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Characterization and Identification of Orchid Mycorrhizal Fungi Enhancing Seed Germination in *Cymbidium* from Southern Vietnam

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ABSTRACT

Background: *Cymbidium* is a genus of orchids that grow in soil, on substrates, or epiphytically, forming symbiotic associations with orchid mycorrhizal fungi (OMF). These fungi play a crucial role in facilitating water and nutrient uptake, which is essential for seed germination in natural environments. This study aimed to characterize and identify OMF strains associated with *Cymbidium* orchids cultivated in Southern Vietnam and to evaluate their effects on symbiotic seed germination on tree fern fibre medium.

Methods: Roots and growing media from thirty *Cymbidium* accessions, collected in Southern Vietnam, were stained with Trypan Blue and examined under a light microscope. OMF isolates were cultured on potato dextrose agar (PDA) medium and tested for their ability to promote seed germination on tree fern fiber. The fungal isolates that significantly enhanced germination rates were identified based on morphological characteristics and sequencing of the internal transcribed spacer (ITS) region using ITS1/ITS4 primers, followed by BLAST analysis against the NCBI database.

Result: Five distinct fungal morphotypes were observed in *Cymbidium* roots, with occurrence rates ranging from 3.3% to 40.0%. Two isolates, designated as OMF-S1 and OMF-S3, significantly promoted seed germination, achieving rates of 15.3% and 13.7%, respectively. Molecular identification results showed that OMF-S1 had high similarity to *Fusarium* sp., while OMF-S3 had high similarity to *Trichoderma* sp. These findings highlight the potential application of specific OMF strains in *Cymbidium* cultivation, propagation and conservation efforts.

Key words: *Cymbidium*, *Fusarium*, Orchid mycorrhizal fungi (OMF), Symbiotic germination, *Trichoderma.*

INTRODUCTION

Orchidaceae is one of the largest plant families, comprising over 26,000 species distributed worldwide (Phillips *et al.,* 2020). The diversity of species is reflected in the vast range of size, shapes and flower colors, making orchids highly popular and economically valuable in many countries (Koh *et al.,* 2014). The genus *Cymbidium* is a group of evergreens, flowering plants in the Orchidaceae family, naturally distributed across Asia and Oceania (Matsuda and Sugiura, 2019; Pridgeon *et al.,* 2009). The hot and humid climate of Vietnam provides favorable conditions for the growth and development of *Cymbidium* orchids (Tran, 1998).

Plant-fungal symbiosis refers to the interaction between a beneficial fungus and a plant species (Amina and Hamida, 2025; Lugo *et al.,* 2019). This type of relationship provides several advantages for crop plants, including enhanced water and nutrient absorption, improved tolerance to unfavorable environmental conditions and increased resistance to various pathogens (Amina and Hamida, 2025; Chaubey *et al.,* 2025). Orchids (Orchidaceae), in particular, establish a unique symbiotic relationship with fungi known as orchid mycorrhizal fungi (OMFs) (Abdullah, 2018). OMFs play a crucial role in the developmental stages of orchids (Oja *et al.,* 2015). In addition to facilitating water and nutrient uptake, OMFs are essential for orchid propagation by promoting seed germination (Stöckel *et al.,* 2014).

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Orchid seeds are extremely small, lightweight and produced in large quantities within the orchid capsule (Arditti and Ghani, 2000). A single capsule contains up to millions of seeds (Chen *et al.,* 2014). Nevertheless, orchid seeds lack an endosperm, which means they do not have the necessary energy required for germinating independently (Zhao *et al.,* 2021). Therefore, for natural germination, orchid seeds must form a symbiotic association with mycorrhizal fungi, which, in turn, will support the seed absorb water and essential nutrients for embryo development and germination (Utami and Hariyanto, 2020; Pradhan *et al.,* 2014).

The penetration of compatible fungal hyphae into the protocorm tissue occurs through the epidermal hairs (Smith and Read, 2010). The symbiotic process start immediately after the embryo absorbs water and swells, then breaking the seed coat, facilitating the invasion of OMF into the orchid seed (Abdullah, 2018). Within approximately 20 to 36 hours after OMF contact with the seed, mycorrhizal fungi can colonize the epidermal hairs and spread hyphae from cell to cell, forming a dense region of cortical cells containing coils of hyphae known as pelotons (Smith and Read, 2010). Initially, newly formed pelotons contain fungal hyphae rich in ribosomes and glycogen (Hadley and Williamson, 1971). However, as their structure develops, the fungal cytoplasm becomes vacuolated and glycogen is degraded (Peterson and Currah, 1990). More than one fungus can produce pelotons at the same time in the same orchid tissue (Moore *et al.,* 2011, Smith and Read, 2010). The surviving fungal hyphae re-colonise the orchid cells. This reinfection cycle occurs several times in each cell (Abdullah, 2018). In this way, the peloton increases the surface area between the orchid and fungus for the exchange of nutrients between both partners, supplying minerals, water and carbon (Herrera *et al.,* 2017). During this process, OMF supply water and essential minerals to the embryo, enabling the germination of orchid seeds (Kauth *et al.,* 2008). Furthermore, OMF enhance plant photosynthesis and biomass accumulation by supplying various macronutrients and micronutrients, such as Zn and Cu (Mitra *et al.,* 2020; Chen *et al.,* 2017; Bonfante and Genre, 2010; Smith and Read, 2010), as well as improving plant water-use efficiency (Chauhan *et al.,* 2022). Besides their ability to facilitate seed germination and nutrient absorption in Orchidaceae, OMFs have also been observed in association with members of the Fabaceae family (Shi *et al.,* 2020; Azarnia *et al.,* 2020). The presence of OMF in orchid root zones is abundant and they perform diverse functions. Thus, further research on identifying and characterizing different OMF strains specific to *Cymbidium* orchids is essential for optimizing their application in propagation and cultivation, ultimately improving germination success and plant development.

MATERIALS AND METHODS

Time and location of the study

The experiments in this study were conducted from May 2024 to February 2025 at the Laboratories of the Faculty of Agronomy, Nong Lam University, Ho Chi Minh City, Vietnam.

Cymbidium orchid collection

Cymbidium plants used for OMF isolation were collected from 10 different orchid gardens in Southern Vietnam. In each garden, three mature *Cymbidium* plants, measuring 50-60 cm in height and possessing at least five pseudobulbs, were selected. A total of 30 plants were transferred to the laboratory for OMF screening. For each orchid plant, fresh green-colored root segments were collected and the root tips were removed. The remaining root segments were cut into pieces approximately 1.2-1.5 cm in length, with a total sample weight of 1.0 g. The root samples were then rinsed under running tap water for 60 seconds to remove surface debris. Following cleaning, the root samples were longitudinally sectioned into thin slices (<2 mm thick) and stained with trypan blue (Phan *et al.,* 2024).

Isolation of OMFs from *cymbidium* roots

After collection, root samples were rinsed under running tap water to remove debris, then transferred to a biological safety cabinet for surface sterilization. The samples were immersed in 1% $NaClO_3$ for 3 minutes, followed by thorough rinsing with sterile distilled water. They were then soaked in 70% ethanol for 30 seconds and washed 3-5 times with sterile distilled water to ensure complete sterilization. The OMFs in the root samples were isolated on PDA in Petri dishes (Suryantini *et al.,* 2016). The morphology of isolated OMF strains on PDA was also compared with fungal structures observed in orchid roots to ensure that OMFs in root samples and isolated OMFs on PDA were similar.

Germination of *cymbidium* seeds treated with isolated OMF strains

Five fungal strains isolated from *Cymbidium* sp. root samples were successfully cultured on PDA medium and designated as OMF-S1, OMF-S2, OMF-S3, OMF-S4 and OMF-S5. These strains, along with a sterile distilled water control, were tested for their effects on *Cymbidium* seed germination using the following protocol.

Preparation of growth medium

Tree fern fibre was autoclaved at 121°C and 1 atm for 15 minutes. After cooling, 10 g of the sterilized substrate was transferred into each Petri dish.

Seed sterilization and sowing

Orchid capsules were surface sterilized with 70% ethanol for 30 seconds, then longitudinally split open. In each experimental unit, 100 seeds were counted and evenly distributed across five designated positions on the Petri dish (20 seeds per position).

Preparation and application of fungal suspensions

Hyphae of OMF strains cultured on PDA were collected and suspended in 30 mL of sterile distilled water in Falcon tubes. The suspension was shaken at 250 rpm for 30 minutes to disperse the fungal hyphae. A 10 mL aliquot of the fungal suspension was evenly applied to each Petri dish containing *Cymbidium* seeds. For the control treatment, 10 mL of sterile distilled water was applied instead.

Treatment application

The fungal suspensions and sterile distilled water were reapplied to the corresponding experimental units every two days.

Germination parameter assessment

Germination rate (%)

The percentage of germinated seeds was recorded at 30 days after sowing using the formula:

$$\text{Germination rate (\%)} = \frac{\text{Number protocorms formed}}{\text{Total number of seeds sown}} \times 100$$

Identification of OMF strains promoting *cymbidium* seed germination

OMF strains that significantly promoted seed germination were selected for DNA extraction using a DNA extraction kit from ABT Biomedical Solutions Co., Ltd. Qualified DNA samples were amplified using primers targeting the internal transcribed spacer (ITS) region: forward primer ITS1 (TCCGTAGGTGAACCTGCGG) and reverse primer ITS4 (TCCTCCGCTTATTGATATGC) (White *et al.,* 1990). PCR amplification was performed in a thermal cycler under the following conditions: an initial denaturation at 94°C, followed by 35 cycles of denaturation at 94°C, annealing at 59°C and extension at 72°C, with a final extension at 72°C (Lievens *et al.,* 2003).

RESULTS AND DISCUSSION

Morphological characterization of orchid mycorrhizal fungi in *cymbidium* roots

The analysis of OMF presence in the roots of collected *Cymbidium* plants in Table 1 revealed that all five hyphal types shared common characteristics, including branching, constrictions at branching points, the presence of septa and nuclei within the hyphae. Additionally, all five OMF types formed hyphae within root cells and established connections with adjacent cells. However, each hyphal type exhibited distinct morphological and structural traits. Notably, OMF-S5 hyphae had a larger diameter than the other hyphal types and only OMF-S5 hyphae contained spherical yellow structures within the hyphal nuclei. Furthermore, the results indicated that multiple OMF forms can coexist within the roots of *Cymbidium* orchids. This finding aligns with the observations of Cevallos *et al.* (2017), who reported that orchids inhabiting the same location tend to associate with distinct OMF communities. Such specific associations may provide an adaptive advantage by reducing competition for water or nutrients.

Effects of OMF on the germination of *cymbidium* seeds

Germination time and rate are critical parameters influencing orchid propagation. In natural environments, orchid seed germination is entirely dependent on OMFs, with different fungal genera exerting varying effects on germination (Tsulsiyah *et al.,* 2021). Therefore, germination time and rate serve as essential indicators for evaluating the ability of OMF strains isolated from orchid roots to promote seed germination.

The results presented in Fig 1 indicate that different OMF strains from *Cymbidium* roots influenced seed germination time and rate. When supplemented with only OMF-S1 or OMF-S3, seeds could germinate on tree fern fiber medium (Fig 2) within 30 days after sowing, with germination rates of 15.3% and 13.7%, respectively. However, by 30 days after sowing, seeds treated with OMF-S2, OMF-S4, OMF-S5 or the control (without OMF) did not germinate under the same conditions. These findings suggest that not all OMF strains facilitate orchid seed germination; successful germination occurs only when seeds establish symbiosis with suitable OMF strains. The study of Xiang *et al.* (2018) demonstrated that both fungal strains successfully induced *Cymbidium mastersii* seed germination within 30 days of inoculation. Over the following 30 days, 48.0% of the protocorms developed their first leaves, whereas most protocorms either died or ceased development in the absence of continued symbiotic fungal supplementation.

Identification of OMF strains promoting *cymbidium* seed germination

When cultured on PDA medium, OMF-S1 hyphae exhibited a cottony, white appearance with dense, tightly packed growth (Fig 3A), resembling fungal characteristics of the genus *Fusarium* (Zemanková and Lebeda, 2001). In contrast, OMF-S3 formed smooth, white hyphal clusters that later turned green as spores developed, producing concentric rings on the agar surface. The reverse side of the agar plate displayed a white-yellow coloration (Fig 3B), which aligned with characteristics of fungi related to the genus *Trichoderma* (Langa-Lomba *et al.,* 2022; Kumar *et al.,* 2019).

Comparison of the OMF-S1 sequence with GenBank data confirmed its affiliation with the genus *Fusarium* (Table 2). The ITS region sequence of OMF-S1 exhibited 95.45% similarity to *Fusarium* sp. LN828164.1, previously isolated from the roots of *Cymbidium ensifolium* (Jin-Ai *et al.,* 2018). The findings of Çığ *et al.* (2018) demonstrated that *Fusarium* sp., isolated from the roots of ten terrestrial orchid species in Spain, promoted seed germination in *Orchis spitzelii* (73.9%), *Ophrys straussii* (91.6%) and *Dactylorhiza umbrosa* (93.5%). Additionally, *Fusarium*

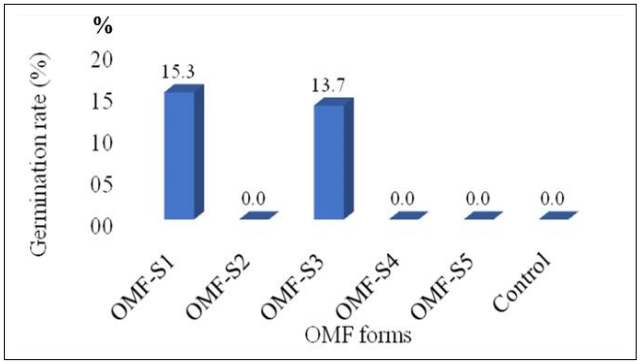
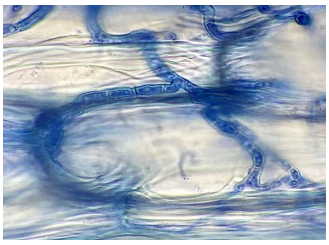
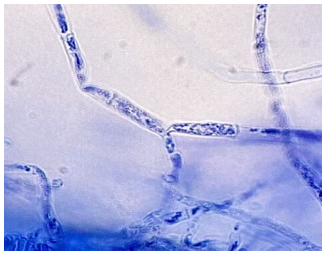
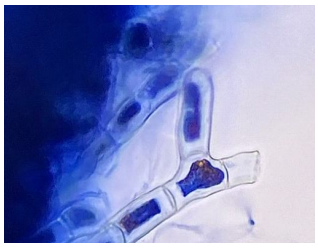


Fig 1: Germination rate of *Cymbidium* sp. orchid seeds applied OMFs at 30 days after sowing.

oxysporum, isolated from *Bletilla striata* roots, has been reported to enhance plant growth by significantly increasing height, fresh weight and dry weight (Jiang *et al.,* 2019). Although certain *Fusarium* species are known root rot pathogens in various crops others establish beneficial symbiotic relationships with orchids, facilitating seed germination and promoting plant growth in natural environments.

Comparison of the OMF-S3 sequence with GenBank data confirmed its affiliation with the genus *Trichoderma* (Table 2). The ITS region sequence of OMF-S3 exhibited 97.24% similarity to *Trichoderma* sp. KF896896, specifically *Trichoderma spirale*, previously isolated from the roots of *Cymbidium goeringii* and *Cymbidium faberi* (Yu *et al.,* 2015). Additionally, *Trichoderma* sp. isolated from the roots of *Cymbidium atropurpureum* has been shown to significantly enhance seedling growth parameters, including plant height, stem diameter, leaf number, root length, fresh weight and dry weight. Moreover, it contributed to improved disease resistance during the seedling stage (De Medeiros *et al.,* 2023).

Table 1: Morphology of OMFs fungal hyphae in *Cymbidiu*m sp. roots observed under a microscope at 100x magnification.

OMFs	Hyphae of OMFs under a microscope at 100x magnification	Characterictics
OMF-S1		The branched hyphae with angles: 40°-60°. The hyphal diameter: 1.9±0.4 μm. The hyphae exhibited constrictions at branching points, possessed septa and contained nuclei.
OMF-S2		The branched hyphae with angles: 30°-60°. The hyphal diameter: 1.8±0.2 μm. The hyphae exhibited constrictions at branching points, possessed septa and contained nuclei.
OMF-S3		The branched hyphae with angles: 45°-90°. The hyphal diameter: 1.4±0.4 μm. The hyphae possessed septa, contained nuclei and were distributed along the root length.
OMF-S4		The branched hyphae with angles: 15°-90°. The hyphal diameter: 2.5±0.6 μm. The hyphae possessed septa, contained nuclei and densely colonized orchid root cells.
OMF-S5		The branched hyphae with angles: 60°-90°. The hyphal diameter: 10.6±1.7 μm. The hyphae possessed septa, contained nuclei and displayed spherical yellow structures within the

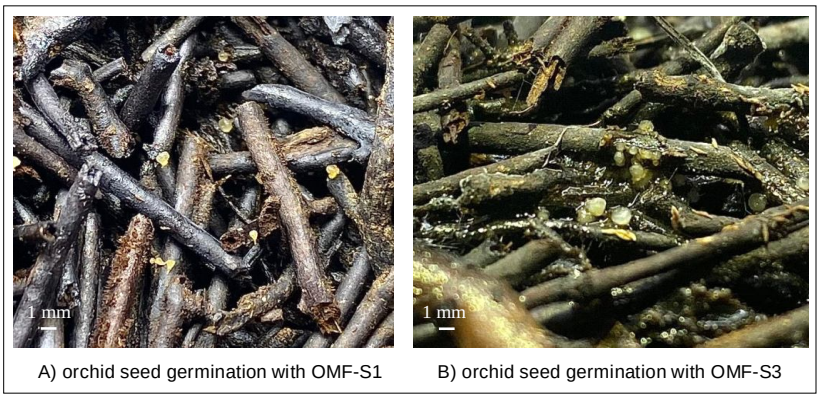


Fig 2: Germination of *Cymbidium* orchid seeds with OMF-S1 (A) or OMF-S3 (B) on tree fern fiber at 30 days after sowing.

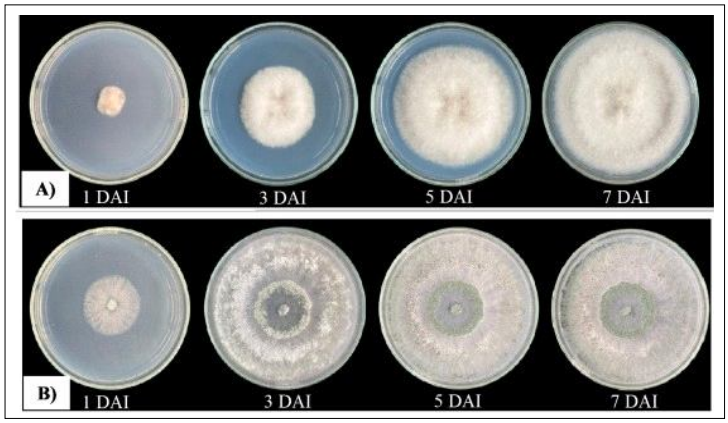


Fig 3: Formation of OMF-S1 hyphae (A) and OMF-S3 hyphae (B) on PDA medium at 1, 3, 5 and 7 days after inoculation (DAI).

Table 2: Similarity of OMF types in *Cymbidium* roots promoting orchid seed germination compared with other publications.

OMF types	Identification results	Similarity ratio on BLAST (%)	Reference OMF from other studies
OMF-S1	*Fusarium* sp.	95.45	*Fusarium* sp. LN828164,1 (Jin-Ai *et al.,* 2018)
OMF-S3	*Trichoderma* sp.	97.24	*Trichoderma* sp. KF896896 (Yu *et al.,* 2015)

CONCLUSION

Five OMF forms were identified in the roots of *Cymbidium* based on an analysis of 30 root samples. OMF-S1 and OMF-S3 effectively promoted orchid seed germination on tree fern fiber medium, achieving germination rates of 15.3% and 13.7%, respectively. Molecular identification using ITS1/ITS4 primers indicated that OMF-S1 related to the genus *Fusarium*, while OMF-S3 is associated with the genus *Trichoderma*.

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Disclaimers

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish or preparation of the manuscript.

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