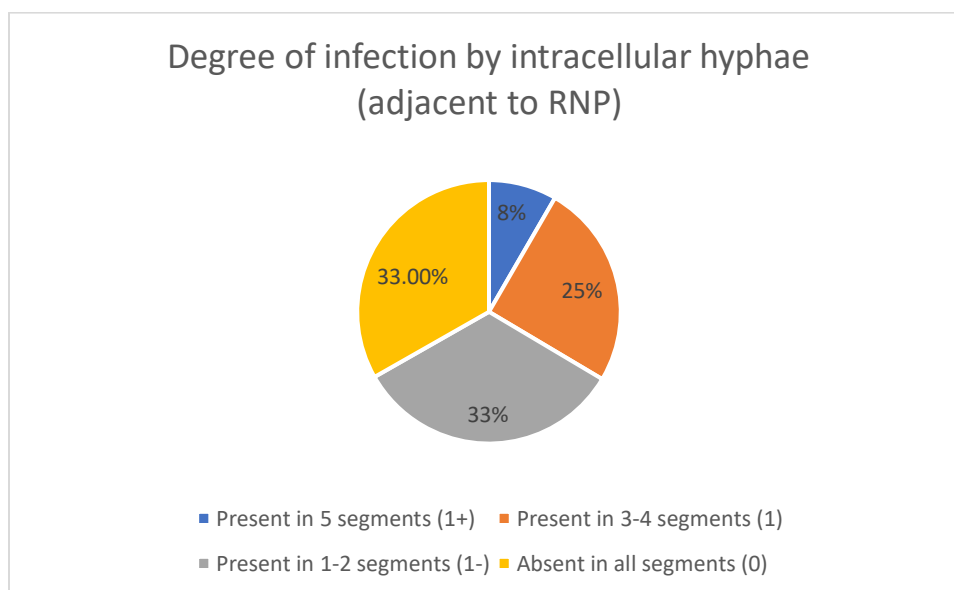
Fig. 12: Depicts the proportion of AM infection in the analyzed tree species by observation of intercellular hyphae adjacent to the protected area; shows that intercellular hyphae were absent in most samples (consistent with the data collected from the three sites combined)
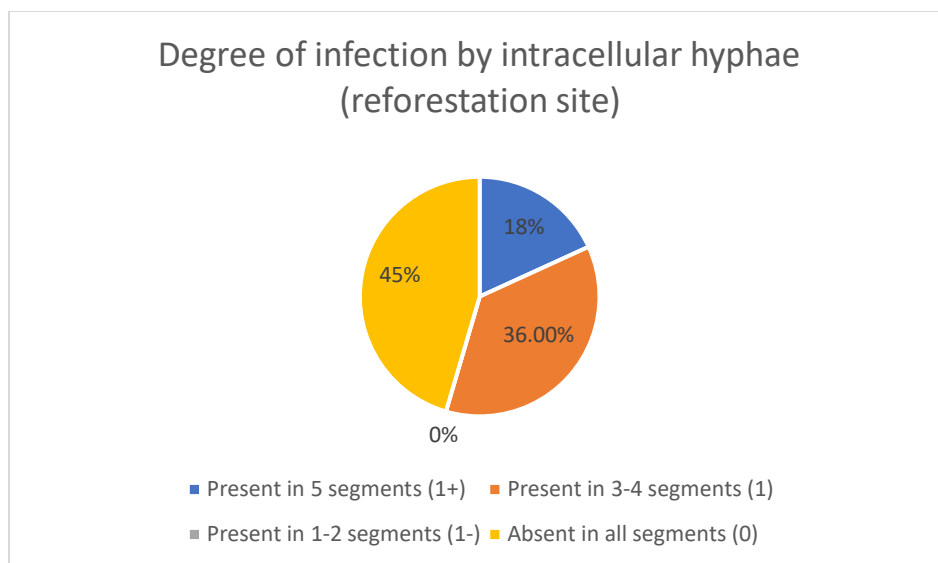

Fig. 13: Depicts the proportion of AM infection in the analyzed tree species by observation of intercellular hyphae in the reforestation sites; shows that intercellular hyphae were absent in most samples (consistent with the data collected from the three sites combined)
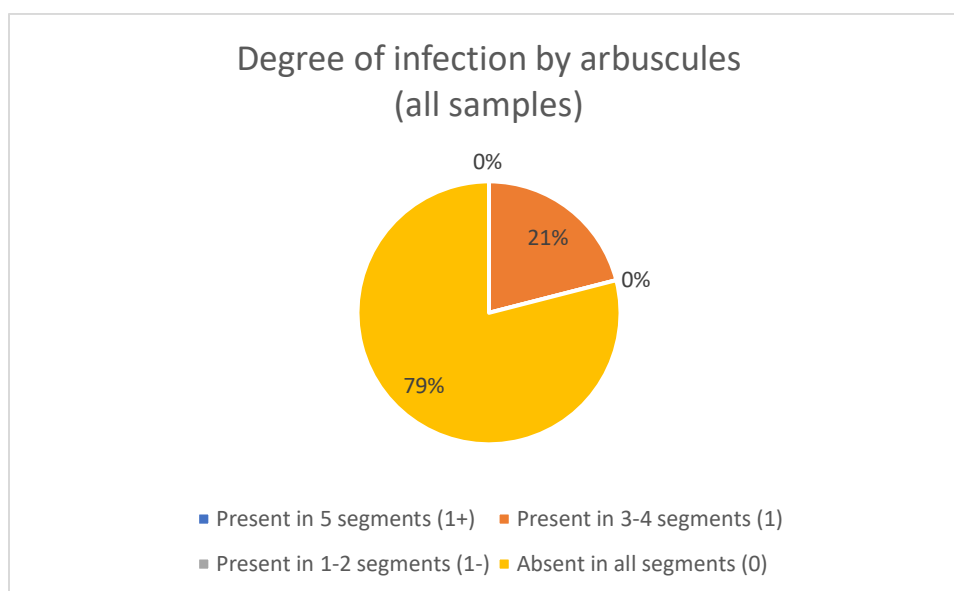
Fig. 14: Depicts the proportion of AM infection in the analyzed tree species by observation of intracellular hyphae in all samples across the three sites; shows that intracellular hyphae were observed in 68% of the species.
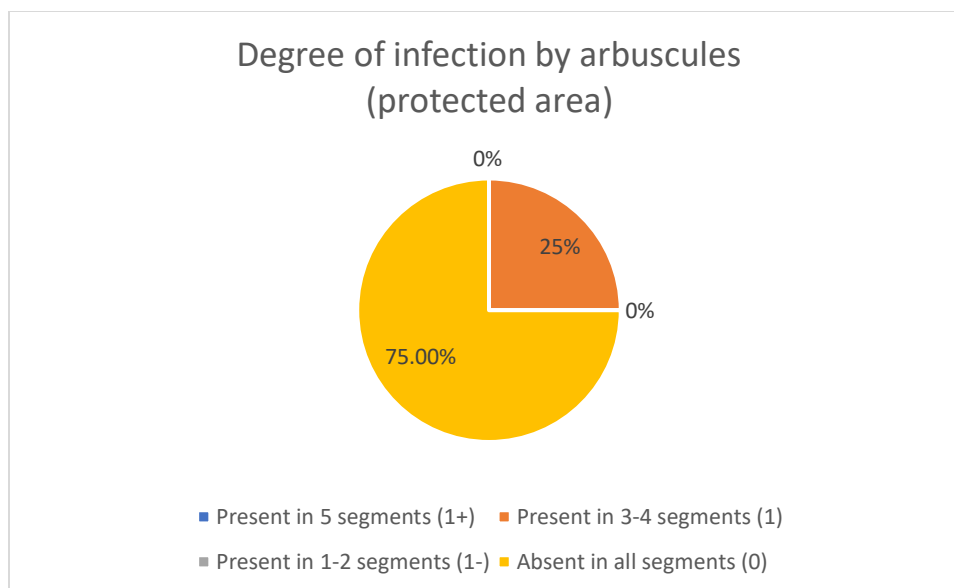

Fig. 15: Depicts the proportion of AM infection in the analyzed tree species by observation of intracellular hyphae adjacent to the protected area; structures were not visible in one third of samples and only occasionally observed in another third, suggesting lighter infection by hyphae.
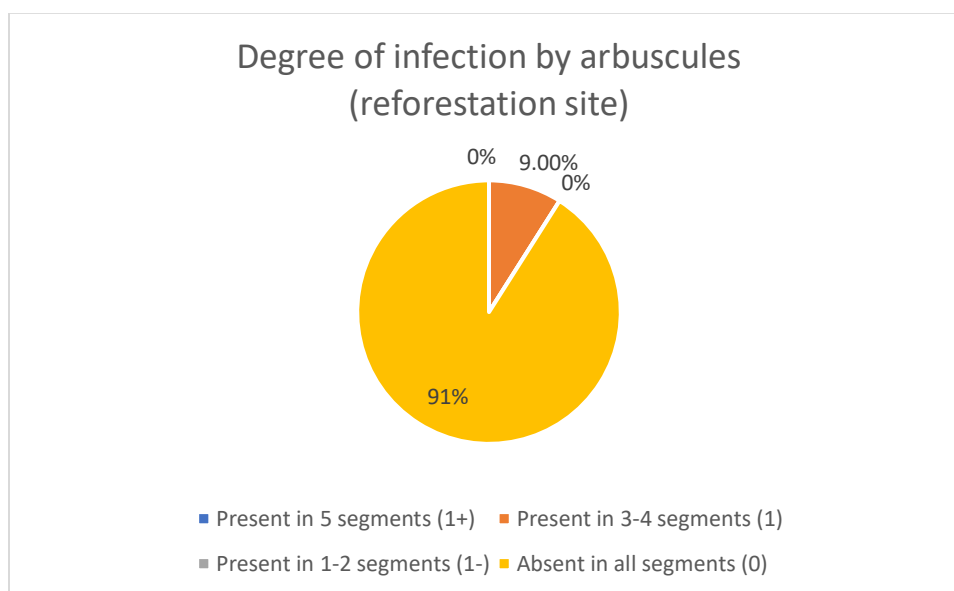
Fig. 16: Depicts the proportion of AM infection in the analyzed tree species by observation of intracellular hyphae in the reforestation sites; no hyphae were observed in almost half of the samples, indicating possibly lighter infection rates.

Fig. 17: Depicts the proportion of AM infection in the analyzed tree species by observation of arbuscules in all samples across the three sites; shows a low abundance of arbuscules.

Fig. 18: Depicts the proportion of AM infection in the analyzed tree species by observation of arbuscules adjacent to the protected area; shows only one fourth of samples containing visible arbuscules.

Fig. 19: Depicts the proportion of AM infection in the analyzed tree species by observation of arbuscules in the reforestation sites; shows only 9% of samples containing visible arbuscules, less than that of the protected area.

Fig. 20: genus *Marasmius* (family Marasmiaceae); observed several times on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing on leaves of *Cathariostachys madagascariensis* (volohosy, giant bamboo) (Pirot, 2006).

Fig. 21: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing near *Dypsis linearis* (fanikara), *Albizia gummifera* (volomborona) *Tambourissa sp.* (ambora), and *Psychotria sp.* (fohananasity).

Fig. 22: genus *Entoloma* (family Entolomataceae); observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing near *Dypsis linearis* (fanikara).

Fig. 23: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing near *Dypsis linearis* (fanikara), *Aphloia theiformis* (fandramanana), and *Psychotria sp.* (fohananasity).

Fig. 24: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914').

Fig. 25: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing near *Xylopia* buxifolia (ramiavona), Garcinia *tsaratananensis* (kimbaletaka), *Chassalia ternifolia* (voananananala), and *Dypsis linearis* (fanikara).

Fig. 26: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing near *Garcinia tsaratananensis* (kimbaletaka), *Chassalia ternifolia* (voananananala), and *Dypsis linearis* (fanikara).

Fig. 27: genus *Marasmius* (family Marasmiaceae); observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing near *Beguea apetala* (lanary madinka) and *Alberta sp.* (fantsikahitra) (Pirot, 2006).

Fig. 28: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914') growing on dead twig of *Cathariostachys madagascariensis* (volohosy, giant bamboo).

Fig. 29: observed on 4/13/23 in RNP (outside specific study area).

Fig. 30: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914').

Fig. 31: observed on 4/13/23 in RNP (outside specific study area).

Fig. 32: observed on 4/13/23 in RNP (outside specific study area).

Fig. 33: genus *Hygrocybe* (family Hygrophoraceae); observed on 4/13/23 in RNP (outside specific study area); they are not often seen in recently burnt areas (Pirot, 2006).

Fig. 34: observed on 4/13/23 in the survey area in RNP (S21˚ 15.6581' E047˚ 25.1914').

Fig. 35: genus *Clavaria* (family Clavariaceae); observed on 4/14/23 in RNP (outside specific study area) (Pirot, 2006).

Fig. 36: genus *Hygrocybe* (family Hygrophoraceae); observed on 4/14/23 in RNP (outside specific study area) (Pirot, 2006).

Fig. 37: genus *Dictyophora* (family Phallaceae, class Gasteromycetes); observed on 4/17/23 in RNP (outside specific study area) (Pirot, 2006).

Fig. 38: observed on 4/17/23 in RNP (outside specific study area).

Fig. 39: genus *Hygrocybe* (family Hygrophoraceae); observed on 4/17/23 in survey area in RNP (S21˚ 15.6581' E047˚ 25.1914'); they are not often seen in areas that have recently been burnt (Pirot, 2006).

Fig. 40: observed on 4/17/23 in RNP (outside specific study area).

Fig. 41: observed on 4/18/23 at the Akopa reforestation site.

Fig. 42: observed on 4/18/23 at the Akopa reforestation site; seen growing near bright red, young roots of *Cryptocarya spp.* (tavolo).