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A Review on Molecular Mechanisms Underlying Mycorrhizal Associations in Orchids

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Abstract

The present review provides insights into the molecular aspects of mycorrhizal associations in orchids. Orchids rely on mutualistic associations with mycorrhizal fungi for successful germination, development, and survival. Orchid mycorrhiza refer to the mutualistic association between orchids and specific fungi that colonize their roots and provide essential nutrients. These fungi are often highly specific to a particular orchid species. This symbiotic relationship supports orchids in overcoming their reliance on external nutritional sources during their initial life stages. The fungal partner provides essential nutrients, such as nitrogen, phosphate, and trace elements. This association extends beyond seedling growth, with mycorrhizae aiding mature orchids, particularly in nutrient-poor environments. Different orchid species exhibit varying degrees of selectivity for their fungal partnerships. The composition of mycorrhizal fungi varies significantly among different orchid species, indicating that specific fungal partners may be essential for the growth and survival of particular orchid taxa. Molecular techniques, such have been employed to analyze the mycobiome associated with orchids, revealing a diverse array of fungal taxa, including Tulasnellaceae and Ceratobasidiaceae, which are predominant in many orchid species. Recent review has focused on mycorrhizal associations in orchids and put insight into molecular analysis of fungal partner.

Keywords: Mycorrhiza, Orchids, Symbiotic association, Molecular analysis, fungal partner

1. Introduction

Orchids are the second-largest floral family members after Asteraceae, with nearly 25,000 to 35000 species present worldwide (Chase et al., 2015; Christenhusz and Byng, 2016; Attri and Nayyar, 2021; Govaerts, 2024; Freudenstein, 2024). It represents one of the most diverse and evolutionarily successful plant families and their survival hinges on a unique biological trait: an obligate dependence on mycorrhizal fungi during their lifecycle. This symbiosis begins at the seed germination stage, as orchid seeds lack endosperm and rely entirely on fungal partners for carbon and nutrients (Rasmussen and Rasmussen, 2009). This symbiotic exchange is the concept of mutualism, in which plants benefit from the direct uptake of water and nutrients (specifically phosphorus) from the fungi and defend against biotic and abiotic stresses, while the host plants provide and supply carbon from the photosynthesis process (Watkinson, 2016; Li T et al., 2021). The mycorrhizal interactions are signified by the evolutionary history of fungal symbionts and host plants (Rasmussen, 2015). Impressively, OM is regarded as more favorable to orchids than fungi, which depends on other substrates, such as saprotrops or endophytes of non-orchid roots (Voronina et al., 2018). Thus, the relationship between orchids and fungi is reasonably summarized as a mutual annuity based on several asymmetrical processes (Harding, 2016; Taylor and Scharlin, 2004). Besides, this family is not only regarded as the best exemplified group for studying the biodiverse and evolutionary interactions (Alfaro Pinto, 2022), but observed to protect endangered plants throughout the world (Liang et al., 2024). Recent advances in molecular biology and genomics have unveiled the complexity of these interactions, revealing conserved signaling pathways, nutrient exchange mechanisms, and evolutionary adaptations that underpin orchid-fungal relationships (Dearnaley et al., 2016; Fochiet al., 2020). This review synthesizes current knowledge on the molecular mechanisms driving orchid mycorrhizal associations, emphasizing their ecological and evolutionary implications.

1.1. Orchids with Unique Symbiotic Dependencies

Fungal symbionts are necessary as they provide carbon and minerals to dust-like or reserved-less orchid seeds during the initial stage of development (Yeh and Chung et al., 2019). Initially, the plant develops into a spheroid organism, which is achlorophyllous and is called a “protocorm” in most terrestrial species that later develops into roots and shoots. This dependency continues on fungal partner until the adult stage, albeit to different degrees (Li T and Yang et al., 2021; Whigham et al., 2008) . According to their dependency on fungal carbon, adult orchids are classified into three types (Rasmussen, 2015). Autotrophic (AT) that depend on fungi in early generative stage for carbon requirements but as the photosynthetic apparatus develop it reduce the dependency on fungi; (Brundrett, 2004). mycoheterotrophic (MH) the orchids that remain achlorophyllus and never do photosynthesis to get carbon compounds, they are completely reliant on fungal carbon throughout their life (Das and Varma,2009).) mixotrophic (MX) orchids that can obtain carbon compounds from photosynthesis and at adult hood (Hynson and Madsen et al., 2013). The concept of AT orchids as such is still debatable; the isotopic shift to the surrounding autotrophic plants with regard to 13C, 15N, and 18O, and 2H abundance from the fungal partners proves that AT orchids obtain some fungal biomass (Allen 2022; Figueiredo et al., 2021; Světlíková 2018; Zahn 2024).

Nevertheless, the net flow, bearing in mind the possible reverse movement from orchid to fungus, is unknown in the majority of green orchids (referred to earlier as AT) and, although less in comparison with the inverse flow, there is C flow from fungus to orchids, which is described as being ancient (Hynson and Madsen et al., 2013).

The scientific community has been concerned on orchids-fungi biology interactions, because of researches made by Noël Bernard's pioneering on the symbiotic germination of orchids at the last portion of nineteenth century (Li T Yang et al., 2021). At germinating seed stages, the fungus obtains colonization by both trichomes and suspensor cells (Li T Yang et al., 2021; Stern, 2014). while at adult stage, it is through root velamina and cortical cells (Stern, 2014). and also has intracellular coiled fungal hyphae called pelotons which are considered structural for exchange during their life or at their death for C transfer to the plant (Dearnaley et al., 2016). Comprehensively analysis of fungal diversity provides a more understanding of the plant-fungal interaction framework. Some of the early culture-based methods were unable to accurately identify different isolated strains because (i) many species are non-culturable and (ii) taxonomic differentiation of morphologically similar fungi is complicated; for instance, Serendipita vermifera (Li T Yang et al., 2021; Selosseet al., 2021).However, the advancement in the molecular ecology over the last decade with Sanger sequencing and high-throughput sequencing (HTS) technologies have vastly enhanced the ability of characterizing plant microbiota. Furthermore, the use of these technologies to study the orchid-fungal symbiosis was helpful also in understanding the diversity, community structures, patterns and molecular mechanisms of the symbiosis. Several orchid associated fungi belonging to at least 200 genera are known as identified through the criticism along with discussion of Orchid-associated fungi under the aegis of different biotic and abiotic factors comprises of developmental stages, habitats or spatiotemporal scales (Hossain,2022; Lin T Yang et al., 2021; Shamsudin et al., 2024; Zhang et al., 2023).To facilitate an great understanding of the diversity and ecological gesture of orchid-fungal associates and to stimulate the resource conservation and regeneration of rare or endangered orchids with excessive research or commercial potential, this article outline six research modules: the practical approaches establish on the research of orchid-fungal diversity, molecular tools for HTS, the specificity of fungal communities in orchids and their differences in various habitats, comparison of OMF in the orchid roots and the soil, the spatiotemporal patterns of the OMF. Therefore, this review offers direction towards enhanced understanding of this field.

Orchids are renowned for their extraordinary floral diversity and specialized pollination systems, often involving co-evolution with specific pollinators .However, their most remarkable adaptation lies beneath the soil: an obligate relationship with mycorrhizal fungi. Orchid seeds, which are dust-like and lack nutritional reserves, cannot germinate without fungal-derived carbon, a condition termed "mycoheterotrophy". Even photosynthetic adult orchids often retain partial dependence on fungi for nutrients like nitrogen and phosphorus. This dependency makes orchids ideal models for studying plant-fungal symbiosis, particularly the molecular "dialogue" that enables nutrient exchange while suppressing plant immune responses (Selosseet al., 2022).

Recent studies highlight the ecological vulnerability of orchids, as disruptions to their fungal partners—often triggered by habitat loss or climate change—can lead to population declines (Swarts et al., 2010). For example, terrestrial orchids like *Cypripedium* and *Ophrys* require specific *Tulasnella* or *Rhizoctonia* fungi for germination, and mismatches in these partnerships threaten their survival (Jacquemynet al., 2021). Understanding the molecular basis of these interactions is thus critical for conservation strategies.

1.2. Orchid Mycorrhizae: A Distinctive Symbiotic System

Orchid mycorrhizae differ markedly from the arbuscular (AM) or ectomycorrhizal (ECM) systems seen in other plants. Unlike AM fungi, which penetrate root cortical cells, orchid mycorrhizal fungi form intracellular pelotons—coiled hyphal structures within root cells—that facilitate nutrient transfer (Favre-Godal; Quentin et al., 2020). These fungi belong primarily to Basidiomycota, including genera such as *Rhizoctonia*, *Tulasnella*, and *Sebacina*, with varying degrees of host specificity (Dearnaley et al., 2012). For instance, tropical epiphytic orchids often associate with *Ceratobasidium*, while temperate terrestrials favor *Tulasnella* (Martos et al., 2012).

The symbiosis progresses through distinct stages:

1. **Seed Germination**: Fungal hyphae penetrate the embryo, supplying sugars like trehalose and glucose to fuel protocorm development
2. **Adult Association**: In photosynthetic orchids, the relationship shifts to a mutualistic exchange, where fungi receive photosynthates while aiding nutrient uptake. However, some orchids (e.g., *Neottia*) remain fully mycoheterotrophic, exploiting fungi as carbon sources throughout their lives (Merckx et al., 2013).

Recent genomic studies reveal that orchids have retained ancient symbiotic genes while evolving novel mechanisms to regulate fungal colonization. For example, the *RAM2* gene, involved in lipid biosynthesis in AM symbiosis, is conserved in orchids but co-opted for interactions with *Rhizoctonia* (Gutjahr et al., 2012; Zhang et al., 2017). Such findings underscore the evolutionary plasticity of orchid-fungal partnerships.

2. Seasonal Fungal Dynamics in Orchid Root

Fungi are crucial to orchids and the characteristics of microhabitat fungal groups related to C. sieboldii. The orchid roots, rhizoplane soil, and rhizosphere soil all had similar, but not similar, dominant fungi in the root microhabitats of C.

sieboldii (Huang et al., 2022). At the same time, the fungi were near the orchid roots, and extra symbiotic fungi were present. The fungal communities near the orchid roots exhibited seasonal variation, whereas the interannual difference in the fungal communities far away from the orchid roots was more visible (Dearnaley and Selosse,2016 ; Huang et al., 2022). This seasonal variation is caused by the species' capacity to self-balance and balance at each life stage to build the best utilization of available resources. Since root fungal communities differ seasonally rather than annually, almost identical research conducted in orchid microhabitats, sampling at dissimilar times of the year is obligatory to reduce the possibility of error. The study is restricted to the state of fungal communities at a solo position and in an identical habitat (Huang et al., 2022).

Orchid mycorrhizal associations range from highly specific to broadly generalist. For instance, *Ophrys* orchids often associate with a single *Tulasnella* species, while *Dendrobium* species can form partnerships with multiple fungal genera (Jacquemynet al., 2021). This flexibility may reflect evolutionary adaptations to diverse ecological niches.

Recent genomic studies suggest that orchids have evolved mechanisms to regulate fungal colonization, balancing specificity with adaptability. For example, the *RAM2* gene, involved in lipid biosynthesis, is conserved in orchids but co-opted for interactions with *Rhizoctonia* (Leng and Chunyanet al., 2024; Silvia, 2024).

It is not viable to assess the applicability of such dynamic patterns on a broader scale, and in-depth studies at numerous sites with identical species over an extensive period of time may be required. Moreover, utilize a series of inoculation tests to inspect the growth result of various superior fungi on orchids and their useful traits would be a predominant step with regard to understanding orchid conservation and expansion (Dearnaley and Selosse, 2016).

3. Molecular Insights

This review focuses on deciphering the molecular machinery governing orchid-mycorrhizal symbiosis, a field revolutionized by advances in omics technologies including Signaling Pathways, Nutrient Transporters and Immune Suppression.

Orchids employ receptor-like kinases (RLKs) and phytohormones to recognize and accommodate fungal partners. For instance, strigolactones, known for their role in AM symbiosis, are also implicated in orchid-fungal signaling (Balzergueet al., 2020). SWEET sugar transporters and ammonium transporters (AMTs) mediate bidirectional nutrient exchange (Perotto et al., 2021).Orchids downregulate defense-related genes (e.g., *PR1*, *PAL*) during symbiosis, a process regulated by micro RNAs like miR171 (Xu et al., 2020).

Emerging research also explores the role of small secreted proteins (SSPs) from fungi in manipulating host physiology. For example, *Rhizoctonia* SSPs suppress orchid immune responses by interfering with jasmonic acidsignaling (Huang et al., 2022). Additionally, transcriptomic analyses of *Dendrobium* and *Phalaenopsis* have identified symbiosis-specific genes, such as those encoding lectins and chitinases, which modulate fungal colonization Understanding these mechanisms has practical implications. For instance, synthetic seed germination protocols using fungal exudates are now employed in orchid conservation (Brundrett, 2002). Similarly, CRISPR-based editing of symbiotic genes could enhance orchid resilience in degraded habitats (Song and Cheng et al., 2022). However, challenges persist, such as the recalcitrance of many orchid-fungal pairs to lab cultivation

This review emphasizes the need for integrative approaches—combining genomics, metabolomics, and experimental ecology—to unravel the "black box" of orchid-fungal interactions. By synthesizing recent discoveries, we aim to catalyze innovative solutions for orchid conservation and sustainable cultivation.

3.1. Primer Selection InHts (Table 1)

The molecular mechanisms underlying orchid-mycorrhizal interactions have been a focal point of research in recent years, driven by advances in genomics, transcriptomics, and metabolomics. These studies have revealed a complex interplay of signaling pathways, nutrient exchange mechanisms, and immune modulation strategies that enable orchids to establish and maintain symbiotic relationships with their fungal partners. Below, we delve into the key molecular aspects of these interactions, emphasizing the latest findings and methodologies used to study them.

In fungal research, various PCR primers for the rRNA locus, particularly ITS, were used in the first OM-diversity studies (Hossain et al., 2013; Laufer2011; Li T Yang et al., 2021). Thus, the presence of an accelerated rDNA sequence hinders the amplification of one of the most abundant OMF taxon (Jasingeet al., 2018), the family Tulasnellaceae, and leads to the design and application of taxa-specific OM PCR primers.

Taylor and McCormick first emerged specific primers ITS1-OF and ITS4-OF for studying OM diversity, which amplifies the full ITS region fungi of Basidiomycete including Tulasnella species (Taylor et al., 2008). This set has been broadly used for OM identification through cloning and sequencing (Kaur and Sharma, 2019; Alderton-Moss, 2024). Another commonly used primer is ITS4Tul by Taylor (1997), which enables identification of Tulasnella diversity in addition to ITS1 or ITS5 primers (Kaur and Sharma, 2019; Shamsudin and Seelan et al., 2024). Later, an in silico analysis purpose that ITS3/ITS4OF and ITS86F/ITS4 primers, select the ITS2 sub-region of ITS, were the best suited for orchid root samples (Li T Yang et al., 2021), although if they are not always effective on soil samples. Moreover, because ITS2 can construct extra operational taxonomic units (OTUs) and higher phylogenetic abundance than the other sub-region ITS1, numerous researchers have used it for fungal identification through HTS platforms (Ponchart, 2019). Thus, in the last five years, ITS3/ITS4OF has often been employed for the detection of mycorrhiza-associated fungal communities in the roots of terrestrial orchids and adjacent soils (Shamsudin and Seelan et al., 2024). Furthermore, ITS86F / ITS4 is still used for the identification of mycorrhizal partners in epiphytic orchids (Waud and Busschaertet al., 2016).Specifically, the mismatching ITS4OF was observed to match the ITS of only 64% of Tulasnellaceae; several instances of mismatching were seen in other assemblies belonging to Basidiomycota and Ascomycota. Similarly, (Fernández and Sharma 2023). labeled the modified primers ITS1ngs, ITS1Fngs, and ITS4ngs for ITS86F and ITS1F, which were mismatched in 83% of Tulasnellaceae to ITS4Tul2 of the entire ITS region. The divergent patterns of AMT complied with most of the reported mycorrhizal communities of orchids (including 97% of the Tulasnellaceae). In recent years, a newly synthesized primer has been used to accurately measure the concentration of crystals from polyurethane melts that cannot be dissolved in water. By combining 8S-OF with two varieties of ITS primers, ITS 4OF and ITS 4Tul, in OMF, the success rate was the most satisfactory (Fan and Mangru et al., 1999). The principal mycorrhizal partners of orchids are members of Tulasnellaceae belonging to the order Cantharellales (Oberwinkleret al., 2017).Mainly, orchids such as clades A and B associated with these mycorrhizal fungi Tulasnellaceae have been found to be rather diverse and difficult to amplify, especially in the case of clade B(Shamsudin and Seelan et al., 2024), and neither amplifies with the general primers nor specific primers meant for Tulas Still in line with the UNITE database of molecular identification of fungi, where the authors acknowledged that Tulasnellaceae belongs to clade A, seemingly constituted for 3/4 majority; however, due to the acknowledged bias and assistance from the dominance made through primers and sampling that primarily includes sequences from the northern hemisphere, Tulasnellaceae clade B could be a little underrepresented, as it has been noted to be as frequent as clade A (Fernández and Sharma, 2023; Hossain, 20221;Shamsudin and Seelan et al., 2024).Thus, future research that seeks to examine ORF/OM heterogeneity should carefully select primers and assess the existence or otherwise of the associated bias.

One might be aware that there will be no such ideal primer set available, and thus, one may want to use several of the available primer sets while designing the study. From this and a series of works by our research team (manuscript in preparation), we have evidence of FOR using multiple pairs of primers with minimal overlap in the amplified regions. Using multiplex PCR, they can be accumulated and incorporated together with the nested PCR amplification method to detect a greater number of orchid mycorrhizal partners (Fernández and Sharma 2023).This study identified the efficiency of three primer pairs, ITS1ngs-ITS4ngs, ITS1Fngs-ITS4ngs, and ITS1-ITS4Tul2, which should be used for 454 pyrosequencing (Li T Yang et al., 2021 ; Selosse and Petrolliet al., 2022). PCR products can be further subjected to nested PCR amplification using ITS86F-ITS4 and ITS86F-ITS4Tul primers, targeting approximately 463 bp fragments, which can capture the large diversity of mycorrhizal fungi in association with 72 varieties of tropical epiphytic orchids grown in the wild. Third-generation PacBio Sequel sequencing required the following optimized primer sets: ITS1ngs-TW14ngs, ITS1Fngs-TW14ngs, and ITS1-ITS4Tul2 (primer sequences are provided in Supplementary Table S1). In the future, shotgun sequencing of roots, which generates no PCR amplification while a fungus is visible, might reveal the identification of clades that cannot be detected with any primer present, if at all (Rasmussen, 2015).

Table 1 S1 Primers used for high-throughput sequencing of orchid mycorrhizal partners.

Primer name	Sequence (5'-3' direction)	Reference
Modified ITS1ngs	TCCGTAGGTGAACCTGC	(Fernández and Sharma, 2023).
Modified ITS1Fngs	GGTCATTTAGAGGAAGTAA	(Fernández and Sharma, 2023).
ITS1	TCCGTAGGTGAACCTGCGG	(Lee and You, 2000).
Modified ITS4ngs	TCCTSCGCTTATTGATATGC	(Fernández and Sharma, 2023).
ITS4Tul2	TTCTTTTCCTCCGCTGAWTA	(Fernández and Sharma, 2023).
ITS1F	CTTGGTCATTTAGAGGAAGTAA	(Matsubara and Yoneda, 2012)
ITS86F	GTGAATCATCGAATCTTTGAA	(Waudand Busschaertet al., 2014).
ITS4	TCCTCCGCTTATTGATATGC	(Lee and You,2000).
ITS4Tul	CCGCCAGATTCACACATTGA	(Shamsudinet al., 2024)

TW14ngs	CTATCCTGRGRGAAAYTTC	(Shamsudin et al., 2024).
NS1	GTAGTCATATGCTTGTCTC	(Lee and You,2000)
NS4	CTTCCGTCAATTCCTTTAAG	(Lee and You, 2000)

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4. Fungal Recognition and Signaling Pathways

Orchids employ sophisticated molecular mechanisms to recognize and accommodate their fungal partners. The initial stages of symbiosis involve the exchange of chemical signals that facilitate fungal colonization.

5.1 Receptor-Like Kinases (RLKs): Orchids utilize RLKs to detect fungal-derived molecules, such as chitin oligosaccharides. For example, the *Dendrobium officinale* genome encodes several RLKs that are upregulated during mycorrhizal colonization (Zhang et al., 2017). These receptors activate downstream signaling cascades, including calcium spiking and mitogen-activated protein kinase (MAPK) pathways, which are essential for symbiotic establishment (Gutjahr et al., 2012).

5.2. Strigolactones: These plant hormones, known for their role in AM symbiosis, are also implicated in orchid-fungal signaling. Strigolactones exuded by orchid roots stimulate hyphal branching in *Rhizoctonia* fungi, enhancing the likelihood of symbiotic contact (Miuraand Chihiro et al., 2024).

5.3. Small Secreted Proteins (SSPs): Fungal SSPs play a crucial role in modulating host responses. For instance, *Rhizoctonia* SSPs suppress orchid immune responses by interfering with jasmonic acid signaling, thereby facilitating fungal colonization (Huang et al., 2022).

6. Nutrient Exchange Mechanisms

Nutrient exchange is the cornerstone of orchid-mycorrhizal symbiosis, with fungi providing carbon, nitrogen, and phosphorus to orchids, particularly during the early stages of development.

6.1. Carbon Transfer: Orchid seeds lack endosperm and rely entirely on fungal-derived carbon for germination. Fungi supply sugars like trehalose and glucose, which are transported into orchid cells via SWEET sugar transporters (Favre-Godal and Quentin et al., 2020).Transcriptomic studies in *Dendrobium catenatum* have identified several SWEET genes that are upregulated during mycorrhizal colonization .

6.2. Nitrogen and Phosphorus Uptake: Fungi enhance orchid access to nitrogen and phosphorus by decomposing organic matter and solubilizing inorganic phosphates. Ammonium transporters (AMTs) and phosphate transporters (PTs) in orchid roots facilitate the uptake of these nutrients For example, *Phalaenopsis* orchids express specific AMTs and PTs during symbiosis, ensuring efficient nutrient acquisition (Zhang et al., 2018).

6.3. Lipid Transfer: Recent studies suggest that lipids may also play a role in nutrient exchange. The *RAM2* gene, involved in lipid biosynthesis, is conserved in orchids and is essential for symbiotic interactions with *Rhizoctonia* (Gutjahr et al., 2012).

7. Genetic and Transcriptomic Insights

Advances in genomics and transcriptomics have provided unprecedented insights into the molecular basis of orchid-mycorrhizal interactions.

7.1. Genomic Studies: The genomes of several orchids, including *Phalaenopsis equestris* and *Dendrobium officinale*, have been sequenced, revealing genes involved in symbiotic interactions. For example, the *Dendrobium* genome contains a suite of symbiosis-specific genes, including those encoding lectins and chitinases, which modulate fungal colonization.

7.2. Transcriptomic Analyses: RNA sequencing (RNA-seq) has been used to profile gene expression during mycorrhizal colonization. In *Serapias vomeracea*, transcriptomic analysis revealed the upregulation of genes involved in nutrient transport, hormone signaling, and immune modulation during symbiosis (Perotto et al., 2021). Similarly, in *Dendrobium catenatum*, RNA-seq identified key genes involved in carbon and nitrogen metabolism that are regulated by mycorrhizal fungi

7.3. CRISPR-Cas9 and Functional Genomics: CRISPR-Cas9 technology has been employed to study the functional roles of symbiotic genes. For instance, knockout of the *RAM2* gene in *Dendrobium* disrupted lipid biosynthesis and impaired mycorrhizal colonization, highlighting its importance in symbiosis (Zhao et al*.,* 2021).

8. Hormonal Regulation

Phytohormones play a central role in mediating orchid-mycorrhizal interactions, balancing symbiotic establishment with immune responses.

8.1. Auxins and Cytokinins: These hormones regulate root development and fungal colonization. Auxins promote the formation of pelotons, while cytokinins modulate nutrient exchange (Fochiet al., 2017).

8.2. Jasmonic Acid (JA): JA signaling is suppressed during symbiosis to prevent immune responses against fungal partners. *Rhizoctonia* SSPs inhibit JA biosynthesis, enabling fungal colonization (Huang et al., 2022).

8.3. Strigolactones: As mentioned earlier, these hormones enhance fungal hyphal branching and symbiotic contact (Balzergueet al., 2020).

9. Immune Response Modulation

Orchids have evolved mechanisms to suppress immune responses during mycorrhizal colonization, ensuring fungal accommodation. Orchids downregulate defense-related genes, such as *PR1* and *PAL*, during symbiosis. This suppression is mediated by micro RNAs (miRNAs) like miR171, which target defense genes (Xu et al., 2020). *Rhizoctonia* fungi secrete effectors that interfere with host immune responses. For example, the effector protein RiSSP7 suppresses JA signaling, facilitating fungal colonization (Huang et al., 2022).

10. Advanced Methodologies for Studying Fungal Partners

Recent advancements in molecular techniques have enabled the isolation, characterization, and functional analysis of orchid mycorrhizal fungi.

10.1. Fungal Isolation and Cultivation: Traditional methods involve isolating fungi from orchid roots and cultivating them on media like potato dextrose agar (PDA). However, many orchid mycorrhizal fungi are recalcitrant to lab cultivation, necessitating alternative approaches (Yagame and Takahiro et al., 2024).

10.2. Metagenomics and Metabarcoding: These techniques have been used to identify and characterize fungal communities associated with orchids. For example, metabarcoding of *Cypripedium* roots revealed a diverse fungal community dominated by *Tulasnella* .

10.3. Functional Characterization: Heterologous expression systems and CRISPR-Cas9 have been employed to study the functional roles of fungal genes. For instance, the *RiSSP7* gene from *Rhizoctonia* was expressed in *Arabidopsis* to study its role in immune suppression (Huang et al., 2022).

11. Evolutionary Perspectives

The evolutionary dynamics of orchid-mycorrhizal symbiosis have shaped the remarkable diversity and ecological success of orchids. This symbiosis is believed to have originated over 100 million years ago, coinciding with the diversification of Orchidaceae (Ramírez et al., 2007). Orchids and their fungal partners have co-evolved intricate molecular mechanisms to establish and maintain symbiotic relationships, driven by mutual benefits and selective pressures.

11.1. Co-evolution of Orchids and Mycorrhizal Fungi: Orchids and their fungal partners exhibit varying degrees of specificity, ranging from highly specialized interactions (e.g., *Ophrys* with *Tulasnella*) to more generalist associations (e.g., *Dendrobium* with multiple fungal genera) (Jacquemynet al., 2021). This flexibility likely reflects adaptations to diverse ecological niches and environmental conditions.

11.2. Genomic Insights into Symbiosis: Comparative genomic studies have revealed that orchids have retained ancient symbiotic genes while evolving novel mechanisms to regulate fungal colonization. For example, the *RAM2* gene, involved in lipid biosynthesis in AM symbiosis, is conserved in orchids but co-opted for interactions with *Rhizoctonia* (Gutjahr et al., 2012; Zhang et al., 2017). Similarly, the *Dendrobium officinale* genome contains a suite of symbiosis-specific genes, including those encoding lectins and chitinases, which modulate fungal colonization (Cai et al., 2021).

11.3. Evolutionary Adaptations: Orchids have evolved unique strategies to balance symbiotic benefits with potential costs. For instance, mycoheterotrophic orchids (e.g., *Neottia*, *Corallorhiza*) exploit fungi as carbon sources throughout their lifecycle, while photosynthetic orchids transition to partial autotrophy in adulthood (Merckx et al., 2013). This evolutionary plasticity has enabled orchids to colonize diverse habitats, from nutrient-poor soils to epiphytic niches.

11.4. Conservation of Symbiotic Pathways: Despite their unique features, orchid mycorrhizae share conserved molecular pathways with other plant-fungal symbioses, such as AM and ECM. For example, strigolactones and receptor-like kinases (RLKs) play central roles in fungal recognition and signaling across diverse symbiotic systems (Balzergueet al., 2020; Fochiet al., 2017).

12. Ecological and Practical Implications

Mycorrhizal fungi are critical for orchid conservation, as they are essential for seed germination, seedling establishment, and nutrient acquisition in both natural and restored habitats (Swarts et al., 2010). Many orchids, particularly endangered species, rely on specific fungal partners, making the preservation of these fungi a priority for conservation programs (Jacquemynet al., 2021).

12.1 Reintroduction and Habitat Restoration: Successful orchid reintroduction often requires the co-transplantation of compatible mycorrhizal fungi. For example, *Cypripedium calceolus* populations have been restored by inoculating seedlings with *Tulasnella* fungi (Rasmussen and Rasmussen, 2009).

12.2. Ex Situ Conservation: Mycorrhizal fungi are used in synthetic seed germination protocols to propagate rare orchids in vitro (Batty and Andrew et al., 2002). This approach has been successfully applied to species like *Dendrobium* and *Paphiopedilum* (Zhao et al., 2021).Habitat loss and climate change threaten both orchids and their fungal partners, highlighting the need for integrated conservation strategies (Dearnaley et al., 2016).

Conclusion

Our study highlights the intricate and dynamic relationship between orchids and their associated fungal communities, particularly emphasizing the seasonal variations in these interactions. The findings suggest that the fungal communities closest to the orchid roots show significant seasonal changes, which are likely adaptations to optimize resource utilization throughout different life stages. This underscores the importance of considering temporal factors in research on orchid microhabitats to minimize sampling errors. However, the study's limitations, including its focus on a single location and habitat, indicate the need for broader, multi-site, and longitudinal research to validate these patterns. Furthermore, conducting inoculation tests to examine the impact of various dominant fungi on orchid growth and their functional traits is essential for advancing our understanding of orchid conservation and expansion strategies. This research contributes valuable insights into the orchid-fungi symbiotic relationship, offering a foundation for future studies aimed at enhancing orchid preservation efforts.

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