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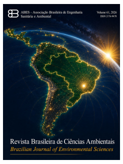

The path towards reversing the red: The mycorrhizal fungus *Tulasnella amonilioides* highly contributes to the *Cattleya intermedia* (Orchidaceae) translocation success

Rumo à reversão do vermelho: O fungo micorrízico *Tulasnella amonilioides* contribui altamente para o sucesso da translocação de *Cattleya intermedia* (Orchidaceae)

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ABSTRACT

Orchids are a key functional group in forest environments that require obligate symbiosis with mycorrhizal fungi at least during the early stages of development. The present study investigated whether *Cattleya intermedia* derived from asymbiotic *in vitro* propagation establishes symbiosis with mycorrhizal fungi after translocation to the wild, and whether this relationship varies along the vertical gradient of phorophytes. Fungi were isolated from plant roots four years after translocation to the trunk and canopy of phorophytes in a subtropical forest in Brazil. The orchid–fungus specificity pattern observed in translocated seedlings and adult plants resembled those reported in the literature for wild seedlings and adult individuals of species in the same environment, revealing the presence of *C. intermedia* mycorrhizal fungi (CiMF) and non-CiMF endophytes of various morphotypes. Isolated CiMF promoted seed germination and *in vitro* seedling development, with 14 isolates obtained from orchids translocated to the canopy and eight from those on the trunk. Plants in symbiosis with canopy-derived CiMF produced a greater number of leaves compared to those associated with trunk-derived CiMF. Phylogenetic analysis of ITS (Internal Transcribed Spacer) region sequences indicated that CiMF belong to Tulasnellaceae, with 21 isolates identified as *Tulasnella amonilioides*, a species also found in symbiosis with wild *C. intermedia* in this environment. The prospecting of compatible mycorrhizal fungi enabled the isolation of new strains, effective for the symbiotic propagation of orchids and contributing to the conservation of *C. intermedia*.

Keywords: conservation; mycorrhiza; Orchidaceae; *ex situ* propagation; forest regeneration.

RESUMO

As orquídeas constituem um grupo funcional chave em ambientes florestais que, para se estabelecer, fazem simbiose obrigatória com fungos micorrízicos, pelo menos nos primeiros estádios de vida. Este estudo investigou se *Cattleya intermedia* provinda de propagação assimbiótica *in vitro* estabelece simbiose com fungos micorrízicos após translocação à natureza e se essa relação varia no gradiente vertical dos forófitos. Os fungos foram isolados de raízes das plantas quatro anos após a translocação ao fuste e à copa de forófitos em floresta subtropical no Brasil. O padrão de especificidade fungo-orquídea nas plantas jovens e plantas adultas translocadas assemelhou-se ao descrito na literatura para plantas jovens e plantas adultas selvagens da espécie neste ambiente, revelando a presença de fungos micorrízicos de *C. intermedia* (FMCi) e de endófitos não FMCi de diferentes morfotipos. Os isolados de FMCi promoveram a germinação e o desenvolvimento de plantas jovens *in vitro*, sendo 14 espécimes oriundos de orquídeas translocadas à copa e oito de orquídeas do fuste. Plantas em simbiose com FMCi da copa apresentaram maior número de folhas em comparação às associadas aos FMCi do fuste. A análise filogenética das sequências da região Internal Transcribed Spacer (ITS) mostrou que os FMCi pertencem a Tulasnellaceae, sendo 21 isolados identificados como *Tulasnella amonilioides*, espécie também associada a plantas selvagens de *C. intermedia* naquele ambiente. A prospecção de fungos micorrízicos compatíveis possibilitou a obtenção de novos isolados eficientes para a propagação simbiótica de orquídeas, contribuindo para a conservação de *C. intermedia*.

Palavras-chave: conservação; micorriza; Orchidaceae; propagação *ex situ*; regeneração florestal.

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Introduction

One of the key characteristics of orchids is the production of fruits containing thousands of minute seeds, referred to as "dust seeds," which consist of an undifferentiated embryo enclosed by a membranous seed coat, lacking an endosperm (Lee and Yeung, 2023). Under natural conditions, the carbon source required for seed germination in early development is derived from a symbiotic association between the embryonic cells and mycobionts, during which orchids exhibit a mycoheterotrophic phase (Zhao et al., 2024). The hyphae of mycobionts that form orchid mycorrhizae create intracellular coils - known as pelotons -within the embryos, basal cells of protocorms, roots, and rhizomes of adult plants (Zahn et al., 2023), thereby distinguishing them from fortuitous orchid endophytes (Rasmussen et al., 2015). These pelotons are degraded within plant cells by enzymes produced by the host tissue, allowing the absorption of nutrients and water for plant metabolism (Zhao et al., 2024).

However, orchid mycorrhizal fungi are not equally compatible with all orchid species (Phillips et al., 2020), nor do they remain the same throughout the orchid's life cycle (Qin et al., 2021; Freestone et al., 2024), resulting in various patterns of mycobiont-orchid specificity as proposed by Rasmussen et al. (2015). Environmental factors such as substrate quality, orchid presence and density, as well as the local microclimate also influence in the distribution and abundance of mycobionts in natural habitats (Li et al., 2021). Given that compatible mycorrhizal fungi are essential for seed germination and initial growth, their presence is critical to the distribution of orchids in their habitats (Rasmussen et al., 2015; Zhao et al., 2021). In addition to mycobiont association, microenvironmental conditions such as substrate pH and moisture where the seeds settle also significantly impact the establishment of the orchids (Pecoraro et al., 2021).

Even when seeds encounter suitable germination conditions and associate with compatible fungi, only a small portion succeed in germinating (~30%), reaching the protocorm stage (0.55%) or developing into seedlings (<0.01%) (Shao et al., 2020). Due to their small size and high surface-area-to-volume ratio, the protocorm and pre-pseudobulb seedling stages are particularly vulnerable, lacking storage organs. This vulnerability is exacerbated in epiphytic species, for which water availability is limited and intermittent (Benzing, 1990). As they grow and develop photosynthetic tissues, orchids shift to autotrophic metabolism but may maintain mycorrhizal relationships throughout their lifespan (Yeh et al., 2019; Fernández et al., 2023).

For endangered orchid species, *ex situ* conservation tools enable controlled development of early life stages (Endres Júnior et al., 2023). Plants may be propagated via *in vitro* germination with symbiotic fungi on culture media (Dixon, 1987) or through asymbiotic techniques using nutrient-rich media with carbon sources (Murashige and Skoog, 1962). These approaches assist the most vulnerable stages of orchid ontogeny under laboratory conditions, allowing the production of healthier plants in greater numbers at a faster rate than it would occur in the wild. Through acclimatization, these plants gain the ability to photosynthesize and survive *ex vitro* conditions (Tikendra et al., 2025).

Orchids propagated *in vitro* from seeds are valuable for translocation programs, contributing to recolonization of areas from which they have been eliminated or enhancing population size and genetic diversity in existing populations, thereby improving their long-term survival (IUCN/SSC, 2013). Translocation for conservation purposes bridges *in vitro* and *in situ* strategies, linking theory and practice in conservation science (Gale et al., 2018). This approach has been applied to the conservation of several epiphytic orchid species across different climate regimes (Wu et al., 2014; Endres Júnior et al., 2024; Hsu et al., 2024). However, the translocation of symbiotically propagated orchids or the establishment of symbiosis in the field by asymbiotically propagated plants remains underexplored. Despite recent advances, gaps remain in the understanding of ecological dynamics behind epiphytic orchid-mycobiont relationships (Phillips et al., 2020). Thus, it is recommended that compatible mycorrhizal fungi be present or introduced at translocation sites (Ramsay and Dixon, 2003).

Cattleya intermedia Grah. is an epiphytic orchid endemic to Brazil (Van den Berg, 2024), most commonly found in the canopy of its phorophytes (Gonçalves and Waechter, 2003). Due to illegal collection and habitat destruction, the species is listed as vulnerable (VU) in Brazil and in the state of Rio Grande do Sul (RS), where this present study was conducted (Menini Neto et al., 2013; Rio Grande do Sul, 2014). A conservation initiative for *C. intermedia* found that individuals propagated asymbiotically *in vitro* exhibited greater development (shoot height, number of leaves, pseudobulbs, and roots) and flowered when translocated to the canopy compared to the trunk of phorophytes in the analysed fragment of the forest in southern Brazil (Endres Júnior et al., 2019; Endres Júnior et al., 2024). The wild *C. intermedia* population in the area is associated with *Tulasnella amonilioides* (P.R.M. Almeida, N. Van den Berg & A. Góes-Neto) S. Fujimori, J.P. Abe, I. Okane & Y. Yamaoka (Fujimori et al., 2019), a fungal species first isolated from the native Brazilian orchids *Encyclia dichroma* (Lindl.) Schltr., *Encyclia ghillanyi* Pabst, and *Brassavola tuberculata* Hook., and originally identified as *Epulorhyza amonilioides* (Almeida et al., 2014). *Tulasnella amonilioides* can induce seed germination and promote *in vitro* growth of *C. intermedia* (Endres Júnior et al., 2023). This mycorrhizal fungus has also been recently identified in other species of the genus, namely *Cattleya bicolor* Lindl., *Cattleya walkeriana* Gardner, *Cattleya jongheana* (Rchb. f.) Van den Berg (Nogueira et al., 2024), and *Cattleya rupestris* (Lindl.) Van den Berg (Almeida et al., 2025).

Considering that environmental factors may vary along the vertical gradient (Kersten, 2010) and influence the distribution of mycorrhizal fungi and the establishment of associated plants (Pecoraro et al., 2021); the critical role of mycorrhizal fungi in orchid population restoration and long-term conservation (Gale et al., 2018); the morphometric differences in *C. intermedia* individuals following translocation (Endres Júnior et al., 2019; Endres Júnior et al., 2024); the established symbiosis between wild *C. intermedia* individuals and *T. amonilioides* in the forest fragment under study (Endres Júnior et al., 2023); and that approximately 36% of endophytes isolated from wild *C. intermedia* are not orchid mycobionts (Endres Júnior et al., 2023), the objectives of this study were to: evaluate whether asymbiotically propagated *in vitro* orchids translocated to the trunk and canopy of phorophytes established symbiotic relationships post-translocation; determine whether the mycobionts found in the translocated plants are the same species as those associated with wild plants in the study area, following the same fungus-plant specificity pattern; and assess whether mycorrhization in the canopy and trunk differs quantitatively. The main hypotheses were: translocated orchids form associations with mycobionts; the mycorrhizal fungi are of the same species (*T. amonilioides*) as those found in wild *C. intermedia* plants from the same environment and follow the same specificity pattern; and roots of plants translocated to the canopy harbour a higher quantity of orchid mycobionts than those on the trunk, positively influencing the establishment of *C. intermedia*.

Material and Methods

Analyses were conducted four years after the translocation of *C. intermedia* plants obtained through asymbiotic *in vitro* seed germination and plant propagation on MS medium (Murashige and Skoog, 1962) (Figure 1A; Sasamori et al., 2015). Prior to translocation, the plants had been acclimatized in the substrate for five months and subsequently maintained for 12 months individually tied to pine bark plaques (5 × 10 cm) (Figure 1B) under controlled laboratory conditions (Endres Júnior et al., 2015). At this stage, individuals were selected for translocation if they had sprouted, produced at least one pseudobulb, and had healthy roots free of injury and/or necrosis, firmly attached to the substrate.

The area that received the orchids is protected (29°41'S, 51°06'W, alt. 16.4 m), located within the urban matrix of the municipality of Novo Hamburgo, in the state of Rio Grande do Sul (RS), Brazil. The regional climate is subtropical (Cfa, Köppen classification), without a defined dry season (Alvares et al., 2013). The vegetation is secondary, exhibiting features of both the Pampa and Atlantic Forest phytogeographical domains (IBGE, 2012), regenerating since the 1990s, when the conservation unit was established (Novo Hamburgo, 1999). This area is located within the natural distribution range of the *C. intermedia* in RS and supports a small wild population (~10 individuals; Endres Júnior et al., 2023), characterizing the translocation as a reinforcement (IUCN/SSC, 2013). A total of 70 plants were translocated to the trunks (3.5–4.0 m in height) and 70 to the canopies (6.5–7.0 m) of 17 phorophytes (Figure 1C; Endres Júnior et al., 2019). At the time of this study, 48 plants remained on the trunks (10 had been removed and 12 had died), while all plants in the canopies were still present.

Fungal isolation and cultivation

Seven *C. intermedia* plants with roots measuring between 20 and 30 cm were selected from the trunks and the canopies of phorophytes (Figures 1C and 1D), where each plant was attached to a different phorophyte. One root was collected from each plant, moistened with sterile distilled water, placed in a sealed plastic bag, labelled, and transported in an ice-filled thermal container to the laboratory for analysis (Endres Júnior et al., 2023). From each root, two opposite ends were removed, each forming a 2 cm segment. These segments were rinsed in running water, surface-sterilized with 70% ethanol for 1 minute, followed by 2% NaClO for 6 minutes, then rinsed with sterile distilled water. Each segment was transversely sectioned under a stereomicroscope (Labomed CZM4), resulting in ten sections containing fungal pelotons within the cortex (Figures 1E and 1F). The cortical region (Figure 1G) of each section was excised and transferred to a PDA (potato-dextrose-agar) medium in 9 cm Petri dishes, with five sections per plate (Figure 1H). In total, 140 root sections (28 plates) from the plants attached to the trunks and canopies, where they were cultured in the dark at 25±1°C and monitored for hyphal growth.

To obtain pure cultures of endophytes, colonies showing differences in growth rate, colony color, and in the presence and abundance of aerial hyphae within the same plate were selected taxonomically informative traits for fungal identification (Airin et al., 2023). This resulted in 19 isolates from trunk-attached plants and 24 from canopy-attached plants. A 1 cm^3 piece from the edge of each fungal colony was transferred to a new Petri dish containing the same medium (Figure 1I) and cultivated under the same conditions described above.

Determination of mycorrhizal fungi associated with *Cattleya intermedia*

All fungal isolates were tested for their ability to promote *in vitro* seed germination and plant growth of *C. intermedia*, in order to determine which ones corresponded to mycorrhizal fungi of this species (CiMF). Seeds were obtained from a natural population of *C. intermedia* located in the municipality of Portão, 9.04 km from the translocation site (29°41'16.02"S, 51°12'9.24"W). 200 mg of seeds were extracted from mature fruits (Figure 1J). The seeds were surface-sterilized (70% ethanol for 1 minute, 2% NaClO for 10 minutes), immersed in 50 mL of sterile distilled water, and pipetted into Petri dishes containing OMA medium (4 g L^{-1} oat, 7.5 g L^{-1} agar, distilled water, pH adjusted to 5.6) (Dixon, 1987). Each plate was inoculated with a 1 cm^3 PDA medium cut from the edge of each fungal isolate (Figure 1K). The negative control consisted of seed cultures on OMA medium without fungal inoculation. The DEJ17 isolate of *T. amonilioides*, previously confirmed as a promoter of germination and early growth of *C. intermedia* (Endres Júnior et al., 2023), was inoculated in a OMA medium with seeds as the positive symbiotic control. Seeds were sown in a MS medium (50% of the original macronutrient formulation, 30 g L^{-1} of sucrose) as the positive asymbiotic control, since this medium had been successfully used for asymbiotic *in vitro* culture of *C. intermedia* (Sasamori et al., 2015; Endres Júnior et al., 2023).

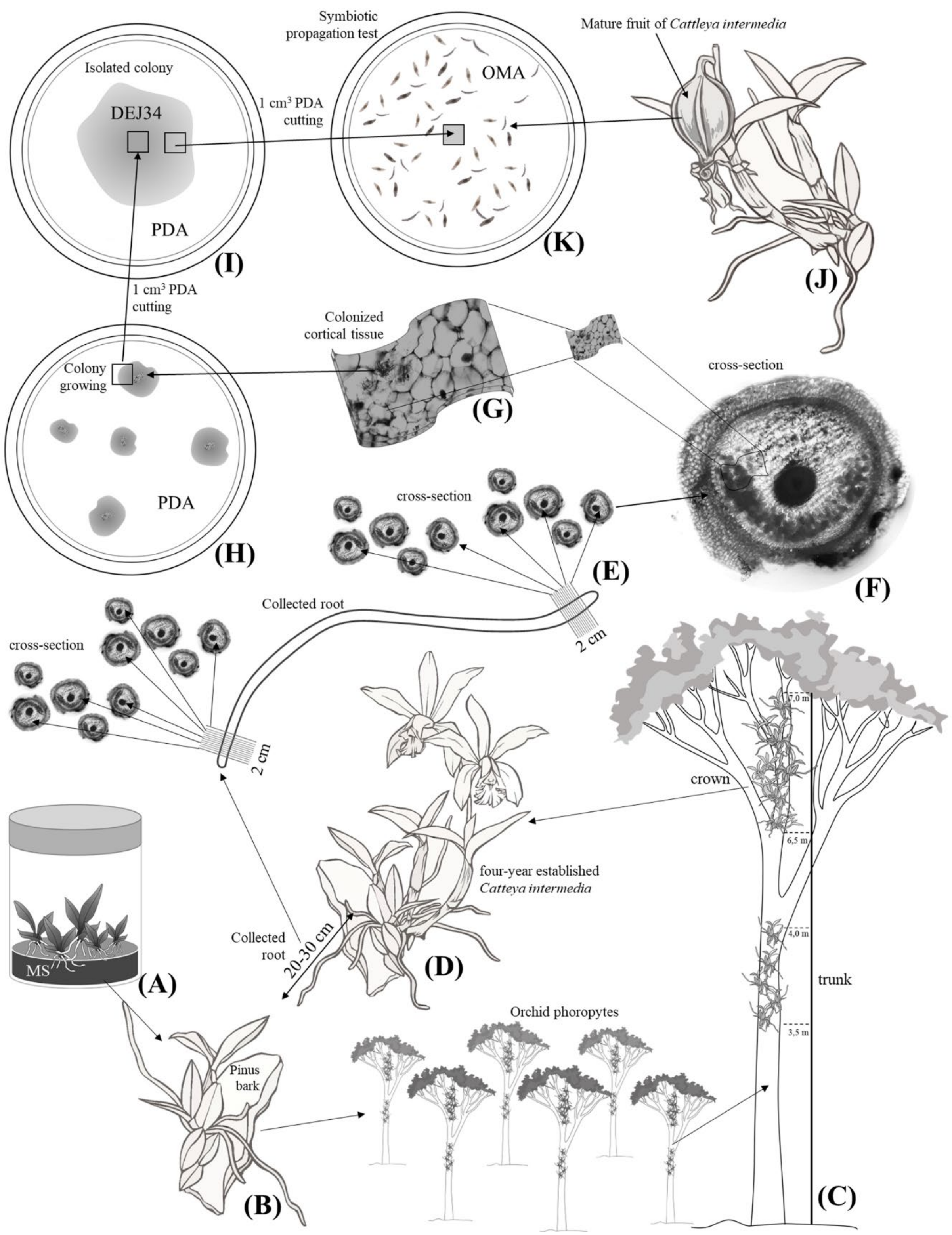

Figure 1 – Stages developed from orchid propagation to the collection of root samples with fungi. (A) *In vitro* plants prior to acclimatization, (B) cultivation of orchids on pine bark plaques, (C) seven phorophytes supporting the orchids selected for root mycorrhization analysis from trunk and canopy strata, (D) plant with root selected for mycorrhizal sampling, (E, F) root tips cut to obtain ten cross sections containing pelotons, (G, H) cortical region containing pelotons removed from the cross section for culturing in Petri dishes, (I) 1 cm³ medium plugs with distinct colonies transferred to fresh PDA medium for fungal isolation, (J) seeds obtained from *C. intermedia* fruits from the study area, (K) seeds pipetted onto OMA medium inoculated with 1 cm³ of PDA medium containing the fungal isolates.

Six plates were prepared for each treatment, sealed with plastic wrap, and maintained at 26±1°C, with a 12:12 h light:dark photoperiod and an irradiance of 100 µmol m^{-2} s^{-1}. Isolates were classified as CiMF if they promoted seed germination (testa rupture) and developed the protocorm stage (rhizoids and promeristem) and the seedling stage (true leaves and roots) (Endres Júnior et al., 2023), as determined over a 90-day monitoring period following germination. Fungi that did not meet these criteria were considered non-mycorrhizal for *C. intermedia* (non-CiMF). The percentages of CiMF and non-CiMF per phorophyte stratum were calculated based on the ratio of the absolute number of isolates in each category compared with the total number of isolates obtained. To assess whether the mycobiont-orchid relationship pattern of the translocated individuals matched that of the wild *C. intermedia* plants in the same environment, the CiMF and non-CiMF percentages obtained in this study were compared with the data reported by Endres Júnior et al. (2023).

Evaluation of germination, growth index, and morphometric development of *Cattleya intermedia* in symbiosis

From the selected CiMF isolates according to the criteria described in the previous section, 12 isolates of distinct origin were selected (five trunk-attached plants and seven canopy-attached plants, with no repetition of individual plant or phorophyte) to quantitatively evaluate *in vitro* seed germination and the growth index of *C. intermedia*, 90 days after sowing. The assessments also included the positive symbiotic and asymbiotic control treatments. Seed viability, germination percentage, and growth index were evaluated under a dissecting microscope, following the method described by Endres Júnior et al. (2023). Seeds were considered viable when they contained a distinct, rounded, and hyaline embryo. Viability percentage (*Vp*) was calculated as Equation 1:

$$Vp = (Nvs \times 100) / Nts \qquad (1)$$

Where:
Nvs: the number of viable seeds;
Nts: the total number of seeds per dish.

Plant development stages were classified on the following scale, from 0 to 5: stage 0=no germination; stage 1=testa rupture by enlargement of the embryo (i.e., germination); stage 2=production of rhizoids; stage 3=appearance of promeristem, multiple rhizoids; stage 4=appearance of the first true leaf (from this stage on, the plants are defined as seedlings); stage 5=formation of a second true leaf and root system. The germination percentage (*Gp*) was calculated as Equation 2:

$$Gp = (NGi \times 100) / Nvs \qquad (2)$$

Where:
NGi: the number of germinated individuals (stages 1–5);
Nvs: the number of viable seeds.

The growth index was calculated as Equation 3:

$$GI = (N1 + N2 + N3 + N4 + N5 \times 5) / (N0 + N1 + N2 + N3 + N4 + N5) \qquad (3)$$

Where:
N0: the number of seeds at stage 0;
N1: the number of plants at stage 1, and so forth.

Plants with two or more leaves and true roots were cultivated in flasks (200 mL volume) containing 30 mL of the culture medium (OMA for plants from symbiotic cultures with 12 isolates and the symbiotic control isolate; modified MS medium for asymbiotic control plants), totaling 45 plants per treatment (five plants per flask). One year after sowing, the plants were individually evaluated for shoot height (cm), length of the longest root (cm), number of leaves and roots, and fresh mass (mg) (Endres Júnior et al., 2023).

Phylogenetic characterization of mycorrhizal fungi associated with *Cattleya intermedia*

Portions of mycelium of the 22 CiMF isolates were obtained from pure cultures (eight from trunk-attached plants and 14 from canopy-attached plants) were used for DNA extraction, amplification, and sequencing. Total DNA was extracted using the modified protocol of Góes-Neto et al. (2005). The nuclear ribosomal region (nuc-r) comprising the internal transcribed spacers (ITS1 and ITS2) and the 5.8S gene (ITS: ITS1+5.8S+ITS2) was amplified by a PCR (Polymerase Chain Reaction) using the primer pair ITS8F/ITS6R (Dentinger et al., 2010). DNA fragments were stained with the SYBR™ Safe DNA Gel Stain and subjected to horizontal electrophoresis on 2% agarose gel using a loading buffer. Positive results were identified as single, homogeneous bands under UV light. Sequencing was performed by Macrogen Inc. (Geumcheon-gu, Korea).

The generated sequences were manually curated using Geneious v9. Sequences obtained in this study were aligned using MAFFT v7 and manually verified in MEGA v7 along with others retrieved from GenBank. The alignment and resulting phylogenetic tree were deposited in the Harvard Dataverse (Endres Júnior et al., 2025). All phylogenetic analyses were performed on the CIPRES Science Gateway. Maximum Likelihood (ML) analyses were conducted using RAxML 8.2.9. The dataset construction followed Endres Júnior et al. (2023), incorporating a broad set of sequences, including recently published species from Brazil (e.g. Freitas et al., 2020; Arifin et al., 2021; Arifin et al., 2022; Crous et al., 2023). The ITS sequences obtained from wild *C. intermedia* plants in the same study area, first reported by Endres Júnior et al. (2023), were also included. The phylogenetic tree was edited using the Toytree Python library and Inkscape® (Eaton, 2020).

Statistical analyses

Data were subjected to the Shapiro-Wilk normality test, where germination rates (%) and the growth index of *in vitro* propagated individuals were compared among treatments using ANOVA, followed by Tukey's test. Student's *t*-test for independent samples was used to compare germination and growth index values between individuals symbiotically propagated with fungal isolates obtained from the two phorophyte strata. The morphometric data of *in vitro* propagated plants were compared among treatments using the Kruskal-Wallis test, followed by the Student-Newman-Keuls test. Mean morphometric values between plants propagated with fungi from the trunk and canopy, as well as those propagated with the DEJ17 and MS controls, were compared using the Mann-Whitney test. All analyses were performed using the SPSS software version 28, with statistical significance set at 5%.

Results

Isolation, cultivation, and phylogenetic characterization of fungi

A total of 19 and 24 fungal isolates were obtained from the roots of *C. intermedia* growing on the trunks and canopies of phorophytes, respectively. Out of these samples, 8 (42.1%) and 14 (58.3%) were identified as CiMF (compatible mycorrhizal fungi), capable of promoting seed germination and initial plant growth. The information regarding the 22 CiMF isolates is presented in Table 1. The ITS region sequences of the 22 CiMF isolates were used in phylogenetic analyses, resulting in a final alignment comprising 192 specimens and 1,274 base pairs. In the Maximum Likelihood (ML) analysis, the RAxML bootstopping criterion indicated that 250 pseudoreplicates were sufficient to estimate branch support (BS), and the final optimization likelihood was -lnL=23,947.94.

Table 1 – Fungal isolates obtained from the roots of *Cattleya intermedia* plants translocated to the trunk and canopy of phorophytes in a forest fragment, identified based on GenBank sequences.

Isolate	Origin	Sample**	Species	blast	blast value
DEJ54*	**Trunk**	**2A**	***Tulasnella sp.***	**Tulasnella sp. strain DerIV_**	**97pc**
DEJ40	**Trunk**	**3A**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ41	Trunk	3B	*Tulasnella amonilioides*	Epulorhiza_amonilioides_Serrant9_	99pc
DEJ43	**Trunk**	**4A**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ42	Trunk	4B	*Tulasnella amonilioides*	Epulorhiza_amonilioides_Serrant9_	99pc
DEJ44	**Trunk**	**6A**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ45	**Trunk**	**7A**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ46	Trunk	7B	*Tulasnella amonilioides*	Epulorhiza_amonilioides_Serrant9_	99pc
DEJ49	**Canopy**	**1A**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ50	Canopy	1B	*Tulasnella amonilioides*	Epulorhiza_amonilioides_Serrant9_	99pc
DEJ48	**Canopy**	**2A**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ52	Canopy	2B	*Tulasnella amonilioides*	Epulorhiza_amonilioides_Serrant9_	99pc
DEJ57	**Canopy**	**3A**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**98pc**
DEJ58	Canopy	3B	*Tulasnella amonilioides*	Epulorhiza amonilioides strain tiro34_	99pc
DEJ59	Canopy	4A	*Tulasnella amonilioides*	Epulorhiza amonilioides strain orob32_	97pc
DEJ60	**Canopy**	**4B**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ65	Canopy	5A	*Tulasnella amonilioides*	Epulorhiza amonilioides strain orob32_	97pc
DEJ61	**Canopy**	**5B**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ62	Canopy	5B1	*Tulasnella amonilioides*	Epulorhiza_amonilioides_Serrant9_	99pc
DEJ63	Canopy	6A	*Tulasnella amonilioides*	Epulorhiza_amonilioides_Serrant9_	98pc
DEJ64	**Canopy**	**6B**	***Tulasnella amonilioides***	**Epulorhiza_amonilioides_Serrant9_**	**99pc**
DEJ34	**Canopy**	**7B**	***Tulasnella amonilioides***	**Epulorhiza amonilioides strain orob32_**	**98pc**

*Isolates in bold were selected for *in vitro* germination and growth tests; **numbers represent individual roots, and letters represent the two segments of each root.

The majority of CiMF isolates (95.5%) were identified as *T. amonilioides*. The only isolate not closely related to *T. amonilioides* was DEJ54, which was recovered as a sister lineage to an undescribed *Tulasnella* species in Brazil (Figure 2).

The analysis of fungus–orchid specificity patterns observed in *C. intermedia* plants revealed the presence of both CiMF and non-CiMF endophytes exhibiting distinct morphotypes (Figures 3A and 3B). Among the CiMF isolated from translocated plants, which were confirmed as mycobionts through *in vitro* symbiotic propagation tests, *T. amonilioides* was predominant, with one additional isolate (DEJ54), also belonging to *Tulasnella* (Figure 3A).

Germination, growth index, and morphometric development of *Cattleya intermedia* in symbiosis

The average seed viability observed was 60.1%, where all CiMF isolates increased seed germination percentages compared to the negative control and were as effective as the positive controls (Z=10.816; P<0.001) (Table 2). The lowest growth indices (GI) were recorded in the negative control and in the asymbiotic control (MS), respectively (Z=79.703; P<0.001). Isolate DEJ44, identified as *T. amonilioides*, produced a higher GI than the positive control DEJ17. The GI of plants propagated with DEJ54, the only isolate not identified at the species level, did not differ significantly from most GIs of plants propagated with *T. amonilioides*, except for DEJ40 and DEJ44 (trunk-associated plants) and DEJ9 (canopy-associated plant) (Table 2). The germination rates and GI of plants propagated in symbiosis with CiMF from trunk-associated orchids did not differ significantly from those propagated with CiMF from canopy-associated orchids (germination: t=-0.594; P=0.554; GI: t=0.451; P=0.653).

Shoot height (SH) was greater in plants from asymbiotic positive control (MS) than in those from the symbiotic positive control (DEJ17) (U=547.0; P=0.0043) (Figure 4A). Plants propagated with the *Tulasnella* isolates DEJ43 (trunk), DEJ49, and DEJ48 (canopy) exhibited lower SH compared to the symbiotic control DEJ17. In contrast, plants propagated with isolates DEJ40 and DEJ45 (trunk), DEJ57, DEJ61, and DEJ34 (canopy), showed higher SH than the symbiotic control (Figure 4A). Plants propagated with DEJ54, DEJ43, DEJ44 (trunk), DEJ49, DEJ48, DEJ60, and DEJ64 (canopy) showed lower SH than the MS control. There was no significant difference in SH between plants propagated with fungi from trunk- and canopy-associated orchids (U=14824.0; P=0.4219). The number of leaves (NL) in CiMF-propagated plants did not differ significantly from the controls, except for DEJ54 (*Tulasnella* sp.), which had fewer leaves. The controls themselves also did not differ in NL (U=843.5; P=0.8805) (Figure 4B). NL in plants propagated with CiMF from trunk-associated orchids was lower than that of plants propagated with canopy-associated CiMF (U=12887.0; P=0.0050).

Root length (RL) in all CiMF treatments was greater than in the asymbiotic control (Figure 4C), which was also observed in the plants grown with the DEJ17 control (U=563.0; P=0.0068).

Figure 2 – Phylogram of the relationships among *Tulasnella* isolates and allied lineages inferred by Maximum Likelihood analysis based on ITS sequences. The support values for the clades are as follows: black circles ≥90%, gray squares ≥70% and <90%, white diamonds ≥60% and <70%, and inverted white triangles ≥50% and <60%.

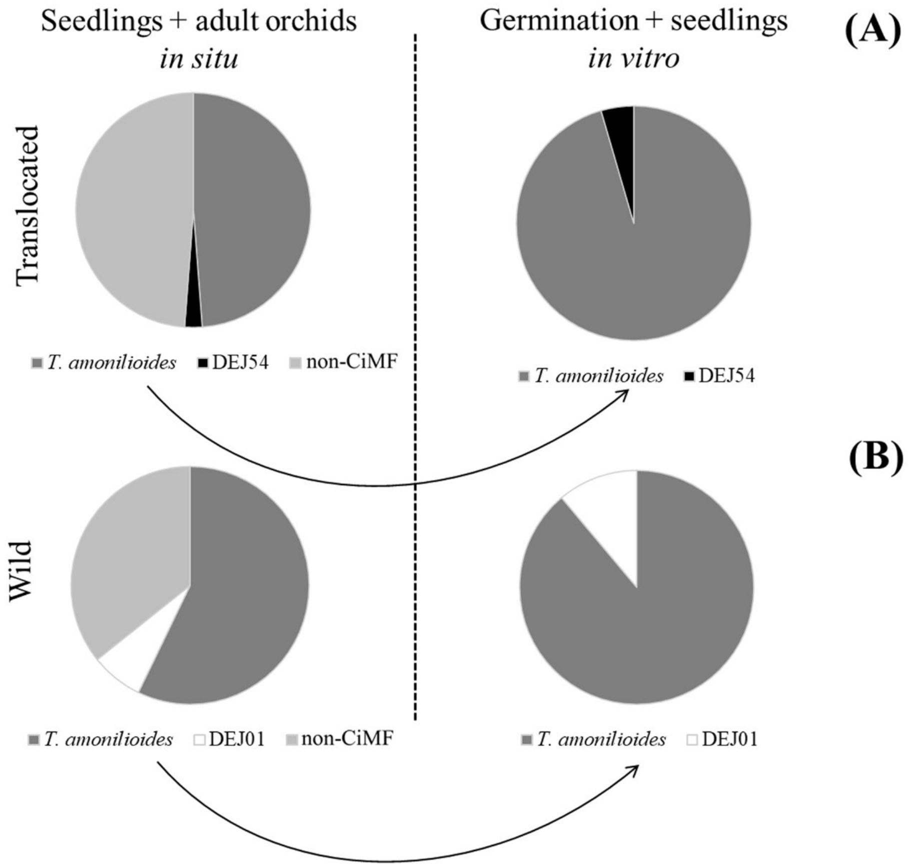

Figure 3 – Pattern of *Cattleya intermedia* mycorrhizal fungi (*T. amonilioides*, DEJ54, DEJ01) and non-CiMF isolated from translocated (A, left) and wild (B, left) seedlings and adult plants of *C. intermedia* and respective proportion of CiMF capable to induce *in vitro* seed germination and seedling development (A and B, right).

Table 2 – Germination and growth index (mean±standard deviation) of *Cattleya intermedia* plants propagated *in vitro* with mycorrhizal fungal isolates obtained from plants translocated to the trunk and canopy of phorophytes, and with positive controls (DEJ17 and MS) and negative control (uninoculated OMA medium)*.

Stratum	Treatment	Seed number	Viable seeds (%)	Germination (G%)	Growth index (GI)	Mean per stratum (%)
Trunk	DEJ54	654	331 (50.5)	96.2±1.5 a*	3.9±0.3 cd	G=97.6
	DEJ40	437	259 (59.2)	96.6±3.4 a	4.4±0.2 ab	
	DEJ43	584	332 (56.6)	98.1±1.3 a	3.9±0.3 d	
	DEJ44	496	337 (67.6)	97.0±2.8 a	4.6±0.1 a	GI=4.2
	DEJ45	384	269 (70.3)	98.8±1.5 a	4.3±0.3 abcd	
Canopy	DEJ49	476	276 (58.0)	97.0±1.3 a	4.5±0.0 ab	G=97.3
	DEJ48	493	272 (55.3)	98.5±1.3 a	3.9±0.7 cd	
	DEJ57	481	293 (61.7)	97.8±1.8 a	4.0±0.0 bcd	
	DEJ60	523	347 (66.0)	98.6±1.7 a	4.3±0.1 abcd	
	DEJ61	535	329 (61.5)	97.0±2.0 a	4.3±0.2 abcd	GI=4.2
	DEJ64	466	281 (60.4)	97.6±3.0 a	4.4±0.2 abc	
	DEJ34	575	351 (61.0)	96.9±1.8 a	4.1±0.1 bcd	

*Means followed by the same letter in the column do not differ according to Tukey's test at a 5% significance level.

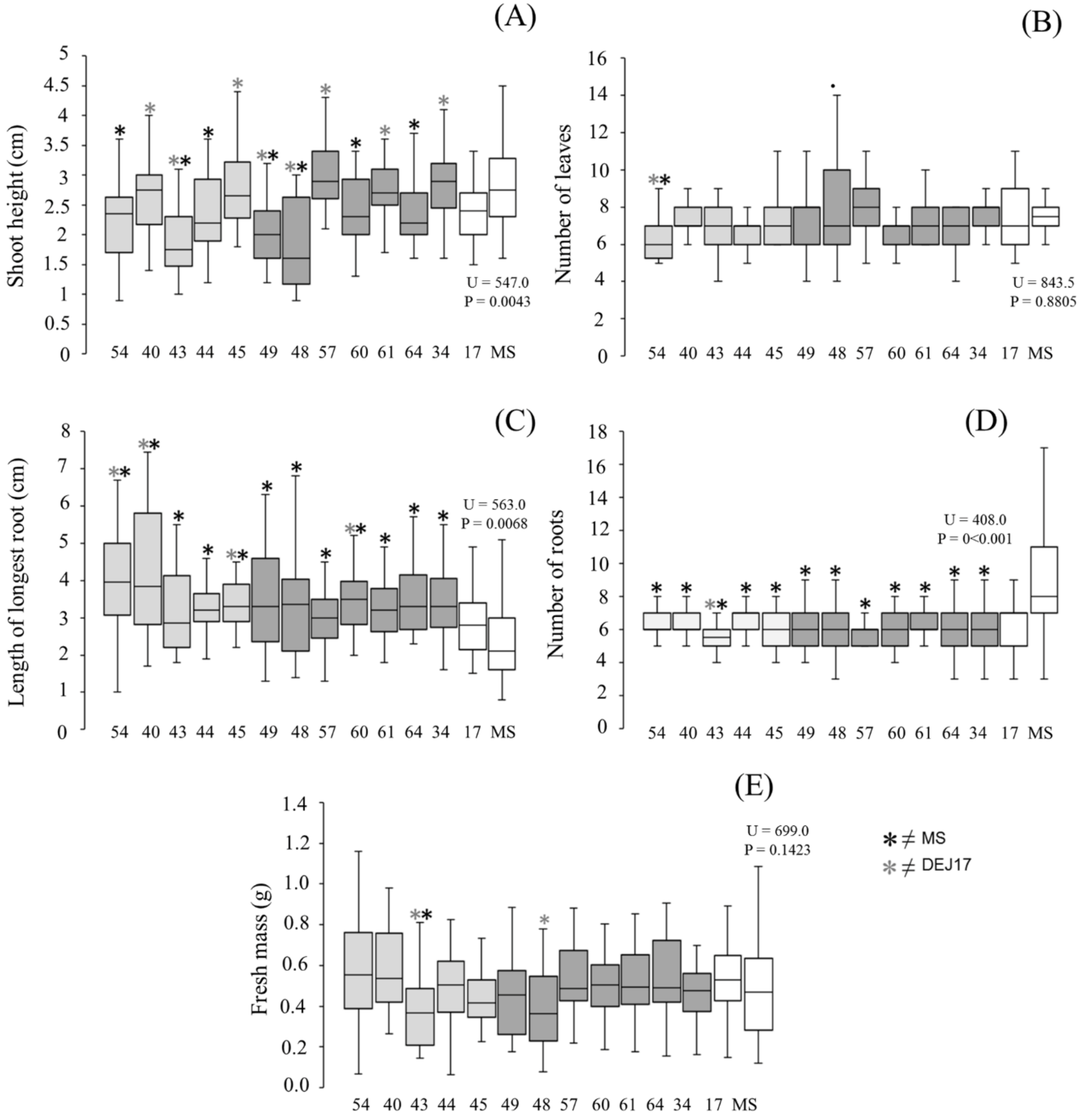

Figure 4 – Boxplot of (A) shoot height, (B) number of leaves, (C) length of the longest root, (D) number of roots, and (E) fresh mass of *Cattleya intermedia* plants propagated *in vitro* with *Tulasnella* isolates obtained from roots of plants translocated to the trunk and canopy of phorophytes. Gray asterisks indicate significant differences compared to the positive control DEJ17, and black asterisks indicate significant differences compared to the positive control MS, according to the Student-Newman-Keuls test at a 5% significance level.

Plants propagated with DEJ54, DEJ40 (trunk), and DEJ60 (canopy) exhibited even greater RL than those in the DEJ17 control. There was no significant difference in RL between plants propagated with trunk-derived and canopy-derived CiMF (U=14444.0; P=0.2315). Root number (RN) was lower in symbiotically propagated plants compared to the MS control (Figure 4D), including those of the DEJ17 control (U=408.0; P<0.001). Plants propagated with DEJ43 had lower RN than those of the DEJ17 control. There was no significant difference in RN between plants propagated with CiMF from trunk or canopy (U=14698.0; P=0.3505).

The fresh mass (FM) of plants propagated with CiMF was similar to those grown in the positive controls, except for DEJ43, which produced lower FM than both DEJ17 and MS, and DEJ48, which produced lower FM than DEJ17 (Figure 4E). There was no significant difference in FM between plants propagated with CiMF from the trunk and the canopy (U=15376.0; P=0.8171), nor between the two positive controls (U=699.0; P=0.1423).

Discussion

Plants obtained through asymbiotic *in vitro* propagation, acclimatized *ex vitro* (Sasamori et al., 2015) and translocated to a forest fragment established associations with *C. intermedia* mycorrhizal fungi (CiMF) as well as with non-CiMF. It is assumed that the transition from the mycoheterotrophic to the autotrophic stage reduces the dependence of the orchid on mycorrhizal associations via their green tissues (Chauhan and Attri, 2024). However, the presence of fungal pelotons (both intact and degraded) in all root segments collected demonstrates that mycobionts remain important for translocated *C. intermedia* individuals even at the autotrophic stage, as previously observed for wild adult individuals of this species (Endres Júnior et al., 2023). Although chlorophyllous tissues are perennial in this species and can be found during different seasons of the year (Endres Júnior et al., 2024), part of the carbon used by photosynthetically capable orchids comes from mycorrhizal fungi (mixotrophy), as well as other nutrients essential for plant metabolism (Selosse et al., 2025). This may be crucial due to the main characteristics of the epiphytic environment, including intermittent water supply and nutrient availability limitations (Benzing, 1990).

Cattleya intermedia showed high fungal specificity regarding seed germination and initial growth *in vitro*, although the large diversity of isolates obtained in the laboratory indicates successful colonization and diversification of relationships established by *C. intermedia* with various fungal groups, characterized by their ability to form intracellular pelotons while not causing necrosis in cortical tissues (Rasmussen and Rasmussen, 2014). The main entry route for mycorrhizal fungi is the seed suspensor, although they can colonize plants through epidermal cells, protocorm rhizoids (Gao et al., 2023), and roots themselves (Attri, 2023). Thus, orchids are constantly colonyzed by other non-mycorrhizal endophytes, whose roles remain poorly understood (Rasmussen and Rasmussen, 2014; Rasmussen et al., 2015) but are believed to be highly diverse and potentially crucial to orchid physiology and adaptation (Li et al., 2021).

The broad diversity of fungi found in the roots of *C. intermedia*, especially considering CiMF, may be related to the translocation (reinforcement) to an area with natural occurrence of the species (IUCN/SSC, 2013). Observations of translocated plants, wild plants in the study area, and seeds propagated *in vitro* allowed for the inference that *C. intermedia* follows the “5” pattern proposed by Rasmussen et al. (2015). In this pattern, the orchid can germinate with a selection of mycorrhizal fungi and develop initially, so that a significant portion of fungi differs at the adult stage, although the initial mycorrhizal relationship is maintained by the persistence of fungi in the roots of adult plants. The same specificity pattern observed in wild plants was recorded in translocated plants, indicating that individuals propagated *in vitro* and taken to the nature successfully established their ecological relationships four years after planting.

The CiMF associated with the translocated plants belong to Tulasnellaceae, one of the main families of rhizoctonia fungi in orchids worldwide, both epiphytic and terrestrial plants (Herrera et al., 2017; Oberwinkler et al., 2017), and are very important for plants from temperate and tropical regions such as Brazil, including the genus *Cattleya* Lindl. (Freitas et al., 2020). Furthermore, most CiMF isolates belong to *T. amonilioides*, the same species isolated from wild plants in the study area (Endres Júnior et al., 2023). Studies discuss the importance of proximity to adult plants of the same species, or even other species, for the availability of individuals in the environment capable of infecting orchid seeds, thus establishing the symbiosis (Batty et al., 2001; Reiter and Menz, 2022).

Our results indicate a higher occurrence of *Tulasnella* in orchids translocated to the canopy compared to those translocated to the trunk, explained by the ability of these fungi to occupy specific niches on the phorophytes, due to the structural compartmentalization of trees, which alters abiotic conditions across different strata (Petrolli et al., 2021; Johnson et al., 2023). Orchid mycorrhizal fungi may occur in nature independently, regardless of the presence of orchids in each microsite, as they feed on organic matter from various sources in the environment (Rasmussen and Rasmussen, 2009). Habitat heterogeneity also affects the distribution and activity of propagules (Batty et al., 2001). Thus, *Tulasnella* lineages likely already occurred in the phorophytes selected for *Cattleya intermedia* translocation, fulfilling specific ecological roles. Due to their ability to degrade organic matter, these organisms may benefit from the greater amount of suspended soil typically found in the canopy or from the specific abiotic conditions in this stratum (Kersten, 2010; Endres Júnior et al., 2024). Therefore, when the orchids were translocated into the habitat, their roots were colonized by both CiMF and non-CiMF fungi, establishing ecological relationships crucial for the success of their translocation (Reiter et al., 2016). Since the translocated *C. intermedia* individuals began flowering from the third year after establishment *in situ* (Endres Júnior et al., 2024), and since these plants are already in association with CiMF, the next generation will likely form a self-sustaining population, thus fulfilling the goals of the translocation (IUCN/SSC, 2013).

The seed germination and growth index of *in vitro* plants inoculated with CiMF isolated from canopy-grown plants were not superior to those inoculated with fungi from trunk-grown plants. However, in the subsequent months of symbiotic culture, not only did CiMF obtained from translocated plants promote better development compared to the positive controls (DEJ17 and MS), but the number of leaves in plants inoculated with canopy-derived CiMF was also higher than those in symbiosis with trunk-derived CiMF. The higher proportion of CiMF present in the roots of canopy-grown plants, combined with the ability of these fungi to promote greater leaf development, may lead to greater success in the establishment of canopy-translocated orchids compared to those on trunks. This hypothesis is supported by the data from the first three years of establishment of the individuals in this study, which show that plants in the canopy stratum exhibited greater vegetative development and reached reproductive maturity (Endres Júnior et al., 2019; 2024).

Previous studies indicate that *C. intermedia* plants establish more successfully and develop more rapidly when translocated to environments with higher light availability (Endres Júnior et al., 2015), where they are likely to make better use of the light for the production of photosynthates. These sugars, in turn, can be utilized by CiMF through the established symbiotic relationship, closing a cycle in which the canopy environment becomes beneficial to the occurrence of fungi, the orchids, and the symbiosis itself. Research has pointed to the interrelation between certain environmental conditions such as the phorophyte structure, canopy openness (Kartzinel et al., 2013), substrate quality, presence of other orchids, and microclimate (Jersáková and Malinová, 2007) — which together form important microsites for orchid recruitment (Reiter and Menz, 2022). Thus, the establishment of new individuals seems to depend not only on the availability of suitable niches for the orchids, but also for compatible mycorrhizal fungi, which is a critical factor for the self-perpetuation of the translocated orchid population (Batty et al., 2001).

Conclusion

The protected area where the orchids were planted, despite consisting of a secondary forest fragment located in an urban matrix and undergoing regeneration for less than three decades has proven to be a suitable environment for the conservation of *C. intermedia*, a species that has suffered a significant population decline in Brazil, from its original endemic status. Our results demonstrate that *T. amonilioides* is not only capable of inducing *in vitro* seed germination but also of promoting the *in vitro* development of *C. intermedia* and generating healthy plants that can be acclimatized and grown *ex vitro*. The data from this study allow us to define the species as mixotrophic in adulthood, benefitting highly by its relationship with fungi of the Tulasnellaceae, especially *T. amonilioides*, a fungus that also belongs to a group known to act as saprotrophs. The fungus-plant specificity pattern was consistent between wild and translocated plants, showing that the *in vitro* propagated individuals successfully rebuilt their ecological relationships four years post-translocation. Thus, this fungus could be translocated to areas where compatible orchid species are intended to be introduced in the future, or it could be introduced together with the *C. intermedia*, potentially triggering beneficial changes that may also facilitate the establishment of other orchid species.

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Authors' Contributions

Endres Júnior, D.: conceptualization; data curation; formal analysis; investigation; methodology; project administration; validation; writing – original draft; writing – review & editing; **Silva**, G. A.: data curation; formal analysis; investigation; methodology; project administration; validation; writing – review & editing; **Sasamori**, M. H.: conceptualization; data curation; formal analysis; investigation; methodology; project administration; validation; writing – original draft; **Silveira**, R. M. B.: funding acquisition; administration; resources; supervision; validation; writing – review & editing; **Droste**, A.: conceptualization; data curation; formal analysis; funding acquisition; methodology; project administration; resources; supervision; validation; writing – original draft; writing – review & editing.

References

Airin, A.A.; Arafat, M.I.; Begum, R.A.; Islam, M.R.; Seraj, Z.I., 2023. Plant growth-promoting endophytic fungi of the wild halophytic rice *Oryza coarctata*. Annals of Microbiology, v. 73, Article 36. https://doi.org/10.1186/s13213-023-01738-3.

Almeida, C.E.A.; Ruppenthal, C.D.; Boniek, D.; Mendes, R.S.M.; Scotti, M.R., 2025. Tripartite Orchid–OMF–AMF symbioses in the Brazilian *campo rupestre*: implications for plant and soil nitrogen dynamics. Rhizosphere, v. 36, Article 101169. https://doi.org/10.1016/j.rhisph.2025.101169.

Almeida, P.R.M.; Van den Berg, C.; Goes-Neto, A., 2014. *Epulorhiza amonilioides* sp. nov.: A new anamorphic species of orchid mycorrhiza from Brazil. Neodiversity, v. 7, (1), 1-10. https://doi.org/10.13102/neod.71.1.

Alvares, C.A.; Stape, J.L.; Sentelhas, P.C.; Gonçalves, J.L.M.; Sparovek, G., 2013. Köppen's climate classification map for Brazil. Meteorologische Zeitschrift, v. 22, (6), 711-728. https://doi.org/10.1127/0941-2948/2013/0507.

Arifin, R.R.; May, T.W.; Linde, C.C., 2021. New species of *Tulasnella* associated with Australian terrestrial orchids in the Cryptostylidinae and Drakaeinae. Mycologia, v. 113, (1), 212-230. https://doi.org/10.1080/00275514.2020.1813473.

Arifin, R.R.; Reiter, N.H.; May, T.W.; Linde, C.C., 2022. New species of *Tulasnella* associated with Australian terrestrial orchids in the subtribes Megastylidinae and Thelymitrinae. Mycologia, v. 114, (2), 388-412. https://doi.org/10.1080/00275514.2021.2019547.

Attri, L.K., 2023. Studies on mycorrhizal associations in an orchid. Asian Journal of Biological and Life Sciences, v. 12, (1), 179-186. https://doi.org/10.5530/ajbls.2023.12.24.

Batty, A.L.; Dixon, K.W.; Brundrett, M.; Sivasithamparam, K., 2001. Constraints to symbiotic germination of terrestrial orchid seed in a mediterranean bushland. New Phytologist, v. 152, (3), 511-520. https://doi.org/10.1046/j.0028-646X.2001.00277.x.

Benzing, D.H., 1990. Vascular epiphytes: general biology and related biota. Cambridge University Press, Cambridge, 376 p.

Chauhan, P.; Attri, L.K., 2024. Mycorrhizal associations in oOrchids: A review. Asian Journal of Biological and Life Science, v. 13, (3), 278-286. https://doi.org/10.5530/ajbls.2024.13.36.

Crous, P.W.; Costa, M.M.; Kandemir, H.; Vermaas, M.; Vu, D. et al., 2023. Fungal Planet description sheets: 1550-1613. Persoonia, v. 51, 280-417. https://doi.org/10.3767/persoonia.2023.51.08.

Dentinger, B.T.M.; Margaritescu, S.; Moncalvo, J.-M.M., 2010. Rapid and reliable high-throughput methods of DNA extraction for use in barcoding and molecular systematics of mushrooms. Molecular Ecology Resources, v. 10, (4), 628-633. https://doi.org/10.1111/j.1755-0998.2009.02825.x.

Dixon, K.W., 1987. Raising terrestrial orchids from seed. In: Harris, W.K. (Ed.), Modern orchid growing for pleasure and profit. Orchid Club of South Australia, Adelaide, pp. 47-100.

Eaton, D.A.R., 2020. Toytree: A minimalist tree visualization and manipulation library for Python. Methods in Ecology and Evolution, v. 11, (1), 187-191. https://doi.org/10.1111/2041-210X.13313.

Endres Júnior, D.; Alves-Silva, G.; Sasamori, M.H.; Siveira, R.M.B.; Droste, A., 2023. Successful *Tulasnella amonilioides* isolation from wild *Cattleya intermedia* and effectiveness of the mycobiont on *in vitro* propagation of this threatened Orchidaceae. Journal of Environmental Analysis and Progress, v. 8, (1), 9-29. https://doi.org/10.24221/jeap.8.1.2023.5160.009-029.

Endres Júnior, D.; Alves-Silva, G.; Silveira, R.M.B.; Droste, A., 2025. Data for "Towards reversing the red: *Tulasnella amonilioides* highly contributes to the *Cattleya intermedia* translocation success". Harvard Dataverse. https://doi.org/10.7910/DVN/ZBYVEZ.

Endres Júnior, D.; Sasamori, M.H.; Petry, C.T.; Droste, A., 2024. Responses of translocated *Cattleya intermedia* (Orchidaceae) to environmental key features in a vertical gradient of subtropical forest, Brazil. Revista Brasileira de Geografia Física, v. 17, (1), 668-688. https://doi.org/10.26848/rbgf.v17.1.p668-688.

Endres Júnior, D.; Sasamori, M.H.; Petry, C.T.; Santos, M.S.; Droste, A., 2019. Host tree bark traits and development of reintroduced *Cattleya intermedia* (Orchidaceae) plants in Southern Brazil. Rodriguésia, v. 70, Article e03092017. https://doi.org/10.1590/2175-7860201970046.

Endres Júnior, D.; Sasamori, M.H.; Silveira, T.; Schmitt, J.L.; Droste, A., 2015. Reintrodução de *Cattleya intermedia* Graham (Orchidaceae) em borda e interior de um fragmento de Floresta Estacional Semidecidual no sul do Brasil. Revista Brasileira de Biociências, v. 13, (1), 33-40.

Fernández, M.; Kaur, J.; Sharma, J., 2023. Co-occurring epiphytic orchids have specialized mycorrhizal fungal niches that are also linked to ontogeny. Mycorrhiza, v. 33, (1-2), 87-105. https://doi.org/10.1007/s00572-022-01099-w.

Freestone, M.; Reiter, N.; Swarts, N.D.; Linde, C.C., 2024. Temporal turnover of Ceratobasidiaceae orchid mycorrhizal fungal communities with ontogenetic and phenological development in Prasophyllum (Orchidaceae). Annals of Botany, v. 134, (6), 933-948. https://doi.org/10.1093/aob/mcae089.

Freitas, E.F.S.; Silva, M.; Cruz, E.S.; Mangaravite, E.; Bocayuva, M.F. et al., 2020. Diversity of mycorrhizal *Tulasnella* associated with epiphytic and rupicolous orchids from the Brazilian Atlantic Forest, including four new species. Scientific Reports, v. 10, Article 7069. https://doi.org/10.1038/s41598-020-63885-w.

Fujimori, S.; Abe, J.P.; Okane, I.; Yamaoka, Y., 2019. Three new species in the genus *Tulasnella* isolated from orchid mycorrhiza of *Spiranthes sinensis* var. *amoena* (Orchidaceae). Mycoscience, v. 60, (1), 71-81. https://doi.org/10.1016/j.myc.2018.09.003.

Gale, S.W.; Fischer, G.A.; Cribb, P.J.; Fay, M.F., 2018. Orchid conservation: bridging the gap between science and practice. Botanical Journal of the Linnean Society, v. 186, (4), 425-434. https://doi.org/10.1093/botlinnean/boy003.

Gao, X.; Wang, Y.; Deng, D.; Luo, Y.; Shao, S. et al., 2023. Morphogenesis changes in protocorm development during symbiotic seed germination of *Dendrobium chrysotoxum* (Orchidaceae) with its mycobiont, *Tulasnella* sp. Horticulturae, v. 9, (5), Article 531. https://doi.org/10.3390/horticulturae9050531.

Góes-Neto, A.; Loguercio-Leite, C.; Guerrero, R.T., 2005. DNA extraction from frozen field-collected and dehydrated herbarium fungal basidiomata: performance of SDS and CTAB based methods. Biotemas, v. 18, (2), 19-32.

Gonçalves, C.N.; Waechter, J.L., 2003. Aspectos florísticos e ecológicos de epífitos vasculares sobre figueiras isoladas no norte da planície costeira do Rio Grande do Sul. Acta Botanica Brasilica, v. 17, (1), 89-100. https://doi.org/10.1590/S0102-33062003000100007.

Herrera, H.; Valadares, R.; Contreras, D.; Bashan, Y.; Arriagada, C., 2017. Mycorrhizal compatibility and symbiotic seed germination of orchids from the Coastal Range and Andes in south centra Chile. Mycorrhiza, v. 27, 175-188. https://doi.org/10.1007/s00572-016-0733-0.

Hsu, R. C.C.; Chen, Y.C.; Lin, C., 2024. The impact of changing climate on an endangered epiphytic orchid (*Pleione formosana*) in a montane cloud forest and the conservation challenge ahead. Plants, v. 13, (17), Article 2414. https://doi.org/10.3390/plants13172414.

Instituto Brasileiro de Geografia e Estatística (IBGE), 2012. Manual técnico da vegetação brasileira. IBGE, Rio de Janeiro, 272 p.

International Union for Conservation of Nature, Species Survival Commission (IUCN/SSC), 2013. Guidelines for reintroductions and other conservation translocations, version 1.0. IUCN Species Survival Commission, Gland, 57 p. (Accessed October 10, 2024) at:. https://portals.iucn.org/library/efiles/documents/2013-009.pdf.

Jersáková, J.; Malinová, T., 2007. Spatial aspects of seed dispersal and seedling recruitment in orchids. New Phytologist, v. 176, (2), 237-241. https://doi.org/10.1111/j.1469-8137.2007.02223.x.

Johnson, L.J.A.N.; Kane, M.E.; Zettler, L.W.; Mueller, G.M., 2023. Diversity and specificity of orchid mycorrhizal fungi in a leafless epiphytic orchid, *Dendrophylax lindenii* and the potential role of fungi in shaping its fine-scale distribution. Frontiers in Ecology and Evolution, v. 11, Article 1057940. https://doi.org/10.3389/fevo.2023.1057940.

Kartzinel, T.R.; Trapnell, D.W.; Shefferson, R.P., 2013. Critical importance of large native trees for conservation of a rare Neotropical epiphyte. Journal of Ecology, v. 101, (6), 1429-1438. https://doi.org/10.1111/1365-2745.12145.

Kersten, R.A., 2010. Epífitas vasculares - histórico, participação taxonômica e aspectos relevantes, com ênfase na Mata Atlântica. Hoehnea, v. 37, (1), 9-38. https://doi.org/10.1590/S2236-89062010000100001.

Lee, Y.I.; Yeung, E.C., 2023. The orchid seed coat: a developmental and functional perspective. Botanical Studies, v. 64, (1), Article 27. https://doi.org/10.1186/s40529-023-00400-0.

Li, T.; Wu, S.; Yang, W.; Selosse, M.-A.; Gao, J., 2021. How mycorrhizal associations influence orchid distribution and population dynamics. Frontiers in Plant Science, v. 12, Article 647114. https://doi.org/10.3389/fpls.2021.647114.

Menini Neto, L.; Barros, F.; Vinhos, F.; Furtado, S.G.; Judice, D.M. et al., 2013. Orchidaceae. In: Martinelli, G.; Moraes, M.A. (Eds.), Livro vermelho da Flora do Brasil. Jardim Botânico do Rio de Janeiro, Rio de Janeiro, pp. 749-818. (Accessed September 11, 2024) at:. https://dspace.jbrj.gov.br/jspui/handle/doc/26.

Murashige, T.; Skoog, F.A., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, v. 15, (3), 473-497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

Nogueira, P.T.S.; Freitas, E.F.S.; Silva, J.A.R.; Kasuya, M.C.M.; Pereira, O.L., 2024. Efficiency of mycorrhizal fungi for seed germination and protocorms development of commercial C*attleya* species (Orchidaceae). Brazilian Journal of Microbiology, v. 56, 589-599. https://doi.org/10.1007/s42770-024-01597-3.

Novo Hamburgo, 1999. Lei Complementar nº 167, de 1 de março de 1999. Aprova o Plano de Manejo do Parque Municipal Henrique Luís Roessler, estabelece as normas de uso e ocupação da área e dá outras providências (Accessed October 15, 2024) at:. https://leismunicipais.com.br/a/rs/n/novo-hamburgo/lei-complementar/1999/17/167/lei-complementar-n-167-1999-aprova-o-plano-de-manejo-do-parque-municipal-henrique-luis-roessler-estabelece-as-normas-de-uso-e-ocupacao-da-area-e-da-outras-providencias.

Oberwinkler, F.; Cruz, D.; Suárez, J.P., 2017. Biogeography and ecology of Tulasnellaceae. In: Tedersoo, L. (Ed.), Ecological Studies. Springer, Cham, v. 230, pp. 237-271. https://doi.org/10.1007/978-3-319-56363-3_12.

Pecoraro, L.; Rasmussen, H. N.; Gomes, S. I. F.; Wang, X.; Merckx, V. S. F. T. et al., 2021. Fungal diversity driven by bark features affects phorophyte preference in epiphytic orchids from southern China. Scientific Reports, v. 11, (1), Article 11287. https://doi.org/10.1038/s41598-021-90877-1.

Petrolli, R.; Vieira, C.A.; Jakalski, M.; Bocayuva, M.F.; Vallé, C. et al., 2021. A fine-scale spatial analysis of fungal communities on tropical tree bark unveils the epiphytic rhizosphere in orchids. New Phytologist, v. 231, (5), 2002-2014. https://doi.org/10.1111/nph.17459.

Phillips, R.D.; Reiter, N.; Peakall, R., 2020. Orchid Conservation: From theory to practice. Annals of Botany, v. 126, (3), 345-362. https://doi.org/10.1093/aob/mcaa093.

Qin, J.; Feng, J.-Q.; Zhang, W.; Zhang, S.-B., 2021. Mycorrhizal fungal partners remain constant during a root lifecycle of pleione bulbocodioides (Orchidaceae). *Journal of Fungi*, v. 7, (11), 994. https://doi.org/10.3390/jof7110994.

Ramsay, M.M.; Dixon, K.W., 2003. Propagation science, recovery and translocation of terrestrial orchids. In: Dixon, K.W.; Kell, S.P.; Barrett, R.L.; Cribb, P.J. (Eds.), Orchid conservation. Natural History Publications, Kota Kinabalu, pp. 259-288.

Rasmussen, H.N.; Dixon, K.W.; Jersáková, J.; Těšitelová, T., 2015. Germination and seedling establishment in orchids: a complex of requirements. Annals of Botany, v. 116, (3), 391-402. https://doi.org/10.1093/aob/mcv087.

Rasmussen, H.N.; Rasmussen, F.N., 2009. Orchid mycorrhiza: implications of a mycophagous life style. Oikos, v. 118, 334-345. https://doi.org/10.1111/j.1600-0706.2008.17116.x.

Rasmussen, H.N.; Rasmussen, F.N., 2014. Seedling mycorrhiza: A discussion of origin and evolution in Orchidaceae. Botanical Journal of the Linnean Society, v. 175, (3), 313-327. https://doi.org/10.1111/boj.12170.

Reiter, N.; Menz, M.H.M., 2022. Optimizing conservation translocations of threatened *Caladenia* (Orchidaceae) by identifying adult microsite and germination niche. Australian Journal of Botany, v. 70, (3), 231-247. https://doi.org/10.1071/BT21132.

Reiter, N.; Whitfield, J.; Pollard, G.; Bedggood, W.; Argall, M. et al., 2016. Orchid re-introductions: an evaluation of success and ecological considerations using key comparative studies from Australia. Plant Ecology, v. 217, 81-95. https://doi.org/10.1007/s11258-015-0561-x.

Rio Grande do Sul, 2014. Decreto nº 52.109, de 1 de dezembro de 2014. Declara as espécies da flora nativa ameaçadas de extinção no Estado do Rio Grande do Sul. Lex-Diário Oficial do Rio Grande do Sul, ano LXXII, (233), 2-11.

Sasamori, M.H.; Endres Júnior, D.; Droste, A., 2015. Asymbiotic culture of *Cattleya intermedia* Graham (Orchidaceae): the influence of macronutrient salts and sucrose concentrations on survival and development of plantlets. Acta Botanica Brasilica, v. 29, (3), 292-298. https://doi.org/10.1590/0102-33062014abb0054.

Selosse, M.A.; Alaux, P.L.; Deloche, L.; Delannoy, E.; Minasiewicz, J. et al., 2025. Mixotrophy in orchids: facts, questions, and perspectives. New Phytologist, v. 246, (5), 1912-1921. https://doi.org/10.1111/nph.70106.

Shao, S.C.; Wang, Q.X.; Beng, K.C.; Zhao, D.K.; Jacquemyn, H., 2020. Fungi isolated from host protocorms accelerate symbiotic seed germination in an endangered orchid species (*Dendrobium chrysotoxum*) from southern China. Mycorrhiza, v. 30, 529-539. https://doi.org/10.1007/s00572-020-00964-w.

Tikendra, L.; Singh, A. R.; Vendrame, W.; Nongdam, P., 2025. *In vitro* propagation of endangered *Vanda coerulea* Griff. ex Lindl.: asymbiotic seed germination, genetic homogeneity assessment, and micro-morpho-anatomical analysis for effective conservation. Agronomy, v. 15, (5), Article 1195. https://doi.org/10.3390/agronomy15051195.

Van den Berg, C., 2024. *Cattleya* in Flora e Funga do Brasil. Jardim Botânico do Rio de Janeiro (Accessed October 15, 2024) at:. https://floradobrasil.jbrj.gov.br/FB11338.

Wu, K.; Zeng, S.; Lin, D.; Silva, J.A.T.; Bu, Z. et al., 2014. *In vitro* propagation and reintroduction of the endangered *Renanthera imschootiana* Rolfe. PLoS One, v. 9, (10), Article e110033. https://doi.org/10.1371/journal.pone.0110033.

Yeh, C.M.; Chung, K.; Liang, C.K.; Tsai, W.C., 2019. New insights into the symbiotic relationship between orchids and fungi. Applied Sciences, v. 9, (3), Article 585. https://doi.org/10.3390/app9030585.

Zahn, F.E.; Söll, E.; Chapin, T.K.; Wang, D.; Gomes, S.I.F. et al., 2023. Novel insights into orchid mycorrhiza functioning from stable isotope signatures of fungal pelotons. New Phytologist, v. 239, (4), 1449-1463. https://doi.org/10.1111/nph.18991.

Zhao, D-K.; Mou, Z-M.; Ruan, Y-L., 2024. Orchids acquire fungal carbon for seed germination: pathways and players. Trends in Plant Science, v. 29, (7), 733-741. https://doi.org/10.1016/j.tplants.2024.02.001.

Zhao, D-K.; Selosse, M-A.; Wu, L.; Luo, Y.; Shao, S-C. et al., 2021. Orchid reintroduction based on seed germination-promoting mycorrhizal fungi derived from protocorms or seedlings. Frontiers in Plant Science, v. 12, Article 701152. https://doi.org/10.3389/fpls.2021.701152.