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Ectomicorrizas de florestas multidiversas

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Amanda Cristina Zanatta

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O presente trabalho em nível de mestrado foi avaliado e aprovado por banca examinadora composta pelos seguintes membros:

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Prof. Paulo Tamaso Mioto, Dr.

Orientador

Florianópolis, 2022.

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“Eu acredito na intuição e na inspiração. A imaginação é mais importante que o conhecimento. O conhecimento é limitado, enquanto a imaginação abraça o mundo inteiro, estimulando o progresso, dando luz à evolução. Ela é, rigorosamente falando, um fator real na pesquisa científica.” Albert Einstein

RESUMO

As ectomicorrizas (ECMs) são associações mutualísticas entre fungos e plantas, sendo encontradas em região temperada, boreal e tropical. As ECMs possuem funções ecossistêmicas extremamente relevantes como sequestro de carbono e formação da rede micelial comum. A evolução dessa associação é complexa. Os fungos ectomicorrízicos surgiram há 200 milhões de anos. Ao longo do tempo, o status ectomicorrízico surgiu 80 vezes ou mais de forma convergente a partir do status saprotrófico. Esta dissertação está dividida em dois capítulos. O primeiro tem como objetivo realizar uma revisão da literatura para discutir dados recentes que indicam que as ECMs estão presentes em regiões com alta diversidade de plantas. Discutiremos a possibilidade do aparecimento das ECMs em florestas multidiversas, focando nas suas particularidades e nas possíveis vantagens de se manter esse tipo de associação. O segundo capítulo trata de tentativas feitas em laboratório de isolar fungos potencialmente ectomicorrízicos nativos de florestas multidiversas. Ambientes tropicais foram locais de origem de importantes linhagens de fungos ectomicorrízicos como /amanita, /clavulina e /inocybe. Atualmente, as ECMs podem ser encontradas em região tropical e temperada. Apesar disso, pouco se sabe sobre as associações ectomicorrízicas em região tropical trazendo a necessidade de mais estudos nessas áreas. As ECMs de regiões tropicais podem ter importância fundamental nos ecossistemas e, futuramente, poderiam ser usadas para a restauração de ecossistemas. Algumas ectomicorrizas tropicais possuem um manto bem desenvolvido, mas não apresentam a rede de Hartig. Essa característica pode ser um sinal de que essa associação pode não estar tão estabelecida como as ectomicorrizas tradicionais. A formação da rede de Hartig envolve mecanismos muito específicos como as *Mycorrhiza-induced Small-Secreted Proteins* (MiSSPs). A falta das MiSSPs prejudica muito a formação da rede de Hartig, mas isso parece não interferir muito na formação do manto. Mesmo sabendo da importância da rede de Hartig, descobriu-se que ela não é necessária para a troca de nutrientes, embora aumente a eficiência do processo. Para formar o manto, o fungo ectomicorrízico utiliza mecanismos que aparentam serem menos específicos e que podem ser encontrados em fungos saprotróficos tendo outras funções. Significativas variações ambientais, como falta de carbono no solo, podem influenciar o fungo saprotrófico a se comportar de forma diferente, possibilitando a formação de estruturas micorrízicas mais simples, como o manto. Além disso, um ambiente com alta diversidade de plantas pode ser propício para o surgimento de novas associações. Nesses ambientes, o fungo tem menores chances de encontrar parceiros específicos, favorecendo um comportamento generalista comparado a regiões com poucas espécies de árvores. Essas características ambientais são encontradas em florestas multidiversas da região tropical, o que pode ser evidência de como algumas linhagens de fungos ectomicorrízicos podem emergir e serem mantidas atualmente na região tropical. As tentativas abordadas no capítulo 2 ainda estão em andamento e, até agora, não foi possível isolar os basidiomicetos que provavelmente formam ectomicorrizas.

Palavras-chave: ectomicorriza; região tropical; evolução; fungos ectomicorrízicos; restauração.

RESUMO EXPANDIDO

Introdução

As ectomicorrizas são associações mutualísticas entre planta e fungo. A planta recebe nutrientes e água do fungo, enquanto este recebe carbono. Além disso, o fungo pode proteger a planta contra estresses bióticos, como patógenos, e abióticos, como alta salinidade, seca e poucos nutrientes no solo. As ectomicorrizas possuem estruturas características como a presença de manto, rede de Hartig e hifas extrarradiculares. As ectomicorrizas podem ser usadas para a restauração ambiental, como a recuperação de solos erodidos, contaminados com metal pesado e alterados pelo fogo. As ectomicorrizas são encontradas em região tropical e temperada. Entretanto, os estudos são escassos em região tropical, resultando em grandes lacunas de conhecimento sobre as ectomicorrizas nesses ambientes. Curiosamente, algumas ectomicorrizas encontradas em região tropical possuem características anatômicas diferentes como a falta ou pouco desenvolvimento da rede de Hartig, importante interface de troca de nutrientes. Essa diferença em relação as ectomicorrizas tradicionais encontradas em região temperada poderia ser uma evidência de que essas ectomicorrizas de região tropical representam associações mais recentes do ponto de vista evolutivo.

Objetivos

Os principais objetivos do estudo foram encontrar evidências de que florestas multidiversas são importantes para o surgimento de fungos ectomicorrízicos e que estes fungos podem ter um papel importante para as plantas desses locais.

Metodologia

No primeiro capítulo realizou-se uma revisão bibliográfica visando a possibilidade de que ectomicorrizas de florestas multidiversas sejam um passo intermediário entre de o estilo de vida saprotrofia-ectomicorriza do fungo. Seguiu-se as seguintes linhas de evidência: evolução ectomicorrízica, passos evolutivos necessários para a formação de ectomicorriza, a formação de uma estrutura típica de ectomicorriza, filogenia e biogeografia das ectomicorrizas, associações ectomicorrízicas não tradicionais de florestas multidiversas em região tropical e características ambientais que favorecem a transição e a manutenção do nicho.

No segundo capítulo buscou-se realizar o isolamento de fungos ectomicorrízicos a partir das raízes de uma planta nativa facilitadora de restinga, *Guapira opposita*, e de basidiomas coletados na restinga. Realizou-se a coleta das sementes da planta e a formação de um banco de plântulas. Verificou-se a presença de ectomicorrizas nas raízes de *G. opposita*. Realizou-se o isolamento do fungo a partir das raízes micorrizadas através do corte das pontas das raízes e transferência das mesmas para diferentes meios de cultura: GYP “Sabouraud”, Priddam-Gotilieb modificado (PGKM), Mellin-Norkrans modificado (MNM) e ágar de batata dextrose (BDA). Após isso, foram incubadas em estufas com diferentes temperaturas (23ºC e 26,3ºC). A coleta dos basidiomas na restinga ocorreu após períodos de chuvas intensas durante os meses de janeiro a março, sendo armazenados em geladeira para conservação das estruturas.

Resultados e Discussão

Primeiro capítulo: existem várias evidências que apontam para um possível status intermediário saprotrofia-ectomicorriza das ectomicorriza de florestas multidiversas, como:

- O surgimento de fungos ectomicorrízicos mais de 80 vezes de forma convergente ao longo do tempo a partir de fungos saprotróficos;
- Linhagens de fungos ectomicorrízicos terem origem em ambiente tropical;
- A formação da rede de Hartig envolver genes mais derivados e alguns serem apenas encontrados em fungos ectomicorrízicos;
- A formação do manto, estrutura encontrada nas ectomicorrizas de florestas multidiversas, envolver genes encontrados também em fungos saprotróficos;
- A falta da rede de Hartig não interferir na ocorrência da associação, ou seja, a troca de nutrientes pode ser realizada, podendo o manto ser suficiente para estabelecer a associação;
- As características ambientais de florestas multidiversas serem mais propícias para a transição saprotrofia-ectomicorriza por serem mais desafiadoras para as espécies envolvidas do que em florestas monodominantes, além de poderem propiciar a manutenção da associação não-tradicional.

Segundo capítulo: um banco de plântulas de *G. opposita* foi estabelecido. Um morfotipo de ectomicorriza foi identificado nas raízes das plantas cultivadas em solo de restinga. O crescimento do micélio das pontas das raízes isoladas foi observado, sendo que após quatro dias foi possível observar o crescimento micelial em cinco réplicas nos meios de cultura de BDA, GYP “Sabouraud” e MNM em temperatura de 26,3ºC. As repicagens das culturas foram realizadas para a purificação das mesmas. Quando observados em microscópio, esses fungos apresentaram estruturas parecidas com fungos nematófagos, indicando não serem ectomicorrízicos. Apenas análises de sequenciamento genético e a inoculação desses fungos na planta podem mostrar uma identificação mais precisa. Basidiomais pertencendo a gêneros ectomicorrízicos foram encontrados na área de interesse: *Amanita*, *Pisolithus* sp. e *Russula puigarii*.

Considerações Finais

As ectomicorrizas de região tropical têm grande potencial de exploração. A história evolutiva das ectomicorrizas tradicionais possui relação com a região tropical. As ectomicorrizas encontradas nesses ambientes com florestas multidiversas podem trazer respostas e/ou questões sobre a complexidade dessas associações. O presente estudo traz evidências que podem direcionar estudos futuros, trazendo novas discussões sobre como classificar as ectomicorrizas e os papéis ecológicos dessas associações ectomicorrízicas diferentes. Devemos sempre considerar que as ectomicorrizas possuem uma história evolutiva complexa, sendo desafiador às pesquisas que buscam entender cada vez mais essa associação.

Palavras-chave: ectomicorriza; região tropical; fungos ectomicorrízicos.

ABSTRACT

Ectomycorrhizae (ECMs) are mutualistic associations between plants and fungi that promote nutrient exchange between both partners. Other functions of ECMs include plant stress resistance and carbon sequestration. The main ectomycorrhizal anatomical characteristics are the extraradicular mycelium, the mantle covering the root and the Hartig net between cortical cells. This dissertation is divided in two chapters. The first one is a review aiming to discuss the peculiarities of ECMs from multidiverse forests, whether they could relate to classical ECMs and what would be the advantages of such association. The second one reports our attempts to isolate potentially ectomycorrhizal fungi. The ectomycorrhizal status evolved independently 80 times or more from saprotrophic fungi. Important fungi lineages emerged in formerly tropical environments, such as /amanita, /clavulina and /inocybe. Currently, ectomycorrhizal association can be found in tropical and temperate regions. However, in the tropical region, little is known about ectomycorrhizal association mainly due to lack of studies. Some ECMs from tropical multidiverse forests show differences from the traditional ECMs. In ECMs from multidiverse forests, it is common that the Hartig net is absent or poorly developed. This feature may be a signal that this association may not be so established as traditional ectomycorrhizas. Hartig net formation involves very specific mechanisms such as Mycorrhiza-induced Small-Secreted Proteins (MiSSPs). With no MiSSPs production, the ectomycorrhizal fungi are unable to form a Hartig net, but the mantle remains mainly unaffected. Even knowing the importance of Hartig net, it seems that it is not essential to the exchange of nutrients but it enhances its efficiency. The mantle formation by ectomycorrhizal fungus is apparently governed by less specific mechanisms that can be found in saprotrophic fungi where they perform other functions. Large environmental variations, such as carbon scarcity in soil, may influence saprotrophic fungi to change their behavior enough to form simpler mycorrhizal structures, such as the mantle. Besides this, an environment with high plant diversity may be propitious for new associations to emerge. In these environments, the fungus has lower chances to find specific hosts, favoring a generalist behavior, when compared to regions with a few tree species. These environmental features are found in tropical region, which may be evidence of how some ectomycorrhizal fungi lineages emerged in tropical region and are currently being maintained. Regarding the second chapter, we are still attempting to isolate ECM-forming basidiomycetes from multidiverse forests.

Keywords: ectomycorrhiza; tropical region; evolution; ectomycorrhizal fungi; restoration.

LISTA DE TABELAS

LISTA DE ABREVIATURAS E SIGLAS

ECM – ectomicorriza (*ectomycorrhizae*);

MiSSP – pequena proteína secretada induzida por micorriza (*mycorrhiza-induced small-secreted protein*);

PCWDE – Enzima degradadora da parede celular vegetal (*plant cell wall degradating enzyme*);

SSP – pequena proteína secretada (*small-secreted protein*).

SUMÁRIO

1 INTRODUÇÃO

As plantas são essenciais para a vida como a conhecemos no planeta. Através da fotossíntese, elas fornecem energia para a grande maioria das formas de vida. Além disso, podem servir como habitat para inúmeras espécies e auxiliar na preservação de recursos hídricos essenciais para a manutenção da vida. Entretanto, dentro de um ecossistema, as plantas precisam ter uma boa capacidade para adquirir recursos e resistir a condições desfavoráveis. Neste sentido, a associação com outros organismos pode ajudar a planta. Dessa forma, o auxílio de organismos como fungos e bactérias se torna fundamental para a sobrevivência das plantas (Maksimov et al. 2015; Begum et al. 2019), tendo implicações ecológicas a nível global.

Acredita-se que mais de 80% das plantas façam associação simbiótica mutualística com fungos, chamada de micorriza. O fungo desenvolve hifas que conseguem alcançar locais do solo inacessíveis às plantas e, assim, aumentar a área de absorção de recursos, como fósforo, nitrogênio e água (Smith e Read 2008). Além de fornecer nutrientes e água, o fungo promove proteção contra patógenos e herbívoros, além de resistência a diversos estresses ambientais. Enquanto isso, a planta fornece uma fonte confiável de carbono para o fungo através da fotossíntese (Bogar e Peay 2017). Como consequência, as micorrizas possuem um papel importante no sequestro de carbono no solo, o que auxilia na diminuição das concentrações de dióxido de carbono (CO_2) na atmosfera (Soudzilovskaia et al. 2019).

Existem quatro classificações principais de micorrizas: micorriza arbuscular, orquidoide, ericoide e ectomicorriza. Especula-se atualmente que as micorrizas arbusculares ocorram em 71% das plantas, com fungos pertencentes a Glomeromycota e Endogonomycetes (Brundrett e Tedersoo 2018). Essa associação apresenta estruturas intracelulares chamadas de arbúsculos, que fazem a interface de troca de nutrientes entre os simbiontes. A micorriza orquidoide ocorre em 10% das plantas, exclusivamente em espécies da família Orquidaceae, a qual surgiu há 112 milhões de anos (Givnish et al. 2015). A maioria dos fungos desse tipo de associação pertence a três linhagens de Basidiomycota (Dearnaley et al. 2012).

Curiosamente, todas as sementes de orquídeas precisam dos fungos para germinação e nutrição através do fornecimento carbono. Além disso, algumas espécies são obrigatoriamente micorrízicas por toda a vida (Givnish et al. 2015). A micorriza ericoide surgiu uma vez em Ericaceae e em Diaspensiaceae (ambas Ericales) há 100 milhões de anos. Atualmente, ocorre em 1,4% das plantas. A estrutura característica dos fungos micorrízicos ericóides nas raízes são os ‘coils’, hifas intracelulares (Tedersoo e Bahram 2019). As ectomicorrizas surgiram há 200 milhões de anos e, até então, são encontradas em 2% das plantas (Brundrett and Tedersoo 2018). As ectomicorrizas estão presentes principalmente em ecossistemas florestais das regiões temperada e boreal (Martin et al. 2001), mas também ocorrem em região tropical com funções e diversidade pouquíssimo conhecidas. Os fungos ectomicorrízicos possuem características como hifas extrarradiculares exploradoras do solo, o manto, que reveste a raiz, e a rede de Hartig, composta por hifas que circundam as células corticais radiculares, servindo como uma interface de troca de nutrientes entre os simbiontes (Smith e Read 2008). Os benefícios mais bem conhecidos que as ectomicorrizas promovem para a planta são a proteção contra patógenos, a melhora na absorção de nutrientes minerais e orgânicos e aumento da resistência contra estresses abióticos (Brundrett e Tedersoo 2018).

As ectomicorrizas possuem funções ecossistêmicas importantes, além de auxiliar individualmente as plantas. Uma delas é exercida através das chamadas redes miceliais em comum (*common mycelial network*) que conectam, através do micélio, as plantas ectomicorrízicas da mesma ou de diferentes espécies no solo (Simard et al. 2012). Estudos mostram que essas redes auxiliam no desenvolvimento de plântulas por meio da resistência contra patógenos através da troca de sinais químicos que podem prevenir ataques futuros (Song et al. 2010) e na troca de nutrientes (Simard et al. 1997; Mcguire 2007; Tedersoo et al. 2020). Essa funcionalidade da associação ectomicorrízica no ecossistema pode estar relacionada a um processo ecológico associado a algumas espécies vegetais chamado de facilitação. A planta facilitadora, ou “*nurse plant*”, pode promover melhora nas condições abióticas locais, como temperatura, disponibilidade de nutrientes e água. A planta facilitadora também pode aumentar a proteção contra herbívoros e patógenos nas espécies associadas. Consequentemente, a planta facilitadora possui papéis ecológicos significativos no ecossistema como o aumento da biodiversidade (Brooker et al. 2008). Assim, a facilitação é um processo essencial para mitigar os efeitos de perturbações ambientais e invasão de espécies exóticas. As plantas facilitadoras podem ser uma ferramenta diretamente relacionada a restauração ecológica (Brooker et al. 2008). Desse modo, estudos que buscam compreender como uma facilitadora se estabelece no seu ambiente é fundamental para processos eficazes de restauração ecológica.

Como já citado, existem ectomicorrizas em região tropical, mas a falta de estudos dificulta o entendimento da associação nessa região. As lacunas são significativas acerca da diversidade de simbiontes, da funcionalidade da associação, dos mecanismos fisiológicos e genéticos de espécies nativas e da sua história evolutiva nesses locais. A dificuldade no entendimento das ectomicorrizas se deve a ideia de que as ectomicorrizas “são ausentes ou raras em região tropical” (Corrales et al. 2018), fazendo com que haja pouco interesse em explorar as ectomicorrizas nesses locais. Entretanto, por meio de estudos abrangendo a região tropical, será possível compreender melhor a importância das ectomicorrizas para esses ecossistemas (Weidlich et al. 2020). Talvez as ectomicorrizas sejam abundantes e essenciais nessas regiões, podendo ter funcionalidades até mesmo diferentes das ectomicorrizas de região temperada. De qualquer forma, para obter essas informações, as ectomicorrizas tropicais precisam ser melhor exploradas antes de serem definidas como iguais às da região temperada e/ou ausentes ou raras nos trópicos. Além disso, levando em consideração que a região tropical é a mais biodiversa do planeta (Barlow et al. 2018), é quase certo que haja uma diversidade de plantas e fungos ectomicorrízicos muito maior do que se conhece hoje.

Com a contínua degradação de ambientes naturais e a iminente crise climática, a restauração ecológica e a produção de ferramentas tecnológicas para solucionar problemas urgentes, como a falta de alimento, tornam-se cada vez mais prioritárias para contornar um destino preocupante para todas as formas de vida no planeta. Entender como os ecossistemas funcionam jamais foi tão importante e urgente como está sendo atualmente. Considerando o que se sabe sobre as associações ectomicorrízicas em região temperada, é muito provável que as ectomicorrizas tropicais também tenham funções ecossistêmicas essenciais. Por isso, estudos que visam descobrir as espécies ectomicorrízicas nativas, suas origens, funções, impactos ecológicos e a forma como elas se relacionam com as ectomicorrizas tradicionais são essenciais para montar estratégias de mitigação das consequências de ações antropogênicas.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Os principais objetivos desse estudo foram encontrar evidências de que florestas multidiversas da região tropical são importantes locais para o surgimento de novos fungos ectomicorrízicos e que esses fungos podem ter um papel importante para a biologia das plantas que habitam esses locais.

2.2 OBJETIVOS ESPECÍFICOS

1. Verificar se as ectomicorrizas tropicais de florestas multidiversas possuem relação com as ectomicorrizas clássicas e por que essa associação seria importante nesses ambientes:
   - Analisar a história evolutiva dos fungos ectomicorrízicos;
   - Verificar os mecanismos necessários para a formação das ectomicorrizas;
   - Elencar as possíveis vantagens e desvantagens dessa associação para ambos os simbiontes;
   - Verificar as condições abióticas e bióticas da região tropical que podem propiciar o surgimento de novos fungos ectomicorrízicos.

2. Verificar a formação de ectomicorriza em *Guapira opposita*, planta nativa facilitadora em ambiente de restinga:
   - Isolamento de fungos ectomicorrízicos a partir da raiz de *Guapira opposita* e de basidiomas;
   - Coleta de fungos em ambiente de restinga no mesmo ambiente de ocorrência de *Guapira opposita*.

3 REFERÊNCIAS

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4 CAPÍTULO 1 - EVOLUTIONARY STATUS OF ECTOMYCORRHIZAE FROM MULTIDIVERSE FORESTS

Multidiverse forests: an important place to the emergence of ectomycorrhizal fungi?

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Summary

Ectomycorrhizae (ECMs) are mutualistic associations between plants and fungi, involving nutrient exchange between both partners. The main ectomycorrhizal anatomical characteristics are the extraradicular mycelium, the mantle covering the root and the Hartig net between cortical cells. This review discusses whether ECMs from multidiverse forests could represent the initial steps in ECM formation and why this type of association would be preserved in mutidiverse forests. Ectomycorrhizal fungi evolved independently 80 or more times from saprotrophic fungi, with important fungal lineages originating in formerly tropical environments such as /amanita, /clavulina and /cantharellus. However, little is known about the current ECMs from the tropical region, especially from multidiverse forests. In these sites, ectomycorrhizal associations are often different from the traditional. It has been repeatedly reported ECMs in which the Hartig net is absent of poorly developed. This feature may be a sign that this association may not be so well established as traditional ectomycorrhizas. Hartig net formation involves very specific mechanisms such as Mycorrhiza-induced Small-Secreted Proteins (MiSSPs). Without the production of MiSSPs, the ectomycorrhizal fungi cannot form Hartig net, but the mantle is mostly unaffected. Data also shows that nutrient transfer is possible without the Hartig net, although not as efficiently. The mechanisms involved in mantle formation seem to be less specific and can be found in saprotrophic fungi performing other functions. Large environmental variations, such as carbon scarcity in soil, may influence the behavior of saprotrophic fungi, pushing them to form simpler mycorrhizal structures, such as the mantle. Besides this, an environment with high plant diversity may be propitious for new associations to emerge. In these environments, the chances of finding specific hosts are low, favoring a more generalist behavior, when compared to regions with fewer tree species. These environmental features are found in tropical region, which may be evidence of how some ectomycorrhizal fungi lineages emerged in multidiverse forests and are currently being maintained over more traditional associations.

Keywords: ectomycorrhiza; tropical region; evolution; Hartig net; ectomycorrhizal fungus.

Introduction

Ectomycorrhizal associations are mutualistic symbiosis between plants and fungi. These associations are found mainly in forest ecosystems and have important ecological functions, such as carbon and nitrogen sequestration and plant communication (Smith and Read 2008; Simmard et al. 1997). It is estimated that ectomycorrhizal association occurs in 60% of all trees around the planet (Steidinger et al. 2019). In the last decades, it has been discovered that ectomycorrhizae (ECMs) occur not only in temperate regions dominated by a few tree species, but also in tropical multidiverse forests (Corrales et al. 2018). However, ECMs of the tropical region are significantly less studied than those of the temperate region (Nouhra et al. 2019).

So far, there are only a handful of studies addressing ECM from multidiverse forests. Nevertheless, in the tropical region there are plant families able to form ECM, such as Dipterocarpaceae, Myrtaceae, Fagaceae, Fabaceae, Nyctaginaceae and subfamily Caesalpinioideae (Haug et al. 2005; Alvarez-Manjarrez et al. 2017; Roy et al. 2017; Strullu-Derrien et al. 2017; Vasco-Palácios et al. 2018; Vanegas-León et al. 2019). There are also fungi belonging to ECM lineages mainly from /amanita, /clavulina, /russula-lactarius, /boletus, /tomentella-thelephora, /cantharellus, /inocybe, /cortinarius and /entoloma (Nouhra et al. 2019). Recently, researchers found out in Guiana region the only known ectomycorrhizal fungal lineage endemic to the tropics: /guyanagarika (Sánchez-García et al. 2016). Although there are studies in tropical ecosystems, there is still an important gap in ectomycorrhizal fungal and plant diversity in these regions impairing the aims to understand the importance of ectomycorrhizal association at a global level.

Some ECMs from multidiverse forests have atypical anatomical structures, being curiously distinct from the traditional “mantle and Hartig net” ECM. For example, ectomycorrhizal anatomy in some plant species from Nyctaginaceae family is different with features such as the lack of Hartig net, compensatory structures (transfer cells) and/or incomplete mantle formation (Ashford and Allaway 1982; Allaway et al. 1985; Cairney et al. 1994; Haug et al. 2005; Furtado et al. 2022). Also, a common ectomycorrhizal feature is the suppression of root hair elongation, yet in Nyctaginaceae plants there is no such inhibition (Haug et al. 2005). Researchers suggest that Nyctaginaceae family may have become ectomycorrhizal only recently (Haug et al. 2005; Balestrini and Kottke 2016). These structural differences may also be an argument to exclude these associations from the ectomycorrhizal status (Furtado et al. 2022). Here we discuss the possibility that the ECM from multidiverse forests could be an intermediate step between a free-living saprotrophic fungus and a fully formed ECM. For this study, we considered the following evidence lines: (1) ectomycorrhizal evolution, (2) evolutionary steps needed to ectomycorrhizal formation, (3) the formation of a typical ECM structure, (4) ECM phylogeny and biogeography (plants and fungi), (5) untraditional ectomycorrhizal association from multidiverse forests in tropical region and (6) environmental features favoring this niche transition and maintenance.

Traditional ectomycorrhizal evolution: who, when and where?

Throughout time, there were three mycorrhizal evolution waves (Brundrett and Tedersoo 2018). The first wave occurred 450 million years ago, in the Devonian period, when arbuscular mycorrhiza emerged (Brundrett 2002). This event apparently allowed plants to colonize terrestrial habitats. The second wave occurred 200 million years ago, with the emergence of ECM, orchidoid and ericoid mycorrhizas. The third wave that started 65 million years ago is related mainly to climate and soil conditions, related to high diversity of ECM (Brundrett and Tedersoo 2018). Currently (Anthropocene), it is possible that a rise in atmospheric carbon, climate changes, habitat degradation and invasive exotic plants are enhancing diversity of ectomycorrhizal plants and fungi and hence affecting local biodiversity (Brundrett and Tedersoo 2018). Usually, the distribution pattern of the symbiotic fungi is strongly tied to the associated plants (Sánchez-Ramírez et al. 2014).

During evolution, it is estimated that ECMs originated around 30 times in plant lineages and 80 or more times among fungi, mainly in Ascomycota, Basidiomycota and Mucoromycotina (Endogonales) lineages. The amount of independent origins of ECM indicates that either this is not a complex transition and/or that there are multiple ways to achieve the same results. Curiously, all ectomycorrhizal fungi lineages have saprotrophic fungi as common ancestors (Tedersoo and Smith 2013; Martin et al. 2016; Tedersoo and Brundrett 2017). The majority of known ectomycorrhizal fungal lineages are restricted to temperate ecosystems, but only a few of them were originated in temperate region. For example, a phylogenetic study of the ectomycorrhizal fungal lineage /sebacina showed its origin in temperate coniferous forest in North America (Tedersoo et al. 2014). These distribution patterns in temperate region may be caused by constraints such as temperature and ectomycorrhizal plant host evolutionary history. For example, Pinaceae, the oldest known ectomycorrhizal plant family, is restricted to some boreal and subtropical habitats, while tropical ectomycorrhizal plant families (e.g. Dipterocarpaceae, Fabaceae and Polygonaceae) emerged more recently (Late Cretaceous or Paleogene) (Tedersoo et al. 2012). These evidences may explain why, currently, this association is more abundant in temperate region. However, we must consider that a higher number of studies in the temperate and boreal regions may influence the result of higher fungal lineages in temperate region, since only recently, we began to look for ECMs in the tropics (Nouhra et al. 2019).

Some well-known ectomycorrhizal fungal lineages are widespread across tropical region (/amanita, /russula-lactarius, /boletus and /tomentella-thelephora), which means that they are adapted to tropical environments (Corrales et al. 2018; Tedersoo et al. 2010; Tedersoo and Smith 2013). Curiously, Strullu-Derrien et al. (2018) noted that the only ECM fossils found (described by LePage et al. 1997 and Beimforde et al. 2011) were from tropical or warm regions. Researchers suggest that most of ectomycorrhizal fungal lineages originated in tropical to subtropical regions, but most of the canonical ECM that we find today are restricted to temperate and boreal forests (Strullu-Derrien et al. 2018). It is possible that the basal lineages remained in the tropics, while a global cooling triggered a new diversification of ECM in temperate/boreal regions (Strullu-Derrien et al. 2018). For example, several ectomycorrhizal fungal lineages such as /russula-lactarius, /clavulina, /inocybe and /guyanagarika seem to have tropical origins (Buyck et al. 1996; Matheny et al. 2009; Kennedy et al. 2012; Sánchez-García et al. 2016). An important hypothesis that may explain the distribution pattern in ectomycorrhizal fungal clades is the boreotropical hypothesis. This hypothesis states that during the Tertiary, tropical broad-leaved forests occurred in higher latitudes in regions that are temperate today (Wolfe 1975, 1980). For example, *Amanita* sect. Caesareae, an important ectomycorrhizal fungal lineage, probably originated in Africa between Paleocene and Eocene period, in a Paleotropical setting. Later, during the Miocene and Pliocene period, *Amanita* sect. Caesareae dispersed to temperate and other tropical areas, corroborating to the boreotropical hypothesis (Sánchez-Ramírez et al. 2014). Interestingly, *Amanita* sect. Caesareae have higher diversification in the American continent than in the others (Sánchez-Ramírez et al. 2014). Althought the Paleotropics is supposed to be the area of origin of some ectomycorrhizal fungal lineages (Alexander 2006), the precise biogeography is still unknown for the most of ectomycorrhizal clades (Sánchez-Ramírez et al. 2014). Ectomycorrhizal fungal lineages /clavulina, /cantharellus and /inocybe are highly diverse in some tropical sites. While /clavulina occurs in several Neotropical forests, the others do not have a well-distributed pattern, missing in some sites, which is probably a consequence of undersampling (Corrales et al. 2018). So far, only the /guyanagarika fungal lineage appears to be endemic to the tropics, but this could also be a reflection of the undersampling when working with tropical ECM (Sánchez-García et al. 2016). Thus, the tropical region seems to be important for the evolutionary history of ectomycorrhizal associations worldwide and we find no reason to assume that this process stopped millions of years ago.

Based on this, it is likely that in the present tropical region saprotrophic-to-ectomycorrhizal transitions have been happening more recently than initially thought. Therefore, we must address what would be necessary for a saprotrophic fungus to engage in ECM formation.

Similarities and differences between saprotrophic and ectomycorrhizal fungi: an intimate evolutionary relation

Saprotrophic fungi (ascomycetes and basidiomycetes) occupy an important ecological niche acting as the main decomposers in natural ecosystems. These fungi recycle large amounts of carbon and other nutrients, markedly affecting the environment (Grinhut et al. 2007). These fungi developed different mechanisms to decompose soil organic matter. Litter-decomposers use a variety of carbohydrate-active enzymes (CAZy) and oxidative enzymes to degrade lignin (Steffen et al. 2000; Liers et al. 2011). Litter-decomposers and white rot wood decayers seem to share the same strategy of cellulose decomposition (Floudas et al. 2020), using a large set of enzymes such as endoglucanases, cellobiohydrolases and lytic polysaccharide monooxygenases (Horn et al. 2012). Brown rot fungi lack enzymes acting on crystalline cellulose, using hydroxyl radicals generated by nonenzymatic Fenton reaction. This mechanism causes oxidative decomposition of carbohydrates and lignin (Arantes et al. 2012). Interestingly, comparative genome studies indicate that fungal genomes have “signatures” of nutritional strategies transitions through evolution, including the saprotrophic-to-ectomycorrhizal transition (Kohler et al. 2015; Floudas et al. 2020).

Ectomycorrhizal fungi emerged 80 times or more independently during evolution from saprotrophic fungi (Tedersoo and Smith 2013). This common emergence of ectomycorrhizal fungi (Baldrian and Kohout 2017) may be related to less known molecular mechanisms that have recently been explored in a few researches. Saprotrophic-to-ectomycorrhizal transition involves a loss of genes encoding plant cell wall degrading enzymes (Miyauchi et al. 2020). A shared trait of many ectomycorrhizal fungal lineages is the loss of most genes that code for CAZymes as lignolytic peroxidases (Class II PODs), cellobiohydrolases glycosil hydrolase family (GH; 6 and 7), and cellulose binding modules and plant cell wall degrading enzymes (PCWDEs) (Pellegrin et al. 2015; Wu et al. 2021). In addition, curiously, some ectomycorrhizal fungi do not have invertase and sucrose transporters, depending almost exclusively on the capacity of the host to convert sucrose to glucose, which could be a mechanism to regulate the amount of carbon destined to the fungus (Miyauchi et al. 2020). Losing PCWDEs genes was a crucial and irreversible step for ectomycorrhizal fungi emerge from saprotrophic ancestors (Wolfe et al. 2012). After associating with the plant, these genes become less necessary, since the plant is able to provide simple carbohydrates to the fungus. Although researchers suppose that loss of the most saprotrophy-related genes is the major process for ectomycorrhizal fungi emergence, we may not discard the possibility that these gene losses may be a consequence and not a cause of the association with plants. Hence, the ectomycorrhizal fungi emergence may begin before these genes are lost (Smith et al. 2017).

Ectomycorrhizal fungi keep plant tissue integrity, an important aspect for maintaining a mutualistic association (Kohler et al. 2015). From a phylogenetic study of Boletales (with known ectomycorrhizal fungal families), Wu et al. (2021) identified a significant gene loss in early ectomycorrhizal clades. There is a gradual increase in gene duplication rates through time, possibly caused by a proliferation of transposable elements. Genome amplification seems to be a pattern in well–established ectomycorrhizal fungi (Miyauchi et al. 2020). Genes involved in ectomycorrhizal association, such as the ones codifying small-secreted proteins (SSPs), proteases and lipases, are close to transposable elements and hence contribute to a higher gene evolution rate. Transposable elements are abundant in ectomycorrhizal fungi (Miyauchi et al. 2020; Wu et al. 2021) and seem to be more related to the ectomycorrhizal lifestyle than phylogeny (Miyauchi et al. 2020). A phylogenomic analysis in ectomycorrhizal fungi *Amanita* showed that the most of the new genes are lineage-specific, after its ectomycorrhizal status emergence (Hess et al. 2018). This aspect may be an indication that ectomycorrhizal association reduces selective pressure on the fungus and enables a higher genome plasticity and the loss of some functions (Selosse et al. 2014).

Despite losing most of PCWDEs genes, ectomycorrhizal fungi conserve a set of them, such as endoglucanases, mannanases, pectinases, oxidoreductase and laccases plus Fenton non-enzymatic reaction, which enable the ectomycorrhizal fungus to forage soil organic matter for elements other than carbon (Shah et al. 2016; Wu et al. 2021). *Laccaria bicolor* conserved a set of saprotrophy-related genes as carbohydrate active enzymes families GH5 (endoglucanases) and GH28 (pectinases), these genes seem important to symbiosis because they might facilitate hyphae penetration in root apoplast (Eastwood et al. 2011). Curiously, some ectomycorrhizal fungi (e.g. *Acephala macrosclerotiorum* and *Gautieria morchelliformis*) have significant amounts of PCDWEs genes similar to saprotrophic fungi and wood decayers, and they must have a facultative saprotrophic lifestyle (Miyauchi et al. 2020). Additionally, ectomycorrhizal fungi conserved or even expanded other gene families from their ancestors. For example, ectomycorrhizal fungi have a significantly higher number of SSPs in their secretome than saprotrophic fungi (Pellegrin et al. 2015). Saprotrophic fungi produce SSPs, which represent about 40% of their secretomes, to enhance degradation of organic matter (e.g. cellulose). A significant portion (20-61%) of the SSPs is regulated by fungal development such as hydrophobins and lectins (Feldman et al. 2020). These proteins can also be important in the early steps of ectomycorrhizal formation, as we will discuss later.

Ectomycorrhizal and saprotrophic fungi have important ecological interactions, some of them related to organic matter decomposition. Some studies pointed that ectomycorrhizal and saprotrophic fungi degrade organic substances resulting in a competition that may slow down decomposition rates known as Gadgil effect (Gadgil and Gadgil 1971; Lindahl and Tunlid 2015). Although this effect is cited in literature, other studies contest this, because they do not occupy the same niche. Saprotrophs tend to dominate surface sites where organic litter and celullose are abundant, while ectomycorrhizal fungi dominate greater depths where they can obtain nitrogen from recalcitrant organic substrates (Lindahl et al. 2007). Ectomycorrhizal fungi have different mechanisms to exploit soil organic matter and obtain nitrogen, while they depend mainly on the host for carbon supply, a result of having lost the ability to fully degrade lignocellulose (Hobbie and Högberg 2012; Lindahl and Tunlid 2015; Nicolás et al. 2019). Variations in these substrates can result changes in decomposition rates and ecological interactions of ectomycorrhizal and saprotrophic fungi (Marañon-Jiménez et al. 2021). In Boletales, for example, the nutrient deficiency promoted the loss of fungus aggressiveness to the root and allowed brown rot fungi to transition to the ectomycorrhizal lifestyle (Eastwood et al. 2011). Variations in environmental conditions may result in implications to species fitness. Therefore, adaption to unpredictable environments lead species to evolve mechanisms to change phenotypic characteristics, although may result in a low fitness in the short term. This bet-hedging strategy may be important to avoid species extinction risk (Childs et al. 2010). We may speculate that something similar to a bet-hedging strategy may happen with a saprotrophic fungus that ‘searches’ for a host through the production of new structures, resulting in mutualistic associations. Selective pressures that lead these changes may be environmental conditions such as enhanced competitiveness, nutrient scarcity, extreme environment and/or increased atmospheric carbon (Baldrian and Kohout 2017; van der Linde et al. 2018) resulting in fungus genome alterations by transposable elements and gene cooption (Miyauchi et al. 2020; Wu et al 2021). Combined selective pressures may result in saprotrophic-to-ectomycorrhizal transition, changing its ecological niche.

Researchers already proposed that ectomycorrhizal fungi occurs along a biotrophy-saprotrophy continuum due to their variable ability to obtain carbon from soil organic matter and the identification of many fungi with a facultative lifestyle (Koide et al. 2008). Indeed, ectomycorrhizal and saprotrophic fungi share a large set of common secreted proteins (Pellegrin et al. 2015). In this hypothesis, the evolution of ECM from saprotrophic fungi likely occurred during shifts along this continuum and not from a pure saprotrophic state to a pure biotrophic state (Koide et al. 2008). This hypothesis does not discard full saprotrophic or ectomycorrhizal habit in some fungi, but it leaves room to assume that transitions to an ectomycorrhizal status may be more common than we think. In another perspective, there are two main hypotheses proposed to the evolutionary trajectory of saprotrophic-to-ectomycorrhizal state. First, saprotrophic fungus can transit directly to an ectomycorrhizal habit and second, commonly referred as the “waiting room hypothesis”, saprotrophic fungi evolve to endophytic habit and, afterwards, to an ectomycorrhizal status (Selosse et al. 2009; Selosse et al. 2018). Therefore, in our study, we consider that ECM from multidiverse forest may represent intermediate points along this continuum, while traditional ECMs could be further down the “ECM pathway”. This tendency can also be found if we look into the process of ECM formation.

Step-to-step ectomycorrhizal formation

A traditional ECM is usually characterized by three main anatomical structures: extraradical hyphae, mantle and Hartig net (Smith and Read 2008). Interestingly, these structures do not form at the same time. First, both fungus and plant have to establish communication with each other in the rhizosphere (Baptista et al. 2011). After ‘understanding’ the signals, they need to change their physiological and anatomical structure to form traditional ectomycorrhizal features, initially by forming the mantle, then the Hartig net (Tschaplinski et al. 2014). Once the ectomycorrhizal structures are established, symbionts begin the nutrient exchange, so a functional association is formed (Baptista et al. 2011).

The preinfection phase is characterized by changes in rhizosphere exudates, attraction and recognition between plant and fungus through signal exchange (Martin et al. 2001). The plant produces signals such as flavonoids (mainly rutin and quercetin), abietic acid and zeatin. These signals do not seem to be species-specific, since they are produced by a wide variety of plants (Baptista et al. 2011; Gajdosová et al. 2011; Ismail et al. 2016; Singh et al. 2021). Ectomycorrhizal fungi recognize potential hosts and grow toward the roots to initiate colonization. They also produce diffusible molecules such as sesquiterpenes, ethylene and auxin to stimulate the formation of lateral roots, which enhances colonization areas (Felten et al. 2010). Suppression of root hair elongation is also typical of ectomycorrhizal roots. Hypaphorine seems to be involved in this process (Balestrini and Kottke 2016). Alterations in root morphology are not exclusive of ectomycorrhizal fungi. Both saprotrophic and pathogenic fungi are able to produce auxin and ethylene, promoting similar changes in the root system (Raudaskoski and Kothe 2015). After the initial exchange of signals, mantle formation starts. The hyphae produce adhesins to interact with host receptors, and lectins and hydrophobins to affix the fungus on the root surface (Martin et al. 1999; Baptista et al. 2011; Raudaskoski and Kothe 2015). Interestingly, saprotrophic fungi also are able to produce these components (Feldman et al. 2020). After hyphal attachment on epidermal cells, the ectomycorrhizal fungus begins to form hyphal layers that will originate a mature mantle (Martin et al. 2001). The mantle morphology is influenced by both symbionts and abiotic factors and requires a process of hyphal aggregation (Martin et al. 1999). During mantle formation, epidermal root cells usually elongate (Peterson and Bonfante 1994). Once the mantle is established, hyphae penetrate between cortical cells (Baptista et al. 2011). A juxtaposition occurs between hyphae and cortical cell walls, installing a common apoplast in which solutes can move through both symbionts. This structure is the Hartig net, a nutrient exchange interface between symbionts, typically found in ECM (Balestrini and Kottke 2016).

Hartig net formation requires a second wave of information exchange, since it is a more intimate relationship than the mantle. It is not surprising that the Hartig net signals are more specific than the mantle (Dell et al. 1994; Martin et al. 1999). During this step, ectomycorrhizal fungi seem to use a set of effector proteins to suppress host immunity and/or remodel the cell wall (Pellegrin et al. 2019). These proteins are key to understand how the plants differentiate between pathogen and mutualistic fungi and do not fight back when infected. Plett et al. (2011) identified an effector protein, named Mycorrhiza-induced Small-Secreted Protein 7 (MiSSP7), crucial to the formation of ectomycorrhizal association between *Laccaria bicolor* and *Populus trichocarpa*. After the first steps of recognition, the host plant produces rutin and quercetin that activate expression of MiSSP7 production genes in the ectomycorrhizal fungus. MiSSP7 targets root cell nuclei, where it inhibits Jasmonate Zim Domain proteins (PtJAZ5 and PtJAZ6), preventing jasmonate perception (Plett et al. 2014). With the inhibition in jasmonate signaling, the Hartig net starts to form. Notably, without jasmonate repression there is virtually no Hartig net formation, although the mantle seems to be less impacted by this alteration (Basso et al. 2020). Another important effector protein that alters host immunity, called MiSSP7.6, was identified in *Laccaria bicolor* during ectomycorrhizal association with poplar (Kang et al. 2020). MiSSP7.6 is a cysteine-rich protein only found in lineage-specific *L. bicolor* and *L. amethystina*. During contact with poplar roots, *L. bicolor* produces MiSSP7.6 in the apoplastic interface. This effector protein reaches the plant cell nuclei and binds to PtTrihelix regulators, which implies changes to expression in genes related to plant immunity. As MiSSP7, MiSSP7.6 seems to alter plant immunity to promote Hartig net formation in the *Laccaria bicolor*/*Populus tremula x alba* association (Kang et al. 2020). In a similar way to MiSSP7, RNAi mutants with reduced *MiSSP7.6* expression showed a significant reduction in Hartig net formation, but little or no changes in mantle formation (Kang et al. 2020). Another effector protein that seems important to a full-structured ECM is PaMiSSP10b, produced by the ectomycorrhizal fungus *Pisolithus albus* in association with *Eucalyptus grandis* (Plett et al. 2020). PaMiSSP10b secreted by *P. albus* enters in host cells and interacts with S-adenosyl methionine decarboxylase in the polyamine pathway, increasing the overall amount of polyamines (Plett et al. 2020). Polyamines are usually produced by the plant to reduce pathogenic infection (Jiménez-Bremont et al. 2014). The ectomycorrhizal fungus seems to produce PaMiSSP10b to influence polyamine concentrations in host and, curiously, it protects plant tissues from diseases. However, how the ectomycorrhizal fungus benefits from increased polyamine production is still unknown. A possible explanation is that the diversion of S-adenosyl methionine to polyamine production inhibits the biosynthesis of ethylene, a hormone capable of inhibiting Hartig net formation (Belles et al. 1991; Plett et al. 2014; Plett et al. 2020).

All these studies showed that manipulation of these MiSSPs resulted in evident changes in the Hartig net formation, but little or no alterations in mantle formation. It is important to point out that some molecules used to form the mantle are also summoned to initiate Hartig net formation (e.g. symbiosis-regulated acidic polypeptides (SRAP) protein family) (Laurent et al. 1999). Interestingly, there is another effector protein identified in *L. bicolor-P. tremula x alba* ectomycorrhizal association, called MiSSP8, that was crucial for both mantle and Hartig net formation (Pellegrin et al. 2019). MiSSP8 is also involved in non-symbiotic processes such as fruiting body tissues composed by pseudoparenchyma (aggregated hyphae) and is similar to some SSPs of saprotrophic fungi (Pellegrin et al. 2019). Therefore, it seems that MiSSP8 was initially used by saprotrophic ancestors for the formation of the fruiting body and afterwards recruited for ectomycorrhizal symbiosis, both processes that involve hyphae aggregation (Pellegrin et al. 2019).

Studies aiming to discover new molecules involved in ectomycorrhizal association are recent and need more efforts to clarify how this association works in molecular level. For example, *Laccaria bicolor* has 98 MiSSPs upregulated in symbiotic tissues (Martin et al. 2008), but the vast majority of these effector proteins have an unknown function. Researchers propose two categories of MiSSPs: the ones that preexisted in saprotrophic ancestors and the ones that are only found in ectomycorrhizal fungi (Pellegrin et al. 2019). Following this classification, the effector proteins used in mantle formation may be less derivative (e.g. MiSSP8) than the ones used in Hartig net formation (e.g. MiSSP7 and MiSSP7.6). Hence, Hartig net formation seems to be the more ‘advanced’ ectomycorrhizal feature. Accordingly, it would require a more specific communication for the host to ‘authorize’ hyphae formation in apoplast (Plett et al. 2011; Pellegrin et al. 2019). Mantle formation, however, is less intrusive (Dell et al. 1994), so the signals are not as specific. Exceptions to this pattern appear to exist, as it is exemplified by the interaction between *Melaleuca leucodendron* and an unknown fungus species (Alexander and Hogberg 1986). In this association, the mantle was poorly formed or even absent, while the Hartig net developed only in the epidermis. As we pointed out above, the “easier” way to the saprotrophic/ectomycorrhizal fungus form an association with a host is through mantle formation. However, considering the high variation in saprotrophic-to-ectomycorrhizal transitions due to convergent evolution, different host species and variation in environment conditions, it is likely that not all the saprotrophic-to-ectomycorrhizal transitions will show the same pattern.

An interesting study made by Smith et al. (2017) tested interactions between several wood-decay basidiomycetes and the ectomycorrhizal plants *Pinus sylvestris* and *Picea abies*. *Phellinus igniarius*, a wood-decay fungus, developed a Hartig net-like structure in root cortical cells of *P. abies* while *Coniophora cerebella* and *Hypholoma capnoides* formed mantle-like structures on *P. sylvestris* roots (Smith et al. 2017). Based on their results, all the interactions that reached inner root tissues (i.e. cortex and vascular tissue) necessarily involved colonization of the epidermis, sometimes forming an ectomycorrhizal-like structure even if the inner root tissues were not colonized. Another example is from a study using *Serpula lacrymans*, a fungal species belonging to brown rot lineages clade that derived ectomycorrhizal fungi in Boletales. *S. lacrymans* forms a loose mantle on pine roots and these hyphae do not produce small-secreted protein, while the ectomycorrhizal lineages inside Boletales expressed a large set of MiSSPs (Kohler et al. 2015). In this study, researchers suppose that the mantle formation by *S. lacrymans* may be a signal of ancestral condition. Interestingly, the large set of oxidative enzymes inherited from saprotrophic ancestors may be capable of reducing host defense effectiveness and provide the initial steps of the association, such as the mantle (Hess et al. 2018).

Although saprotrophic fungi “colonizing” roots may indicate endophytic status, the presence of ectomycorrhizal-like structures may be an evidence of a nutritional exchange interface that is not formed by endophytic lifestyles (Baldrian and Kohout 2017). This means that the fungus colonizing these roots likely is forming mutualistic association. It seems that wood-decay fungi produce ectomycorrhizal-like structures as a response to selective pressures by environment variations, such as nutrient-limited soils where roots are an abundant carbon source and also easier to colonize due to the depletion of host defenses (Baldrian and Kohout 2017). An important question we have to ask is whether the presence of the mantle without Hartig net could bring any advantage to the association. Usually, the main mantle function is to store nutrients such as glycogen, polyphosphate and amino acids during low nutrient availability in soil (Balestrini and Kottke 2016). It can also act as a barrier against soil contaminants (Policelli et al. 2020) and pathogens (Marx 1972). Interestingly, there is a distinct possibility that nutrient exchange is possible with the mantle alone, although maybe not as efficiently as when the Hartig net is present. By inoculating two different strains of *Paxillus involutus*, one that formed the Hartig net and other that did not in *Populus x canescens* roots, Sa et al. (2019) showed that there was little difference in nutrient transfer capabilities, despite the absence of the Hartig net in one of the strains. Future studies aiming nutrient exchange in associations lacking Hartig net are needed.

Another interesting approach to understand the variations in ectomycorrhizal associations is by using compatible and incompatible associations (natural and induced, respectively). Tschaplinski et al. (2014) compared the changes in metabolites production during the interaction between the fungus *Laccaria bicolor* and two species of *Populus*: *P. deltoides* and *P. trichocarpa*. While *Laccaria bicolor-P. trichocarpa* is a compatible association, *Laccaria bicolor-P. deltoides* is incompatible. During the study, *P. trichocarpa* lowered the amounts of defense metabolites, while *P. deltoides* kept its defense mechanisms on guard (Tschaplinski et al. 2014). Another interesting result is that nutrient exchange in these incompatible associations is possible (e.g. trehalose accumulation), although sugar exchange is more efficient in compatible association. Also, in the incompatible association the fungal colonization rate is lower and the Hartig net is underdeveloped. This may happen due to the host maintenance of defensive metabolites, so ectomycorrhizal fungus is rejected by the root system and metabolic changes to a compatible formation are not induced (Tschaplinski et al. 2014). The researchers do not cite alterations in mantle formation, so the mantle is possibly formed. The approach of using a saprotrophic/ectomycorrhizal fungus may bring information to understand what are the main barriers that a fungus has to overcome in order to colonize a new host. In summary, even if not fully formed, ectomycorrhizal-like associations could bring some benefits for the partners. These benefits, however, may not be as strong as in a fully formed ECM, so how these associations could persist nowadays?

Why and where intermediate associations would be maintained?

Considering that ectomycorrhizal fungi evolved dozen of times, each ectomycorrhizal fungus lineage may have different physiological abilities. Host and environment may be the main evolutionary drivers of ectomycorrhizal symbiosis (van der Linde et al. 2018) and a set of these selective pressures (e.g. different host species and abiotic factors) acting on one fungus species may be totally different from another (Pellitier and Zak 2017). These variations in ectomycorrhizal fungi plus host preferences may result in low and high-quality associations. Assuming that an intermediate ectomycorrhizal fungus may not give to the plant a full set of benefits, why would the plant and the fungus keep a low-quality association? Which are the gains and losses?

During lifetime, plants frequently experience environmental variation and, consequently, the set of resources necessary for the plant also changes. Low-quality partners in ECM may be crucial for plant survival in fluctuating environments (e.g. Moeller and Neubert 2016). The rationale behind this is that a high-quality partner tends to be also highly specific, because the partners need to adapt to one another (Carlson et al. 2022). This specificity, however, lowers the number of possible choices for the organisms and this could be critical when the environment is unpredictable. For example, there are some key steps during plant life cycle in which the individual is especially susceptible to the environment, such as in the seedling establishment phase. In this case, specificity of partners may be risky to plant survival because these partners may be absent (Thrall et al. 2007; Moeller and Neubert 2016). This may not be a problem if the environment is “predictable” enough to assure that the correct partner will be quickly found, but in a changing environment, specificity may be deleterious. Moreover, the “high-quality” of the association may be related to specific environmental conditions. If these conditions change, the quality of the association may be reduced. In summary, it may be more advantageous to have a flexible association with low-quality partners than not forming any association (Batstone et al. 2017).

Mutualistic associations of plant-partner show a tendency of less specialization in the tropical region due to high plant diversity (Schleuning et al. 2012). There are no studies directly related to ECM comparing tropical and temperate regions, however, studies in belowground of Europe temperate forests indicates a relatively high ectomycorrhizal fungi specificity to the most common trees (van der Linde et al. 2018). Maybe this specificity is related to a low host plant diversity in these forests and, consequently, a coevolution process between plant and fungus. Europe temperate forests have significantly lower tree diversity and, consequently, more monodominant forests than tropical region (Slik et al. 2015). One example of specific plant-fungus association found in temperate forests is *Suillus,* a known ectomycorrhizal fungal genus, associated with plants from Pinaceae (*Larix, Pinus* and *Pseudotsuga*) (Liao et al. 2016). Researchers suggest that evolution behind *Suillus*-Pinaceae association involves multiple events of convergent host-switching (Nguyen et al. 2016). Specificity may be important in a habitat where a few tree species are available for ectomycorrhizal association because if the plants have full benefit from a few high-quality partners (specialists), the low-quality partners (generalists) may be eliminated (Batstone et al. 2018). This means that in a monodominant forest, where the dominant plant species are specialists, the chances to form association with many fungal species are significantly lower.

In diverse forests, however, specificity could be a problem because individuals of the same species may be few and far apart and finding a specific partner could be a challenge. A saprotrophic/ectomycorrhizal fungus is likely not a specialist because it might not have yet developed the complex set of necessary mechanisms, as commented above, to form an association such as specialist ectomycorrhizal fungi do. This might be due to selectivity and specificity traits that tend to appear in more derived lineages (Batstone et al. 2018). Phylogenetic studies in other mutualistic associations support a tendency of a generalist partner transit to a specialist status through evolutionary time scales (Gilbert and Webb 2007). Whether specificity is the evolutionary apex of ectomycorrhizal association is controversial and may depend on many factors (e.g. extinction rates related to the inability to switch hosts) (Batstone et al. 2018; Chomicki and Renner 2017), which need to be further studied. Considering that generalism is the rule to establish a niche transition (saprotrophy-mutualism) for these fungi, the tropical region may be an interesting spot of saprotrophic-to-ectomycorrhizal transition, because the high diversity of plant species can enhance the chances for a new mutualistic fungus to find a compatible host (Batstone et al.

2018). Brundrett and Tedersoo (2018) specify that dual-mycorrhizal plants (arbuscular-ectomycorrhiza), also found in tropical region (McGuire et al. 2008), may be in an intermediate evolutionary status. This means that due to its ecological flexibility, saprotrophic/ectomycorrhizal fungus may be more frequent in these plants.

In summary, a saprotrophic/ectomycorrhizal fungus in a multidiverse forest might not have developed the complex set of mechanisms necessary to form a “full ECM” and neither have lost its capacity to degrade soil organic matter simply because it cannot afford to lose its generalism in a varied environment. Studies addressing the specificity in these ECM from multidiverse forests are important to understand whether a specific ectomycorrhizal association is a result of a more detailed process of coevolution. These results could bring important evidence to direct future studies about saprotrophic-to-ectomycorrhizal transitions worldwide. In the following section, we compile some of the data available for ECMs in the tropical region.

Untraditional ectomycorrhizae from multidiverse forests

In the tropical region there are more than 53,000 tree species (most of them extremely rare), while in temperate region (Europe) there are only around 124 tree species (Slik et al. 2015). This difference is usually attributed to a higher plant speciation rate in the Neotropical region (Hughes et al. 2013; Slik et al. 2015). Across the Neotropical region, there are lots of plant biodiversity hotspots, and researchers say that Neotropics may be “an evolutionary laboratory” (Hughes et al. 2013). Similarly to tree species, fungi species occurs in all environments of the tropical region, and it seems that the most of the globally undescribed fungal species are precisely in the tropical region (Aime and Brearley 2012). Ecosystemic services mediated by fungi are the main drivers for the maintenance, for example, of the Amazon forest (Ritter et al. 2020). A study made by Ritter et al. (2020) showed that Amazonian fungi present different patterns of diversity depending on habitat and local variables.

Soil characteristics of tropical region are complex and may be challenging for all species. In some tropical ecosystems with higher fungal richness added to higher soil temperature and faster plant growth result in higher decomposition rates reducing organic matter, which may restrict niches available for soil microbiota (Wardle 2002; Tedersoo et al. 2012; Ritter et al. 2020). Corroborating this, soil carbon content is one of the most important factors influencing fungal richness in determinate areas (Liu et al. 2015; Ritter et al. 2020). In the most nutrient-limited soil of Amazonian biome (campinas) a high diversity of ectomycorrhizal fungi was found (Singer et al. 1983; Roy et al. 2016; Ritter et al. 2020). Interestingly, an increasing of habitat and soil complexity may be associated to the second and the third waves of mycorrhizal evolution (Brundrett and Tedersoo 2018). Curiously, these traits can be found in megadiverse forests of tropical region (Cochrane 2006). Therefore, the characteristics of soil in tropical forests plus the evidence of a higher plant speciation rate may favor the appearance of new associations, such as ECM, in the tropical region.

In the tropical region, there are evidence of 283 plant species from 73 genera in 18 families forming ECM (Corrales et al. 2018). These ectomycorrhizal plant families are Achatocarpaceae, Asteropeiaceae, Betulaceae (*Alnus*), Cistaceae (*Pakaraimaea*), Dipterocarpaceae (*Dryobalanops*, *Pseudomonotes*, *Parashorea* and *Shorea*), Fabaceae (*Aldina*, *Dicymbe* and *Gilbertiodenron*), Fagaceae (*Colombobalans* and *Quercus*), Gnetaceae (*Gnetum*), Goodeniaceae, Juglandaceae (*Oreomunnea*), Myrtaceae, Nothofagaceae (*Fuscospora*, *Lophozonia* and *Nothofagus*), Nyctaginaceae (*Guapira*, *Pisonia* and *Neea*), Phyllanthaceae, Pinaceae (*Pinus*), Polygonaceae (*Coccoloba*), Salicaceae (*Salix*) and Sarcolaenaceae. Although the tropical region has higher plant species richness than the temperate region, some tropical forests are dominated by one tree species or tree species from the same family. These monodominant forests are well represented in Africa (Peh et al. 2011). Some important ectomycorrhizal fungi from tropical region belong to the *Clavulina*, *Russula*, *Lactifluus*, *Sebacina*, *Amanita*, *Laccaria*, *Lactarius*, *Leccinum* and *Boletus* genera, in which certain species seem to be Neotropical endemics (Halling and Mueller 1989, 1996, 1999, 2001; Vasco-Palácios et al. 2018; Nouhra et al. 2019). Despite that tropical region having ectomycorrhizal fungal lineages that also occurs in temperate region, some lineages seem to be absent or poor in tropical region likely because of geographical barriers and/or incompatible hosts, such as

/albatrellus, /endogone1, /hygrophorus, /paxillus-gyrodon, /piloderma, /suillus-rhizopogon and /tricholoma (Corrales et al. 2018).

Some ECMs from multidiverse forests (Table 1), as said, are different from the traditional ECM morphology, which in turn may also reflect a higher diversity of partners or a facultative association. In some of these associations, there are compensatory structures for the lack of Hartig net, such as transfer-cells. This peculiar anatomy may be less efficient than the Hartig net because the contact area between the partners is smaller (Allaway et al. 1985). There are other interesting findings in a study about ECMs occurring in tropical angiospermous trees: *Intsia*, *Melaleuca*, *Monotes*, *Neea*, *Tristania* and *Vateria* (Alexander and Hogberg 1986). *Uapaca kirkiana* and *Vateria indica* have different ectomycorrhizal types, some of them producing Hartig net and others not. Additionally, they showed that mantle structure are thicker in the tropical trees studied comparing to the mantle structure of temperate trees, what may be due to high carbon rate in the soil or provided by the host. Also, in this study, when the Hartig net was present, its development was restricted to the epidermal cells (Alexander and Hogberg 1986).

Table 1. ECMs from multidiverse forests found in nature.

Partners species	Anatomical features	References
Pisonia grandis-unknown fungus	Mantle; poor Hartig net development; transfer-cells	Ashford and Allaway 1982; Allaway et al. 1985
Monotes elegans-unknown fungus	Mantle; poor Hartig net development	Alexander and Hogberg 1986
Uapaca kirkiana-unknown fungus	Mantle; poor Hartig development or absent	Alexander and Hogberg 1986
Vateria indica-unknown fungus	Mantle; poor Hartig development or absent	Alexander and Hogberg 1986
Melaleuca leucodendron-unknown fungus	Poor or absent mantle; Hartig net present	Alexander and Hogberg 1986
Ceratopetalum apetalum-unknown fungus	Mantle; no Hartig net	Mcgee and Furby 1992

Pisonia grandis-basidiomycetes	Mantle; little or no Hartig net; transfer-cells	Cairney et al. 1994
Neea sp. 2-*Thelephora/Tomentella* *Guapira* sp.-*Thelephora/ Tomentella*	Mantle incomplete formation; No root hair suppression; transfer-cells	Haug et al. 2005
Citrus maxima-Phlebopus spongiosus	Mantle; rare or discontinuous Hartig net	Pham et al. 2012
Guapira opposita-Trechispora sp.	Mantle; no Hartig net	Vanegas-Léon et al. 2019
Guapira opposita-Amanita viscidolutea	Mantle; no Hartig net	Furtado et al. 2022

Besides saprotrophic-to-ectomycorrhizal transition, what are the other possibilities that explain these different anatomical features found in the tropics? Allaway et al. (1985), Haug et al. (2005) and Furtado et al. (2022) proposed that soil nutrient variation, recent plant family transition to an ectomycorrhizal status or a new type of mycorrhiza might explain why these different structures are formed in this ectomycorrhizal-like association. Despite those possibilities, we still lack data to test these hypotheses. Currently, different anatomical features are classified as ECM subtypes been called as pisonioid (transfer cells in Hartig net) and superficial (sparse mantle with no or poorly developed Hartig net) (Brundrett and Tedersoo 2018).

It is important to highlight that the current studies being made in the tropics, although very important, are likely not enough to show the real ectomycorrhizal fungal and plant diversity (Nouhra et al. 2019). The actual gaps of knowledge about ECM in tropical region makes it difficult to draw definitive conclusions. Taxonomic identifications of both symbionts (plant and fungus species) through sequencing ectomycorrhizal roots, sporomes and basidiomes from more sites are essential to elucidate the diversity and to understand their evolutionary status and phylogenetic history. However, in the study of Henkel et al. (2012) in Guyana, only 40% of the ectomycorrhizal fungi had sporomes. This may be a challenge to find new ectomycorrhizal associations in the tropical region. It is proposed that many symbiotic fungi have a distribution pattern similar to the associated plant (Sánchez-Ramírez et al. 2014), which can help in terms of orientation to future taxonomic studies, but this may not be a rule for multidiverse forests. Nevertheless, amplifying taxonomic studies worldwide by mycologists and botanists is extremely important. From these baseline studies, others can be produced to unveil physiological and genetic mechanisms of the ECM from multidiverse forests and finally understand how these associations are formed and how they function. Considering that ECM occurs mainly in forests, and tropical region have the largest diversity forests on the planet (Slik et al. 2015), it is likely that a large diversity of ectomycorrhizal species and different working mechanisms could be found in these regions impacting biodiversity maintenance at global level. For this reason, if we want to know the role of ECM in tropical ecosystems, we must study their association directly, not only derive data obtained from ECM found in temperate region.

Concluding remarks

Our study presented many theoretical evidence about the characteristics of ECM from multidiverse forests in the tropical region (Table 2). Here, we propose that these associations may exist in a continuum between a pure saprotrophic and a pure ectomycorrhizal lifestyle. However, we highlight that only studies aiming at the nutrient exchange, genome and transcriptome processes, added to taxonomic, phylogenetic and anatomical analysis of both partners in these associations might lead to more definitive conclusions. Studies focusing in ectomycorrhizal-like associations in tropical region are challenging due to many difficulties, such as lack of financial support, general interest and basic information. However, the tropical region may harbor important information about ECM that may elucidate or bring new questions about the complexity of these associations.

Table 2. Possible features differences between ECM from multidiverse forest and traditional ECM.

ECM from multidiverse forest	Traditional ECM
Missing, not fully formed or compensatory structures	Fully formed anatomical structures
Conservation of most saprotrophy-related genes	Loss of most saprotrophy-related genes

Generalist	Specialist
Facultative saprotrophy lifestyle	Obligate biotrophy lifestyle
Low-quality partners?	High-quality partners?

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5 CAPÍTULO 2 – FUNGI ISOLATION FROM ECTOMYCORRHIZAL ROOTS OF A RESTINGA NURSE PLANT

Ectomycorrhizal fungi isolation from a native nurse plant

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Summary

Ectomycorrhizae (ECMs) are mutualistic associations between plant and fungi. Although ECMs are present in tropical multidiverse forests, very little is known about them. Establishing a model to study ECMs from these areas could be an important step towards understanding not only ECMs from multidiverse forests, but ECMs in general. Exploring native ectomycorrhizal fungi and plants is the first step to understand the main functions of ectomycorrhizas and this knowledge can be used to ecosystem restoration. One of the most threatened ecosystems in Brazil is the restinga, which represent the coastal vegetation of the Atlantic Forest. The aim of this study was to isolate ectomycorrhizal fungi from roots of a native restinga nurse plant (*Guapira opposita*) and from basidiomes of possibly ectomycorrhizal partners of *G. opposita*. When we isolated fungi from the plant roots, these fungi showed structures similar to traps made by nematophagus fungi. This first analysis indicated that these fungi are not ectomycorrhizal, but sequencing is currently being performed to confirm their identity. In the same area of *G. opposita* population, we found basidiomes of known ectomycorrhizal genera: *Amanita* (*Amanita viscidolutea* and *Amanita* sp.), *Pisolithus* sp. and *Russula puigarii*. They are currently being isolated by our group, but their association with *G. opposita* needs to be further confirmed. Once our model is established, we intend to explore the function of ECMs to plant survival in stressful conditions.

Keywords: ectomycorrhiza; *Guapira opposita*; ectomycorrhizal fungi; restinga.

Introduction

Ectomycorrhizal fungi are symbionts that help the plant to survive in the environment. By enhancing the contact surface between plant and soil ectomycorrhizae (ECMs) improve the plant's capacity to absorb water and nutrients (Smith and Read, 2008). Moreover, the fungus protects the plant against salinity, drought, low and high temperatures and others (Guerrero-Galán et al. 2019). Ectomycorrhizal fungi are also capable of forming a common mycelial network (Simmard et al. 1997). These mycelial networks connect the plants in soil and enable nutrient exchange. The implications of these ecological functions may be essential to the maintenance and/or restoration of ecosystems. In restoration, ectomycorrhizal fungi may be effective to recover of eroded soils, soils contaminated with heavy metals and soils altered by fire (Policelli et al. 2020). Besides this, ectomycorrhizal fungi have the potential to recover environments invaded by exotic species of plants and fungi (Policelli et al. 2020). The use of native ectomycorrhizal fungi may improve soil quality and help native plants species during an exotic species invasion (Maltz and Treseder 2015). However, the little available information about ectomycorrhizae in the tropical region is a major barrier in the way of these applications.

One of the most threatened Brazilian ecosystems is the restinga, an ecoregion that has been suffering heavily with urbanization, real estate and tourism (World Wide Fund for Nature, 2021). Lack of knowledge about restinga biodiversity and ecology hampers efforts aiming to minimize socio-environmental impacts caused by its destruction. Studies to discover and understand restinga ecological dynamics are essential for recovery and maintenance of its biodiversity. Restinga is an ecosystem belonging to phytogeographic domain of Atlantic Forest. In this environment there are many plants communities growing in sandy and poorly developed soils (Falkenberg 1999). Many of these plants tolerate stressful conditions such as a soil lacking nutrients and water and with high salinity. In such a difficult environment, the first species to establish there are often facilitators (or nurse plants), that improve the local conditions so more sensitive species are able to grow. This process is very important to ecological succession and, consequently, in restoration (Castanho et al. 2015).

Recently, ectomycorrhizal associations were discovered in the restinga of Santa Catarina Island. Recent data shows that is the plant *Guapira opposita* (Vell.) Reitz (Nyctaginaceae) is able to form ECMs (Slodkowski 2020; Furtado et al. 2022). This plant species is characterized as tree or bush, occurring in Dense Ombrophilous Forest and in restinga (Reitz 1970) with morphoanatomical adaptations of its leaves to survive in shaded or illuminated environments (Aranda et al. 2011). *G. opposita* is also a nurse plant and, therefore, a main target for restoration projects. Studies show that there is a higher species richness and abundance below *G. opposita* when comparing to open restinga areas (Dalotto et al. 2018), which means that *G. opposita* has an important function to plant community and, consequently, to the entire ecosystem.

Despite the occurrence of ectomycorrhizae in restinga, very little is known about ectomycorrhiza diversity, physiology and ecology in these environments. It is essential to discover if there are other ectomycorrhizal plants and fungi species and who their partners are. With this knowledge, it is possible to use native ectomycorrhizal fungi as a way of restoring the site with native species in the most effective and quick way possible (Kardol and Wardle 2010; Policelli et al. 2020). The use of fungal inoculation in mycorrhizal nurse plants is an approach that may be very efficient to ecosystem restoration (Weidlich et al. 2020).

To study ectomycorrhizal associations, the isolation of fungi and the establishment of study models is an important step that can help us to uncover the benefits and importance of this association. The main aim of this study was to identify ectomycorrhizal presence in *G. opposita* and to isolate the fungi from *G. opposita* roots and from the basidiome of some possible ectomycorrhizal fungi.

Material and Methods

Fruit collection and seedling bank

G. opposita fruits were collected from a *G. opposita* population in Lagoa do Peri City Park in Florianópolis, Santa Catarina. The fruits were stored in a refrigerator in the Plant Physiology Laboratory at UFSC-Florianópolis. Seeds were removed from the fruits at the time of planting. Some plants were cultivated in restinga soil, while others were placed in substrate containing commercial a mixture of fertilized soil and vermiculite (1:1). The plants grew in a greenhouse.

This research at Municipal Park of Lagoa do Peri has authorization from the City Environmental Foundation of Florianópolis - FLORAM (Nº 017/2018 - DEPUC).

Presence of ectomycorrhizae and isolation of ectomycorrhizal fungi from mycorrhizal roots

G. opposita seedlings that grew in restinga substrate were taken to Laboratory of Microorganisms Promoting Plant Growth at UFSC-Curitibanos, where the sorting was made for identification of ectomycorrhizas in the roots through a stereomicroscope. Cross-sections were made to observe the presence of the mantle and Hartig net.

Ectomycorrhizal roots were cut into fragments of, approximately, one centimeter. In disinfestation phase, the roots were left in 90% alcohol for one minute, in bleach (2.5%) for two minutes and washed six times with sterilized distilled water (adapted from Gardner and Botelho 2020). The root tips were then transferred to culture media of different formulations. These culture media were GYP “Sabouraud”, Priddam-Gotilieb modified (PGKM), Mellin-Norkrans modified (MNM) and potato dextrose agar (BDA) (Andreazza 2006; Gardner and Botelho 2020; Botelho 2020).

The fragments were placed individually on each Petri dish containing the culture mediums. The Petri dishes were divided and incubated in two stoves with different temperatures: 23ºC with photoperiod and 26.3ºC without photoperiod.

Collection of fungi in the restinga

During the months of January to March, basidiomes were collected in restinga, in the same area of *G. opposita* populations. Tweezers, compartmentalized boxes and notebooks were used during collection. The collections were performed mainly after periods of heavy rain interspersed during these months, a condition that favors the formation of mushrooms. After this, the mushrooms were marked and stored in a refrigerator to conserve the structure, that will be used to isolate the fungi.

Results and Discussion

A seedling bank of *G. opposita* was established in the greenhouse (Figures 1 and 2). These plants will be used in future studies.

A morphotype of ectomycorrhiza was identified in the roots of plants cultivated in restinga soil (Figures 3 and 4) that we attempted to isolate. Mycelial growth from isolated root tips with ectomycorrhiza was monitored daily and, after four days, it was possible to observe similar mycelial development in five replicas with BDA, GYP "Sabouraud" and MNM culture medium (Figures 5-8), mainly in temperature of 26,3 ºC. No growth was observed in the PGKM culture medium. Despite mycelial growth, the culture medium showed signs of contamination by other microorganisms. In these cases, the fungi were purified by transferring a healthy non-contaminated part of the mycelium to a new medium (Figures 9-14). Despite mycelial growth in several dishes, the characteristics observed in some cultures, such as structures similar to traps produced by nematophagous fungi (Figure 15; Padilha 1996), may indicate that they are not ectomycorrhizal. However, genetic sequencing analysis could give us a more precise identification of these fungi and their inoculation with the plant could definitely confirm ectomycorrhizal status. These analyses will be performed in the future.

Basidiomes belonging to known ectomycorrhizal genera (Chambers and Cairney 1999; Hess et al. 2018; Geml et al. 2010) were found in our area of interest, such as *Amanita* (*Amanita viscidolutea* and *Amanita* sp.), *Pisolithus* sp. and *Russula puigarii*. Recently, it was discovered that *Amanita viscidolutea* is able to associate with *G. opposita* in the restinga (Furtado et al. 2022). *Amanita* sp. and *Russula puigarii* may be ectomycorrhizal partners of *G. opposita* as well, because they were found close to *G. opposita* individuals. Besides this, further studies such as the inoculation of these fungi in *G. opposita* seedlings may result in the ectomycorrhizal formation between these partners.

The next step is to analyze if the isolated fungi are ectomycorrhizal partners of *G. opposita*. This can be made inoculating the fungus in sterilized soil with the plant. If it is confirmed that *G. opposita* associates with the isolated fungi, we have a model to study the functioning, the structure and the impacts of this association in plant fitness under a series of conditions. These results could pave the way to new restoration strategies and to a better understanding of how restinga communities assemble.

Figures 1-2. *G. opposita* seedling bank in greenhouse.

Figure 3. *G. opposita* ectomycorrhiza (darker part of the root).

Figure 4. Microscopic visualization of mantle structure from *G. opposita* ectomycorrhiza.

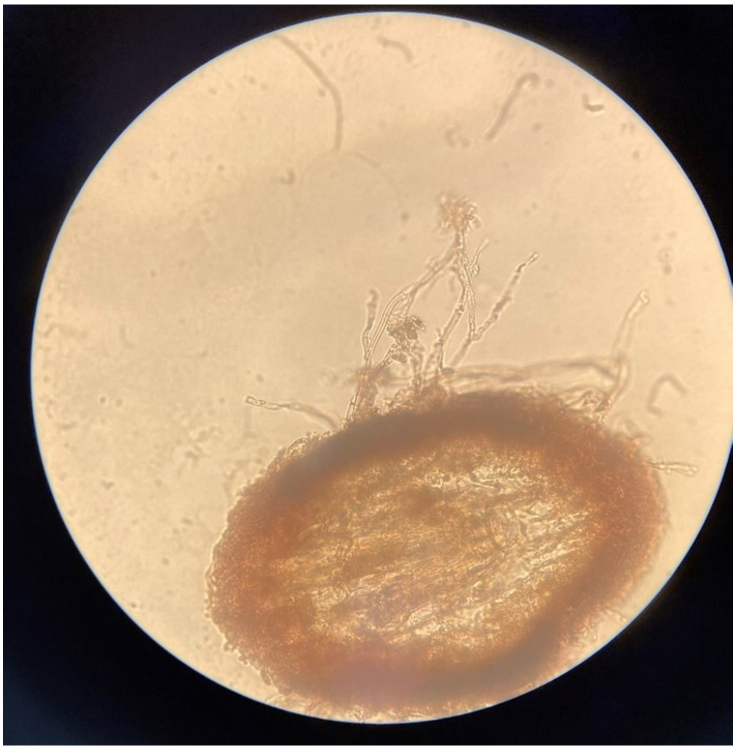


Figura 5. Microscopic visualization of a fungus growing from *G. opposita* mycorrhizal root.

Figure 6. Growing fungus in BDA culture with *G. opposita* mycorrhizal root.

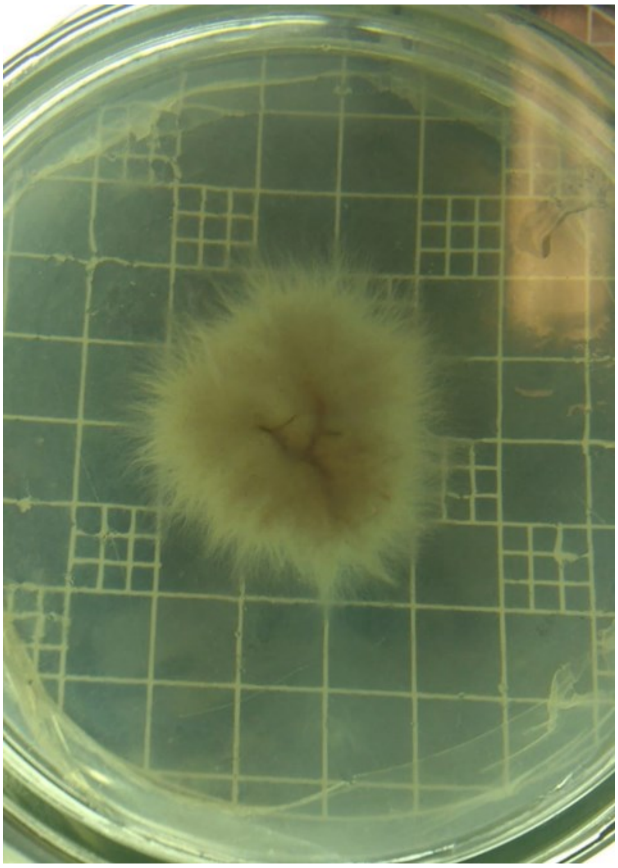


Figure 7. Growing fungus in GYP “Sabouraud” culture with *G. opposita* mycorrhizal root.

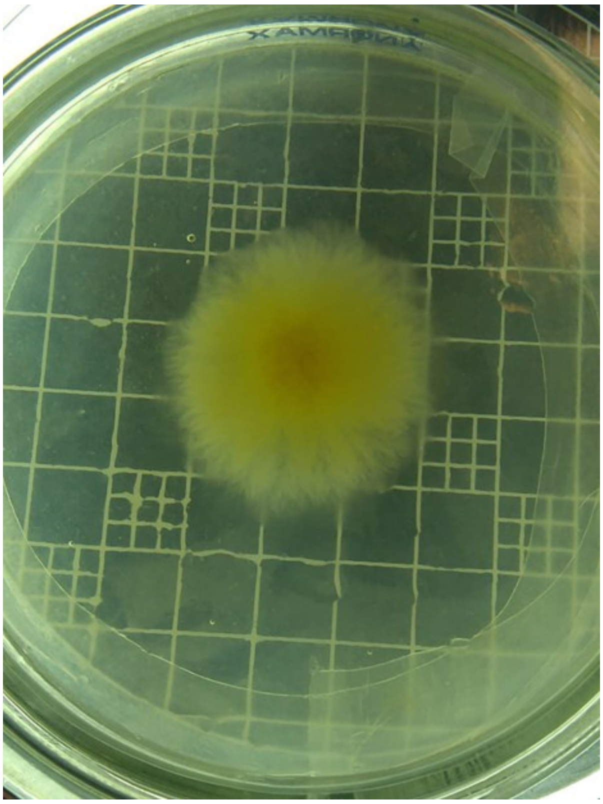
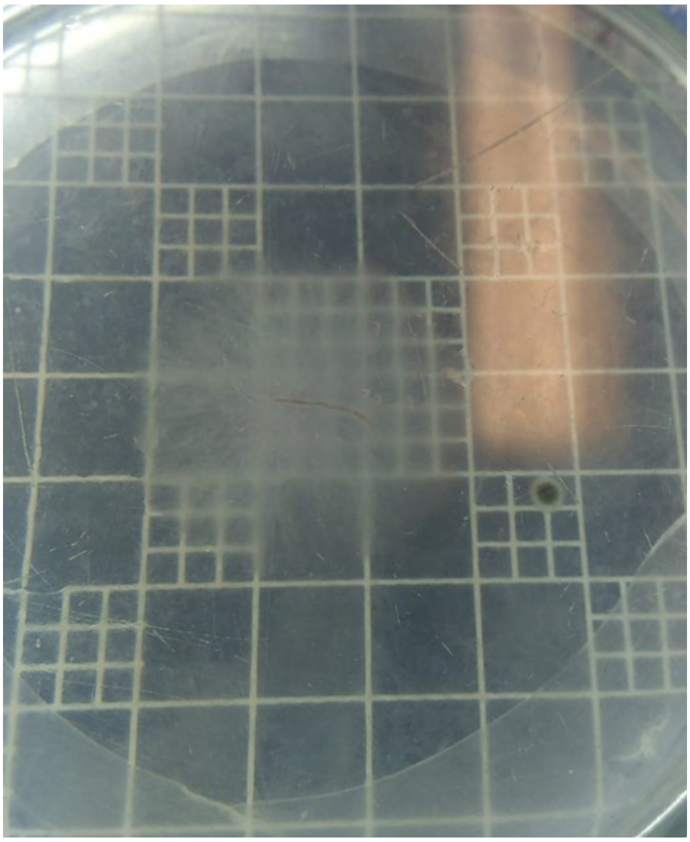


Figure 8. Growing fungus in MNM culture with *G. opposita* mycorrhizal root.



Figures 9-14. Fungal cultures transferred to a fresh medium.

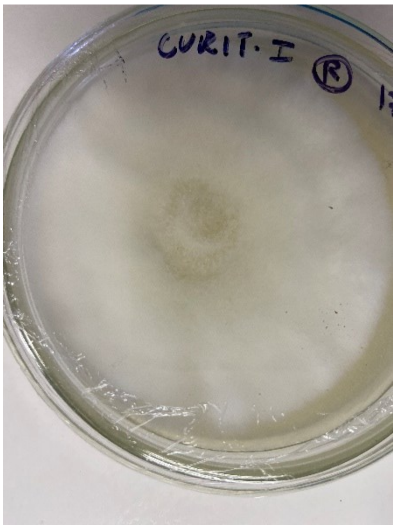


Figure 15. Microscopic structures of isolated fungus from *G. opposita* roots.

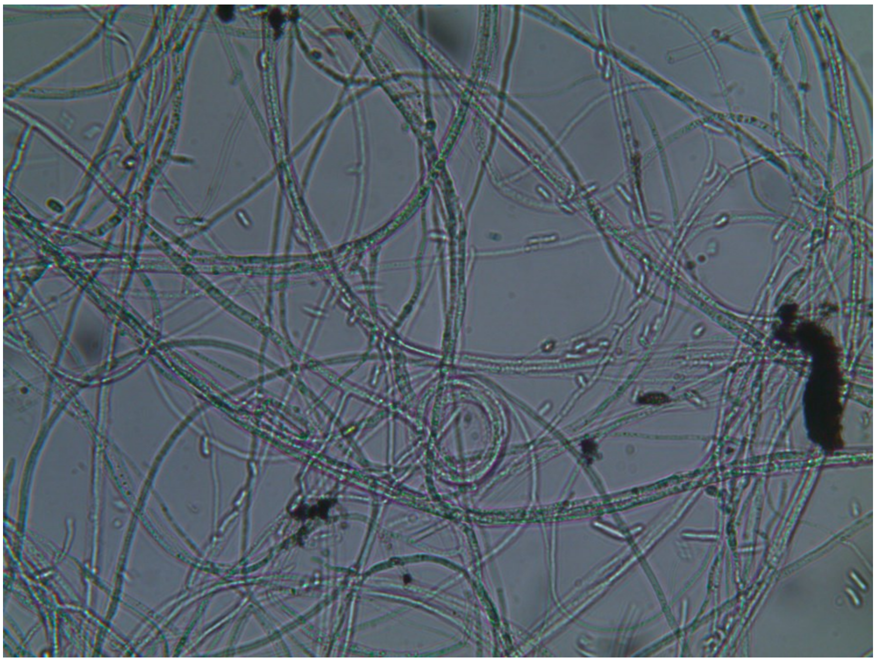


Figure 16. *Amanita* sp. in restinga soil at Lagoa do Peri.

Figures 17-18. *G. opposita* fruiting and flowering in restinga (February 2021 and February 2022 respectively).

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6 CONCLUSÃO

Considerando as ectomicorrizas tradicionais das regiões temperadas e boreais, as ectomicorrizas de região tropical têm grande potencial de exploração. A história evolutiva das ectomicorrizas tradicionais possui relação com a região tropical. As ectomicorrizas de florestas multidiversas podem trazer respostas e/ou novas questões sobre essa associação extremamente complexa. As evidências trazidas neste estudo podem servir como direcionamento para estudos futuros, além de levantar discussões sobre como classificar essas associações e quais os seus papéis ecológicos. A evolução dos fungos ectomicorrízicos é muito complexa, o que devemos sempre considerar em pesquisas futuras. Entender essa complexidade e a sua ocorrência em locais antes descartados, como a região tropical, pode aumentar significativamente o conhecimento sobre as ectomicorrizas.

Devido a pandemia de COVID-19 não foi possível realizar os próximos passos da pesquisa em laboratório como a inoculação do fungo isolado em *G. opposita* para testar a formação de ectomicorriza e, posteriormente, a resistência da planta à salinidade de acordo com o projeto inicial apresentado. Entretanto, apesar das dificuldades impostas pela pandemia, algumas atividades exercidas durante as práticas laboratoriais como a utilização de novos protocolos, estabelecimento de banco de plantas e coleta de fungos possivelmente ectomicorrízicos, auxiliarão estudos paralelos e futuros que buscam entender o papel das ectomicorrizas em *G. opposita* na restinga. Essa pesquisa é inicial e mostrou o tanto que ainda é preciso ser feito para alcançar os objetivos inicialmente estabelecidos, além de ter sido um passo muito importante de aprendizado teórico e prático. As dificuldades inesperadas, devido à pandemia, auxiliaram num maior entendimento e busca pelo que foi inicialmente apresentado, pois tentou-se diversas alternativas para que o que foi proposto fosse realizado. Espera-se que num futuro não tão distante essa pesquisa seja realizada da forma como deveria e que possa agregar conhecimento sobre as ectomicorrizas na restinga, a qual é uma área de pesquisa muito escassa de informações dado o seu enorme potencial de exploração e de importantes resultados.