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# Successful *Tulasnella amonilioides* isolation from wild *Cattleya intermedia* and effectiveness of the mycobiont on *in vitro* propagation of this threatened Orchidaceae

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**ABSTRACT**

This is the first study that reports symbiosis in *Cattleya*, aiming to isolate and identify mycorrhizal fungi capable of promoting the germination of this orchid and to evaluate the development of symbiotically propagated individuals. We compared seed germination percentage, growth index, and morphometric variables of seedlings propagated symbiotically in oatmeal agar (OMA) medium with individuals that were non-symbiotically propagated in Murashige and Skoog (MS) medium. Fungi isolates were identified by phylogenetic analysis and eight of the nine isolates that were efficient in C. *intermedia* propagation were identified as *Tulasnella amonilioides*. The mycobiont improved *C. intermedia* seed germination and plant development when compared with OMA medium without fungi (negative control). Seedlings propagated by symbiotic culture with *T. amonilioides* produced more leaves and longer roots, while shoot height and a number of roots were lower than for seedlings propagated in MS medium with the addition of activated charcoal. The fresh mass of seedlings propagated by symbiotic and a symbiotic techniques was equal, except when seedlings were grown in MS without activated charcoal. *T. amonilioides* enhance the *in vitro* propagation of *C. intermedia* and provide plants that facilitate symbiotic processes in reintroduction environments.

**Keywords:** Epiphytic orchid, *Epulorhiza* sp., orchid conservation, phylogenetic analysis, symbiosis.

## Introduction

Orchids produce small seeds that lack nutritional content for germination and the initial development of the seedlings (Arditti, 1967). In nature, such seeds are infected by hyphae of mycorrhizal fungi that grow inside the parenchyma cells forming pelotons, intracellular structures that characterize the orchid-fungus interaction (Peterson, Massicotte & Melville, 2004). These plants associate with diverse fungal taxa, including nonmycorrhizal endophytic fungi as well as mycorrhizal fungi (Selosse, 2014; Novotná et al., 2018), generally members of Atractiellomycetes, Ceratobasidiaceae, Serendipitaceae and Tulasnellaceae (Suárez et al., 2006; Cevallos et al., 2017; Herrera et al., 2017; Zettler & Dvorak, 2021).

Degradation and nutrient consumption of pelotons provide the energy for protocorm development, which is considered a heterotrophic stage of the orchid's life cycle (Rasmussen, 1995). This strategy is denominated mycoheterotrophy,

and the plants may retain the mutualistic relationship as a dynamic process during adulthood (Merckx, 2013). In this stage, the mycorrhizal fungi can be found mainly inside root cortical cells performing an important role in nutrient supply (Cameron et al., 2007, 2008), as a source of energy supplementary to photosynthesis (mixotrophy or partial mycoheterotrophy), and as an inoculum for seeds that can be dispersed by the mother plant (Rasmussen, 1995; Pereira et al., 2005).

Most epiphytic orchids are easily *in vitro* propagated using complex culture media composed of inorganic salts and sucrose, which provide the nutritional conditions for seed germination and plant development (Arditti, 1992; Knudson, 1921; Otero & Bayman, 2009). However, symbiotic germination is an important tool for epiphytic orchid species propagation for conservation purposes (Zettler et al., 2013; Meng et al., 2019). The establishment of mycotrophy under laboratory conditions (Zettler, 1997) allows a better understanding of the relationships between orchids and fungi (Zettler et al., 1999). Symbiosis and orchid germination occur by inoculation in oatmeal-agar medium (OMA-Dixon, 1987), which acts as a complex carbon source for fungus nutrition. Symbiosis may induce faster germination and a higher growth index in non-epiphytic orchids, making protocorms stronger, more robust, and resistant to infections (Brundrett et al., 2003; Guimarães et al., 2013; Jiang et al., 2015; Pereira et al., 2015; Alomía et al., 2017; Durán-López et al., 2019).

Over the last two decades, studies have appeared about symbiotic cultivation of rupicolous and epiphytic species (Otero, Bayman & Ackerman, 2005; Otero et al., 2007; Zettler, Poulter & McDonald, 2007; Aggarwal et al., 2012; Sathiyadash et al., 2014; Decruse et al., 2018), including Brazilian orchids from the Atlantic Forest (see Freitas et al., 2020; Bazzicalupo et al., 2021). However, most of them focused on the taxonomy, cultivation, and isolation stages of fungi, and on the initial stages of orchid development (protocorm and plant with the first leaves and roots) (Pereira et al., 2003; 2005; 2009; 2014; Freitas et al., 2020; Bazzicalupo et al., 2021).

*Cattleya intermedia* Graham is one of the 97 species of *Cattleya* Lindl. that is endemic to Brazil (van den Berg, 2020). The species has high genetic variability and is one of the most variable of the genus considering flower color and shape (Machado Neto & Vieira, 2011). The multi-flowered inflorescences start being produced precociously, within three years after sowing (Fowlie, 1977; Withner, 1988), and due to these desirable characteristics for breeding and trading, the species has been used in interspecific and intergeneric hybridization worldwide (OrchidRoots, 2022). *Cattleya intermedia* is a threatened species due to direct and indirect anthropogenic pressures, such as loss of suitable habitat, irregular collection for ornamental purposes, and introduction of exotic herbivores to its habitats (Endres Júnior et al., 2015; Menini Neto et al., 2013). As result, this species is classified in the Vulnerable (VU) category on the national red list. Remnant populations of this epiphytic species have been declining, with more than 30% being lost in the last 50 years (Menini Neto et al., 2013).

*In vitro* culture is an important tool for *C. intermedia* propagation, and this species can develop well in a symbiotic MS medium (Murashige & Skoog, 1962; Sasamori et al., 2015). However, as the maintenance of orchids in restored habitats requires the presence of an appropriate fungus for plant recruitment (Zettler, 1997), the reintroduction of individuals obtained by a symbiotic process can favor the establishment of new populations since these plants can serve as inoculum source for the infection of seeds (Stewart & Zettler, 2002).

Knowledge about the relationship between *C. intermedia* and mycorrhizal fungi in nature and even under controlled conditions is lacking. Thus, to isolate and identify mycorrhizal fungi capable of promoting germination of *C. intermedia*, as well as to evaluate the development of symbiotically propagated individuals, we (1) isolated fungal strains from plant roots of a wild population in South Brazil; (2) performed a phylogenetic analysis of the fungal isolates; and (3) evaluated seed germination and plant growth with the isolated mycorrhizal fungi.

## Material and Methods

*Study site and plant source*

The roots of *C. intermedia* were collected in the Henrique Luís Roessler Area of Relevant Ecological Interest (29°41'S, 51°06'W, alt. 16.4 m), a municipal conservation unit inserted in the urban matrix of the municipality of Novo Hamburgo in the state of Rio Grande do Sul (RS) Brazil. The conservation unit encompasses an area of 51.4 ha, composed of grasslands, wetlands, and a portion of secondary forest, in an area of transition between the Pampa and Atlantic Forest phytogeographic domains (IBGE & MMA, 2004). The isolation of endophytic fungi from *C. intermedia* roots and in vitro seed germination evaluation was conducted at the Laboratory of Plant Biotechnology at Feevale University in Novo Hamburgo, RS.

*Fungal isolation and culture*

Roots were collected in September 2017 from seven mature specimens of *C. intermedia* that were growing on arboreal phorophytes located in an area of secondary forest (Figure 1A). A 30 cm root sample was collected from each plant and immediately taken to the laboratory, where its extremities (segments of 2 cm) were washed with tap water. The samples were then surface sterilized in a laminar flow chamber for 1 min in 70% ethanol and 6 min in 2% NaClO solution followed by three rinses in sterile distilled water. The root segments were cut manually in the transverse direction in sterile dishes under a stereomicroscope (Labomed CZM4) to observe fungal pelotons, whose presence was confirmed by observation under 400x magnification (Olympus CX4 microscope; Figure 1B). The cortical layer from 10 cross-sections of each root segment was removed with a scalpel and transferred to two Petri dishes (9 cm diameter) containing potato dextrose agar (PDA) medium. Thus, the sampling effort was as follows: root sample from each of the seven plants x two segments of each root x ten transverse sections distributed in two dishes with PDA, for a total of 140 cuts containing pelotons. Dishes were incubated in the dark at 25ºC for a few days until fungal hyphae could be observed growing from the pelotons (Sharma et al., 2003). Pure cultures were obtained by excising hyphal tips with a sterile scalpel, transferring them to dishes with PDA, and incubating them under the same conditions.

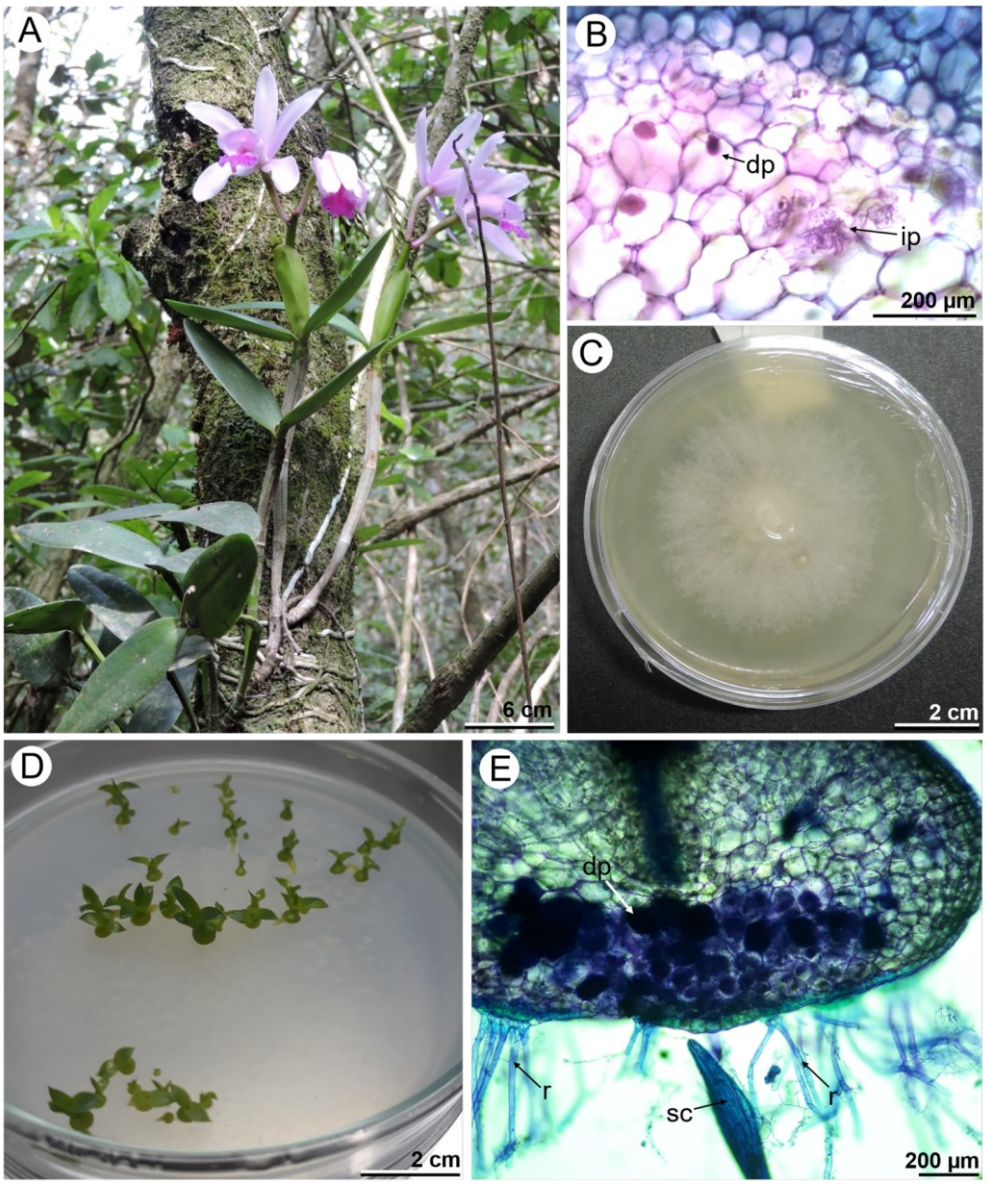


Figure 1. (A) Flowering individual of *Cattleya intermedia in situ* on an arboreal phorophyte; (B) degraded (dp) and intact (ip) pelotons observed in cortical cells of *C. intermedia* root; (C) obtained isolate growing in potato dextrose agar (PDA) in a 9 cm diameter Petri dish; (D) plants of *C. intermedia* after 91 days growing in oatmeal agar (OMA); and (E) transversal cut of a protocorm with pelotons at the basal region. Font: Endres Júnior et al. (2023).

*Fungal molecular characterization: DNA extraction, amplification, and sequencing*

Molecular analysis was performed in the Laboratory of Mycology at the Federal University of Rio Grande do Sul, Porto Alegre, RS. A portion of mycelium was excised from the pure cultures of each of the nine isolates and DNA was extracted by the CTAB method, according to Góes-Neto, Loguercio-Leite & Guerrero (2005). The primer pair ITS8F-ITS6R (Dentinger Margaritescu & Moncalvo, 2010) was used to amplify the nuclear rDNA internal transcribed spacer region, ITS1-5.8S-ITS2 (ITS). Polymerase chain reaction (PCR) was performed with a total volume of 40 µL, containing 20 µL of 2X PCR Taq MasterMix (Applied Biological Material, Vancouver, Canada), 0.8 µL of primer (10 pM), 1 to 2 µL of DNA, and q.s. sterile distilled water. All PCR products were purified with PEG 20% [Poly (ethylene glycol) 8,000 plus NaCl 2.5M] and sequenced by Macrogen (Geumcheon-gu, Korea).

*Fungal molecular characterization: phylogenetic analysis*

Sequences were assembled and manually corrected with Geneious 9 (Kearse et al., 2012), then automatically aligned with MAFFT 7 (Katoh & Standley, 2013) under the "auto" mode strategy. When necessary, the alignment was manually adjusted with MEGA 7 (Kumar, Stecher & Tamura, 2016). Ambiguously aligned regions with high proportions of gaps were manually excluded. Single-gene phylogenetic analyses were carried out with ITS sequences. Two data sets were used. One of them (1) was more inclusive and exploratory to comprehend the diversity available in the group and to cover the heterogeneity previously reported for ribosomal DNA of the genus *Tulasnella* (Moncalvo et al., 2006; Cruz et al., 2014).

First, through the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov), using the Somewhat Similar Sequences (blastn) option under Program Selection, the 100 closest specimens to each one of the sequences found in this study were obtained. In addition, searches of GenBank database with the following keywords were carried out: "(*Epulorhiza*) AND 5.8s", "(*Tulasnella*) AND 5.8s", and "(Tulasnellaceae) AND 5.8s." All recovered sequences were downloaded as ".gb" files and a final ".xlsx" file was built with "Merging_gbfiles_to_xlsx.py" script, which was implemented in Python and is available at https://github.com/genivaldo-alves-silva/Phyloroom (Endres Júnior et al., 2022). Based on closely related species and other supported clades (considering the Maximum likelihood tree) from the abovementioned data set (Endres Júnior et al., 2022), representative specimens of the recovered clades were selected for the second (2) data set (Table 1).

Alignment data are available in Endres-Júnior et al. (2022). All sequences are available at GenBank. We designated *Ceratorhiza* sp. (Ceratobasidiaceae, Cantharellales) and *Serendipita vermifera* (Sebacinaceae, Sebacinales) as outgroup taxa, according to Gónzalez et al. (2016) and Moncalvo et al. (2006).

All phylogenetic analyses were performed online at CIPRES Science Gateway (Miller Pfeiffer & Schwartz, 2011). We analyzed both data sets using Maximum likelihood (ML), and Bayesian inference (BI) for the second data set (2). Maximum likelihood analysis was carried out in RAxML 8.2.9 (Stamatakis, 2014). We provided a partition file to force RAxML software to search for a separate evolution model for each data set. To assess the reliability of the nodes, we computed rapid bootstrapping replicates under the same model, allowing the program to halt bootstrapping automatically by the majority rule extended (MRE)-based boot-stopping criterion (Pattengale et al., 2009). Bootstrap (BS) values above 80 were considered significant (high support) and above 70 were considered moderately supported.

Bayesian inference was performed with Mr. Bayes v. 3.2.6 (Ronquist et al., 2012), with the evolutionary model for BI being estimated using the Akaike Information Criterion (AIC) for each partition, as implemented in MrModeltest 2.3 (Nylander, 2004). The best fitting model identified was General Time Reversible + Proportion Invariant + Gamma (GTR + I + G). We set two independent runs, each with four simultaneous chains for 50,000,000 generations, sampling trees at every 100th generation. The convergence diagnostic was calculated every 10,000th generation, and its critical value was set to stop the analysis automatically when the standard deviation of the split frequencies reached the value defined by the stopval command (stoprule = yes, stopval = 0.01). The first 25% of trees from each run were discarded as burn-in and the 50% majority-rule tree with branch lengths and posterior probabilities (BPP) was calculated from the remaining trees. A BPP value above 0.99 was considered significant (high support) and above 0.95 was considered moderately supported.

Table 1. Summary of specimens included in molecular analyses. New sequences generated in this study are marked in bold. Font: Endres Júnior et al. (2023).

| Species | Genbank | Voucher | Location | Host | Reference |
|---|---|---|---|---|---|
| *Tulasnella albida* | KC152379 | K(M)120788 | United Kingdom | N/A* | Cruz et al. (2014) |
| *Tulasnella albida* | AY373294 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnella amonilioides* | JF907599 | Brass | Brazil | *Brassavola tuberculata* | Almeida, Van den Berg & Góes-Neto (2014) |
| *Tulasnella amonilioides* | JF907600 | 3S | Brazil | *Encyclia dichroma* | Almeida, Van den Berg & Góes-Neto (2014) |
| *Tulasnella amonilioides* | JF907601 | 09ghy | Brazil | *Encyclia ghillanyi* | Almeida, Van den Berg & Góes-Neto (2014) |
| ***Tulasnella amonilioides*** | MZ156778 | **DEJ10** | **Brazil** | ***C. intermedia*** | **This study** |
| ***Tulasnella amonilioides*** | MZ156780 | **DEJ13** | **Brazil** | ***C. intermedia*** | **This study** |
| ***Tulasnella amonilioides*** | MZ156781 | **DEJ15** | **Brazil** | ***C. intermedia*** | **This study** |
| ***Tulasnella amonilioides*** | MZ156782 | **DEJ16** | **Brazil** | ***C. intermedia*** | **This study** |
| ***Tulasnella amonilioides*** | MZ156776 | **DEJ03** | **Brazil** | ***C. intermedia*** | **This study** |
| ***Tulasnella amonilioides*** | MZ156777 | **DEJ07** | **Brazil** | ***C. intermedia*** | **This study** |
| ***Tulasnella amonilioides*** | MZ156779 | **DEJ11** | **Brazil** | ***C. intermedia*** | **This study** |
| ***Tulasnella amonilioides*** | MZ156783 | **DEJ17** | **Brazil** | ***C. intermedia*** | **This study** |
| *Tulasnella anaticula* | EU218891 | UAMH 5428 | Canada | N/A | Taylor et al. (2008) |
| *Tulasnella asymmetrica* | DQ388047 | MAFF P305808 | N/A | *Thelymitra epipactoides* | Suárez et al. (2006) |
| *Tulasnella asymmetrica* | DQ388048 | MAFF P305809 | N/A | *Thelymitra epipactoides* | Suárez et al. (2006) |
| *Tulasnella asymmetrica* | MH134553 | N/A | Australia | *Thelymitra epipactoides* | Reiter et al. (2018) |
| *Tulasnella asymmetrica* | KC152348 | MAFF305808 | Australia | root of terrestrial orchid | Cruz et al. (2014) |
| *Tulasnella asymmetrica* | KC152355 | MAFF305809 | Australia | root of terrestrial orchid | Cruz et al. (2014) |
| *Tulasnella asymmetrica* | MH134555 | N/A | Australia | *Thelymitra epipactoides* | Reiter et al. (2018) |
| *Tulasnella bifrons* | AY373290 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnella calospora* | HQ833210 | N/A | N/A | N/A | unpublished |
| *Tulasnella calospora* | JQ713577 | N/A | China | *Eria coronaria* | unpublished |
| *Tulasnella calospora* | EF393621 | N/A | China | *Cymbidium floribundum* | unpublished |
| *Tulasnella calospora* | EU218888 | N/A | N/A | N/A | Taylor et al. (2008) |
| *Tulasnella calospora* | GU166421 | N/A | N/A | N/A | Nontachaiyapoom, Sasirat & Manoch (2010) |
| *Tulasnella* cf. *albida* | KC152378 | K(M)118140 | United Kingdom | N/A | Cruz et al. (2014) |
| *Tulasnella* cf. *pinicola* | KC152363 | DC309 | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella* cf. *pinicola* | KC152357 | DC309 | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella cumulopuntioides* | LC175322 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella cumulopuntioides* | LC175326 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella cumulopuntioides* T** | NR 160570 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella danica* | AY373297 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnella deliquescens* | LC175331 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella deliquescens* | LC175332 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella deliquescens* | LC175333 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella dendritica* | LC175307 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella dendritica* | LC175311 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella dendritica* T | NR 160569 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella eichleriana* | AY373292 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnella eichleriana* | KC152381 | K(M)143600 | United Kingdom | N/A | Cruz et al. (2014) |
| *Tulasnella ellipsoidea* | LC175313 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella ellipsoidea* | LC175318 | N/A | Japan | *Spiranthes sinensis* | Fujimori et al. (2019) |
| *Tulasnella epiphytica* | JF907598 | AERO 3.2 | Brazil | N/A | Almeida, Van den Berg & Góes-Neto (2014) |
| *Tulasnella irregularis* | EU218889 | N/A | N/A | N/A | Taylor et al. (2008) |
| *Tulasnella irregularis* | GU166413 | N/A | N/A | N/A | Nontachaiyapoom, Sasirat & Manoch (2010) |
| *Tulasnella irregularis* T | NR 160166 | CBS 574.83 | Australia | N/A | Vu et al. (2019) |
| *Tulasnella pruinosa* | AY373295 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnella pruinosa* | DQ457642 | DAOM 17641 | N/A | N/A | Matheny et al. (2006) |
| ***Tulasnella* sp.** | MZ156775 | **DEJ01** | **Brazil** | ***C. intermedia*** | **This study** |
| *Tulasnella* sp. | KC152440 | FO24380a | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella* sp. | KC152383 | FO24462a | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella* sp. | JN015192 | BB0002 2 A | N/A | *terrestrial orchid* | unpublished |

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|---|---|---|---|---|---|
| *Tulasnella* sp. | KP056306 | N/A | Norway | *Goodyera repens* | Liebel et al. (2015) |
| *Tulasnella* sp. | EF176486 | Kings Park D46 | Australia | *Disa bracteata* | Bonnardeaux et al. (2007) |
| *Tulasnella* sp. | JQ713574 | N/A | China | *Ascocentrum himalaicum* | unpublished |
| *Tulasnella* sp. | JQ713595 | N/A | China | *Dendrobium williamsonii* | unpublished |
| *Tulasnella* sp. | KC152374 | FO35532 | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella* sp. | KC152370 | FO35532 | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella* sp. | KP050605 | HD-2014 | China | *Dendrobium officinale* | unpublished |
| *Tulasnella* sp. | KC928352 | mara51 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928353 | mara52 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928360 | tiro23 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928365 | tiro29 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928366 | mara13 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928375 | Serrnova12 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928384 | Serrap11 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928385 | Serrap12 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928386 | Serrap21 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928387 | Serrap22 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928388 | Serrap23 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | KC928389 | Serrap31 | Brazil | *Encyclia ghillanyi* | unpublished |
| *Tulasnella* sp. | AJ313438 | N/A | Singapore | *Dendrobium crumenatum* | Ma et al. (2003) |
| *Tulasnella sphagneti* | KY445922 | N/A | Australia | *Chiloglottis turfosa* | Linde et al. (2017) |
| *Tulasnella sphagneti* | KY095117 | N/A | Australia | *Chiloglottis* aff. *valida* | Linde et al. (2017) |
| *Tulasnella tomaculum* | KC152380 | K(M)123675 | United Kingdom | N/A | Cruz et al. (2014) |
| *Tulasnella tomaculum* | AY373296 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnella violea* | KC152437 | N/A | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella violea* | KC152415 | N/A | Ecuador | N/A | Cruz et al. (2014) |
| *Tulasnella violea* | KC152435 | N/A | Germany | N/A | Cruz et al. (2014) |
| *Tulasnella violea* | AY373293 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnella violea* | DQ457643 | FCUG 125 | N/A | N/A | Matheny et al. (2006) |
| *Tulasnella violea* | AY373303 | N/A | N/A | N/A | McCormick et al. (2004) |
| *Tulasnellacea* | JX138572 | 16 MB-2012 | Australia | *Microtis media* | Sommer et al. (2012) |
| uncultured *fungus* | AB506858 | N/A | Japan | *Cymbidium goeringii* | Ogura-Tsujita et al. (2012) |
| uncultured *Tulasnella* | HM802323 | RW12 | New Zealand | *Nematoceras trilobum* | Watkins (2012) |
| uncultured *Tulasnella* | FJ788862 | N/A | N/A | *Pterygodium catholicum* | Waterman et al. (2011) |
| uncultured *Tulasnella* | AY192451 | N/A | N/A | rhizoid of *Cryptothallus mirabilis* | Bidartondo et al. (2003) |
| uncultured *Tulasnella* | AY192452 | N/A | N/A | thallus of *Cryptothallus mirabilis* | Bidartondo et al. (2003) |
| uncultured *Tulasnella* | MH064467 | N/A | N/A | *Encyclia tampensis* | unpublished |
| uncultured Tulasnellaceae | KX387592 | N/A | China | *Cymbidium bicolor* | Downing (2016) |
| uncultured Tulasnellaceae | KC243936 | N/A | Czech Republic | *Gymnadenia conopsea* | Těšitelová et al. (2013) |
| uncultured Tulasnellaceae | KC243944 | N/A | Czech Republic | *Gymnadenia conopsea* | Těšitelová et al. (2013) |
| uncultured Tulasnellaceae | KC243957 | N/A | Czech Republic | *Gymnadenia conopsea* | Těšitelová et al. (2013) |
| uncultured Tulasnellaceae | KC243956 | N/A | Czech Republic | *Gymnadenia densiflora* | Těšitelová et al. (2013) |
| uncultured Tulasnellaceae | KC243950 | N/A | Czech Republic | *Gymnadenia conopsea* | Těšitelová et al. (2013) |
| uncultured Tulasnellaceae | KJ188451 | N/A | Czech Republic | *Neottia cordata* | Těšitelová et al. (2015) |
| uncultured Tulasnellaceae | KX587478 | N/A | China | *Dendrobium nobile* | unpublished |
| uncultured Tulasnellaceae | MH005882 | N/A | N/A | epiphytic orchid | Xing et al. (2019) |
| **Outgroup** | | | | | |
| *Ceratorhiza* sp. | JX456554 | OB1.2G | Brazil | *Oncidium barbaceniae* | Pereira et al. (2014) |
| *Ceratorhiza* sp. | JX456555 | OB1.3H | Brazil | *Oncidium barbaceniae* | Pereira et al. (2014) |
| *Ceratorhiza* sp. | HQ127084 | N/A | N/A | N/A | Pereira et al. (2011) |
| *Serendipita vermifera* | DQ983815 | MAFF305837 | Australia | *Caladenia dilatata* | Deshmukh et al. (2006) |
| *Serendipita vermifera* | AF202728 | CBS 572.83 | Australia | N/A | Taylor et al. (2003) |
| *Tulasnella eremophila* | KJ701188 | MA-Fungi 88007 | Morocco | *Euphorbia officinarum* | Crous et al. (2015) |

* N/A = Not available; **T = Type-specimen.

*Seed collection and sowing*

Mature fruits of *C. intermedia* were collected in January 2018 from wild individuals of the same population from which the root samples were collected. The fruits were surface-sterilized, and seeds were accessed (Sasamori et al., 2015). About 200 mg of seeds were immersed in 50 mL of sterile distilled water under constant agitation and 1 mL of the suspension with the seeds was pipetted onto 9-cm Petri dishes containing 20 mL of OMA culture medium (oatmeal 4 g $L^{-1}$, agar 10 g $L^{-1}$, distilled water, pH 5.6; Dixon, 1987; Pereira et al., 2015). The OMA medium of each Petri dish was inoculated with a 1 $cm^3$ block of PDA medium containing fungal mycelium obtained from the borders of the colonies of the nine isolates. Four replicates were inoculated with each isolate. Four Petri dishes containing the same volume of modified MS medium (50% of the original formulation of macronutrient salts, 30 g $L^{-1}$ of

sucrose, 4 g $L^{-1}$ of Phytagel™, and pH 5.7; according to Sasamori et al. (2015) were used as a positive control. Four uninoculated dishes with OMA medium were used as a negative control. The dishes were sealed with PVC film and kept in a growth chamber at 26 ± 1°C, under a 12:12 light:dark photoperiod, with 100 μmol $m^{-2}$ $s^{-1}$ irradiance.

*Seed germination and plant development*

Germination and plant development were observed weekly. Contaminated dishes were discarded, and the formation of structures that characterize the stages of orchid development, such as rhizoids, promeristem, leaves, and roots, was observed (Sharma et al., 2003). Thirteen weeks (91 days) after sowing, the dishes were removed from incubation (Figure 1D). Seed germination and plant development were evaluated under a dissecting microscope. Seeds were considered viable when containing a distinct, rounded, and hyaline embryo (Sharma et al., 2003; Stewart & Kane, 2006; Guimarães et al., 2013). Viability percentage (Vp) was estimated by the formula: Vp=(Nvs x 100)/Nts where Nvs is the number of viable seeds, and Nts is the total number of seeds in a dish. Development was scored based on specific literature (Pereira et al., 2015; Durán-López et al., 2019) with some modifications, according to differences that *C. intermedia* presents when compared to other species. Plant development stages were determined on a 0 to 5 scale: 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 (for this study, from this stage on, the plants are defined as seedlings); and stage 5 = formation of a second true leaf and root system. Germination percentage (Gp) was calculated as Gp= (NGi x 100)/Nvs where NGi is the number of germinated individuals (stage 1 through 5), and Nvs is the number of viable seeds. The growth index was estimated based on Otero, Bayman & Ackerman (2005), by the formula: GI = (N1+N2x2+N3x3+N4x4+N5x5)/(N0+N1+N2+N3+N4+N5) where N0 is the number of seeds in stage 0, N1 is the number of plants in stage 1, and so on.

After seed germination and growth index evaluation, the presence of mycorrhizal fungi in *C. intermedia* tissues was confirmed by microscope examination (Olympus CX4) of hand-made longitudinal sections of the plants, which were stained with toluidine blue (Figure 1E). Stage 4 and 5 individuals were selected and transferred to flasks (volume of 200 mL) containing 30 mL of the same medium used for germination for further growth (OMA and MS). MS medium was prepared with and without 10 g $L^{-1}$ of active charcoal. After 90 days, the seedlings were again transferred to flasks containing a fresh culture medium, and the MS medium was prepared to contain 100% of the macronutrient salts and 60 g $L^{-1}$ of sucrose (Sasamori et al., 2015). Each flask received five seedlings and nine replicates were prepared, for a total of 45 individuals per fungal isolate in OMA and MS media.

Seedlings were kept for six additional months, totaling one year *in vitro* under the same conditions of light intensity and temperature as in the initial stage of culturing. Thus, seedlings were removed from the flasks and washed under running tap water. Shoot height, length of the longest root, number of leaves, number of roots, and fresh mass were determined for each plant by using a pachymeter and a high-precision balance, according to methods described by Sasamori et al. (2015). Seedling survival was observed in each treatment and its percentage was estimated based on the total number of plants at the beginning of the experiment. The presence of mycorrhizal fungi was again confirmed by microscope examination of transversal sections of the roots, which were stained as mentioned above.

*Statistical analyses*

Data normality was tested with the Shapiro-Wilk test. Differences in germination percentage and growth index were tested for significance by ANOVA using SPSS v25 (SPSS Inc., Chicago, IL, USA). Comparisons between the morphometric variables of seedlings propagated with different fungi isolates and MS control were performed by the Kruskal-Wallis's test followed by the Student-Newman-Keuls test with BioEstat software, version 5.3. The significance was set at 5% for all analyses.

## Results

*Fungal isolation and culture*

Fungi growth and isolation with PDA were successful. Of the 140 root cross-sections inoculated, 69 (49.3%) presented hyphae development in the cortex. After the morphological analysis, we selected 14 isolates (10% of the initial number) for the previous evaluation of germination, which resulted in the selection of nine isolates (6.4% of the initial number) used in phylogenetic and plant propagation analyses. The isolates showed colonies with slow culture growth, cream to pale cream color, absent or scattered aerial hyphae, clotted to flat aspects, and submerged colony margins. However, we did not

observe the production of monilioid cells in the cultures.

*Fungal phylogenetic characterization*

We obtained the ITS sequences of the nine isolates efficient *in vitro C. intermedia* seed germination and development (Table 1). BLAST and Genbank-based searches resulted in a data set of 3494-specimens (data set 1), and the final DNA sequence alignment was 2053 bp long. The best-scoring ML tree (data not shown) from data set 1 was manually verified and specimens of supported clades and those closely related to the specimens studied here were selected. For data set 2, the final alignment was 1192 bp long with 105 specimens. In the Bayesian analysis, after 900,000 generations, the runs converged to stable likelihood values (-lnL = 16990.14, 16992.81). A 50% majority-rule consensus tree was computed, and Bayesian posterior probabilities (BPP) were generated for the resulting tree.

The boot-stopping criteria of RAxML indicated that 504 pseudoreplications were sufficient to assess the internal branch support and the final ML optimization likelihood was -lnL = 17455.31. The best-scoring ML tree and 50% majority-rule consensus tree did not show any major conflicts in tree topology and were mostly congruent, which allowed us to combine them. Only the topology from ML analysis is exhibited while both BS and BPP values are shown on the branches (Figure 2). Most of the specimens isolated in this study were recovered conspecific to *T. amonilioides* (P.R.M. Almeida, N. Van den Berg & Góes-Neto) S. Fujimori, J.P. Abe, I. Okane & Y. Yamaoka (Fujimori et al., 2019), except for DEJ01, which was retrieved as sister to *T. irregularis*. Both *T. amonilioides* and *T. irregularis* were recovered as closely related in a fully supported clade (BS ML = 100, BPP = 1), in which unnamed specimens from China, Australia, Czech Republic, and Norway were also retrieved (Figure 2).

*Seed germination and plant development*

Seeds of *Cattleya intermedia* germinated in OMA media, with and without inoculation of the mycorrhizal fungi, and in the asymbiotic MS medium. One week after sowing, seeds began to swell, and then germination started in the second week with the rupture of the testa by the enlargement of the embryo. Visual inspection revealed that 31.8% of the non-germinated seeds were devoid of embryos and thus considered inviable (Figure 3A-D). In those treatments for which plants grew faster, with inoculation of DEJ03 and DEJ07 isolates, rhizoids appeared in the third week (Figure 3E). In the fourth week, a central depression was formed at the top of the protocorm, which showed a considerable size increment, making it larger than the seed coat, with multiple rhizoids (Figure 3F). During the fifth and sixth weeks, protocorms maintained their growth and developed the promeristem (Figure 3G). The first leaf appeared in the seventh week, and a few days later, many seedlings presented well-developed leaves (stage 4) (Figure 3G-I). These individuals remained in this stage for approximately two weeks and then formed true roots (stage 5, ninth week). The passage from stage 4 to stage 5 happened at different times of development according to the isolates used for plant infection. The appearance of roots took 13 weeks for plants growing in MS medium when we chose to analyze germination percentages and plant development. The last treatment that reached this stage was with the *Tulasnella* sp. DEJ01 inoculation (Table 2).

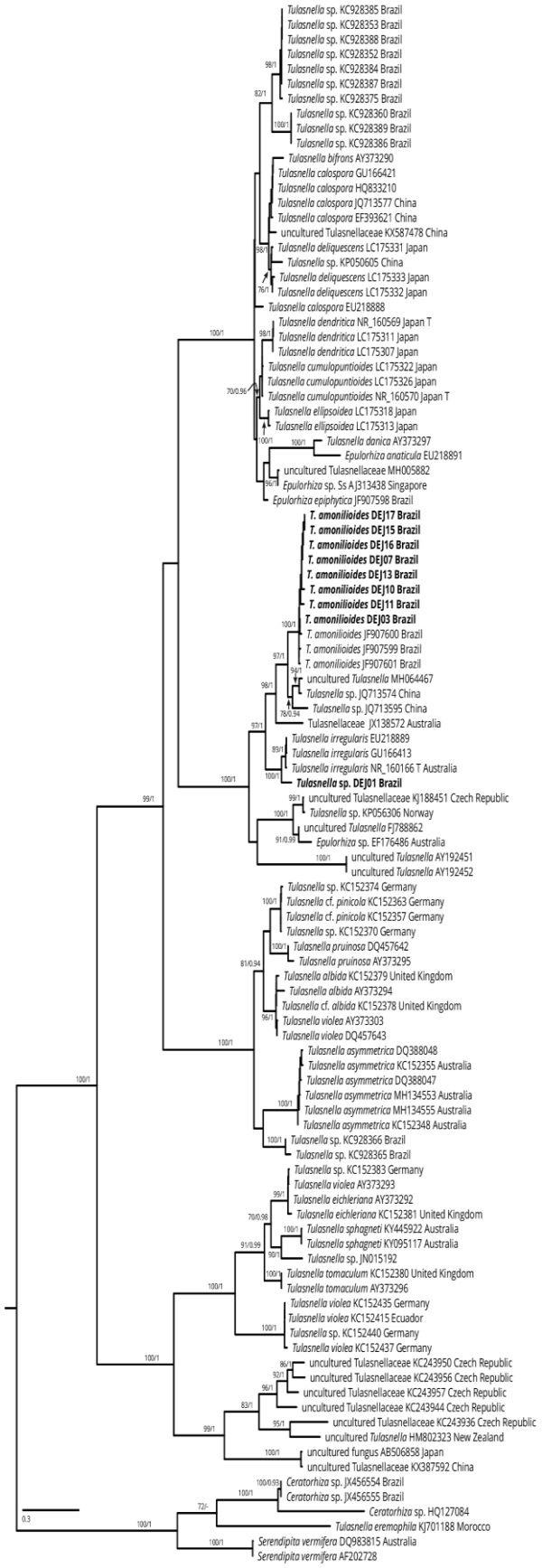


Figure 2. Phylogram of the relationships among *Tulasnella* isolates inferred by Maximum Likelihood analysis based on ITS sequences. Support values on branches are as follows: BS/BPP = 70/0.95 moderately supported, and 99/0.99 or higher highly/fully supported. Sequences provided in this study are in bold. Font: Endres Júnior et al. (2023).

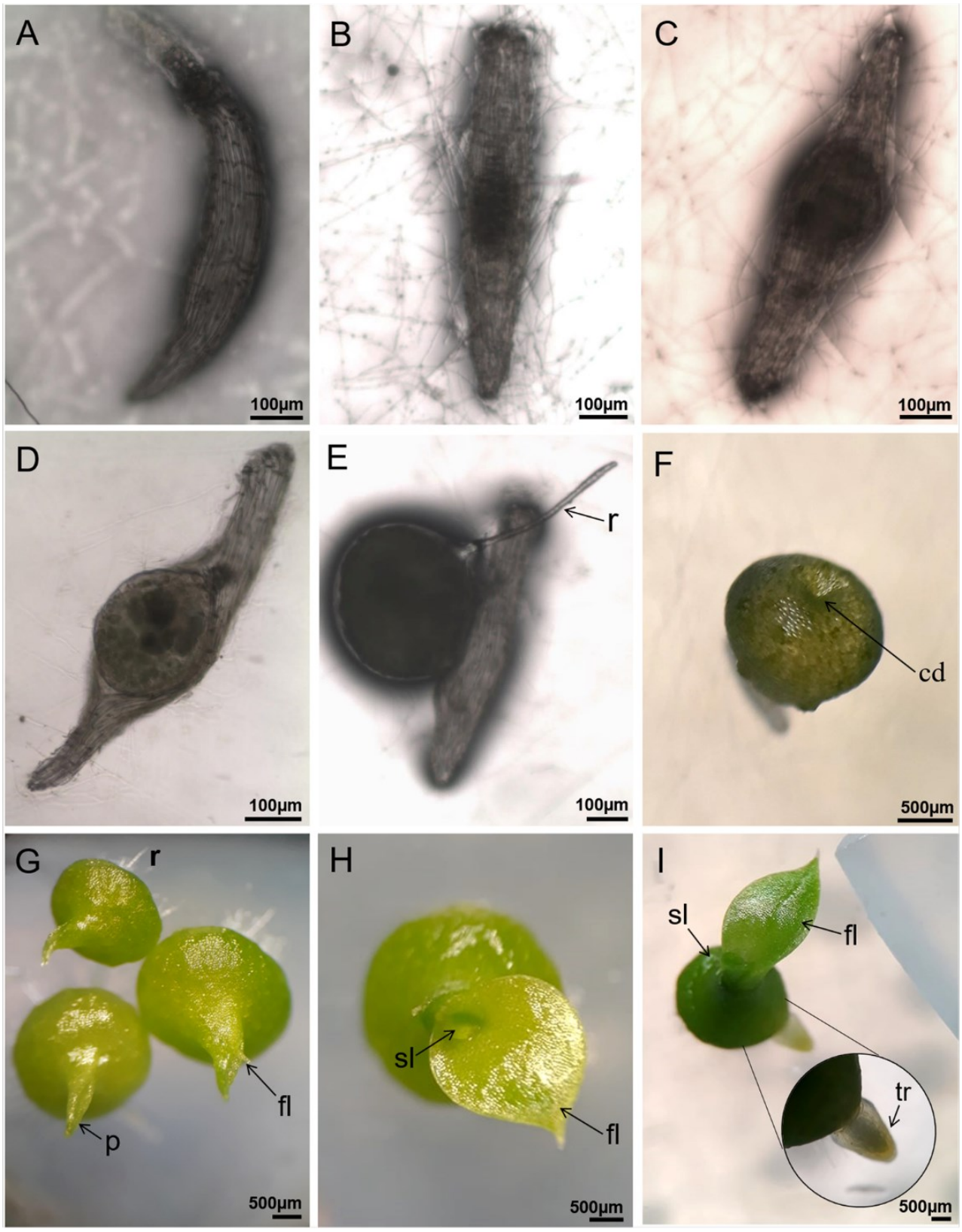


Figure 3. A. *Cattleya intermedia* seed coat (testa) lacking embryo; B. seed with the ovoid hyaline embryo (viable seed); C. stage 0, the swollen embryo inside the coat; D. stage 1, testa ruptured by the enlarged embryo (i.e., germination); E. stage 2, protocorm growing outside the testa with rhizoid (r); F. appearance of a central depression (cd) on the top of the protocorm; G. stage 3, protocorm with promeristem (p) and stage 4, the appearance of the first true leaf (fl); H. seedling with first leaf elongated, second leaf (sl); I. stage 5, true root formation (rt). Font: Endres Júnior et al. (2023).

Seed germination and growth index of *C. intermedia* (13 weeks after sowing) showed significant differences among treatments ($F=9.050$, $p<0.001$; $F=16.597$, $p<0.001$). The germination

percentages with the use of most T. *amonilioides* isolates did not differ among themselves or concerning the MS medium (except DEJ03). These showed higher values for the seeds germinated with the DEJ01 isolate and in OMA without inoculation (Table 2). The seeds were capable of germinating in the OMA medium without inoculation and the protocorms remained in stage 1, with the lowest growth index. The plants with the highest germination percentages were also those with the highest growth index (Table 2). The treatments in which plants reached the highest percentages of individuals in stage 5 (seedlings with true leaves and roots) were DEJ03 (44.3%) and DEJ17 (45.6 %).

Table 2. Seed germination and development of *Cattleya intermedia* plants 91 days after sowing with inoculation of nine *Tulasnella* isolates, oatmeal agar (OMA) without symbiotic fungi, and MS media as control. Font: Endres Júnior et al. (2023).

| Treatment | Nseeds | Vseeds | Germination (%) | Stage of development (%) | | | | | Growth index | Stage 5 week |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 1 | 2 | 3 | 4 | 5 | | |
| **DEJ01** | 517 | 378 | 25.0 ± 7.2 d | 23.7 | 26.0 | 11.5 | 38.9 | 0.0 | 0.9 ± 0.4 cd | 15 |
| **DEJ03** | 407 | 321 | 26.6 ± 2.8 cd | 4.1 | 1.8 | 3.8 | 46.1 | 44.3 | 1.4 ± 0.1 bc | 9 |
| **DEJ07** | 464 | 323 | 42.2 ± 4.3 abc | 10.0 | 14.0 | 7.5 | 49.7 | 18.8 | 1.8 ± 0.2 ab | 9 |
| **DEJ10** | 676 | 474 | 49.0 ± 2.1 a | 5.0 | 11.9 | 7.5 | 64.0 | 11.7 | 2.1 ± 0.0 a | 11 |
| **DEJ11** | 644 | 439 | 51.8 ± 2.6 a | 12.3 | 15.4 | 10.6 | 50.2 | 11.5 | 2.0 ± 0.4 ab | 11 |
| **DEJ13** | 733 | 518 | 50.2 ± 4.0 a | 2.4 | 0.7 | 4.3 | 77.3 | 15.4 | 2.4 ± 0.1 a | 11 |
| **DEJ15** | 644 | 442 | 41.3 ± 4.1 abc | 18.4 | 2.2 | 2.2 | 70.6 | 6.7 | 1.8 ± 0.2 ab | 10 |
| **DEJ16** | 557 | 385 | 45.7 ± 2.2 a | 26.4 | 1.7 | 3.8 | 61.6 | 6.4 | 1.8 ± 0.3 ab | 11 |
| **DEJ17** | 427 | 243 | 43.5 ± 11.1 ab | 4.1 | 1.7 | 3.9 | 44.8 | 45.6 | 2.4 ± 0.5 a | 10 |
| OMA | 750 | 474 | 27.4 ± 7.6 d | 100.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 ± 0.1 d | - |
| MS | 592 | 372 | 42.0 ± 5.9 abc | 12.2 | 4.0 | 17.4 | 63.9 | 2.6 | 1.8 ± 0.3 ab | 13 |

Number of seeds per treatment, Nseeds; number of viable seeds per treatment, Vseeds. Average ± standard deviation followed by the same letter in the column is not different according to the Tukey test, at a significance of 0.05.

Seedlings inoculated with DEJ01 had a survival rate of less than 50% (one year *in vitro*). The highest values for shoot height (SH) and some roots were for seedlings in MS medium with activated charcoal (Figure 4A, D), when compared to most of the symbiotic cultures. The shoot height (SH) of seedlings grown in OMA inoculated with DEJ03 isolate was equal to that of those grown in MS. The number of leaves (NL) was higher in the symbiotic culture than in both asymbiotic treatments (Figure 4B). The length of the longest root (LLR) of the seedlings inoculated with the isolates DEJ13 and DEJ17 (*T. amonilioides*) was greater than in plants inoculated with DEJ01 and those propagated in MS medium. The plants of the other treatments were intermediate (Figure 4C). Fresh mass (FM) of seedlings grown in OMA inoculated with all fungi isolates were equal to the FM of plants obtained in MS medium, and only MS without activated charcoal showed a significantly lower value (Figure 4E). The survival of seedlings propagated in symbiotic cultivation using *T. amonilioides* ranged from 93.3 to 100% and the survival percentages of plants propagated in MS medium with and without activated charcoal were 100% (Figure 4F). The maintenance of symbiotic relationships was confirmed by the observation, at the end of *in vitro* culture, of fungal pelotons in root cross-sections of seedlings grown in all inoculated OMA media.

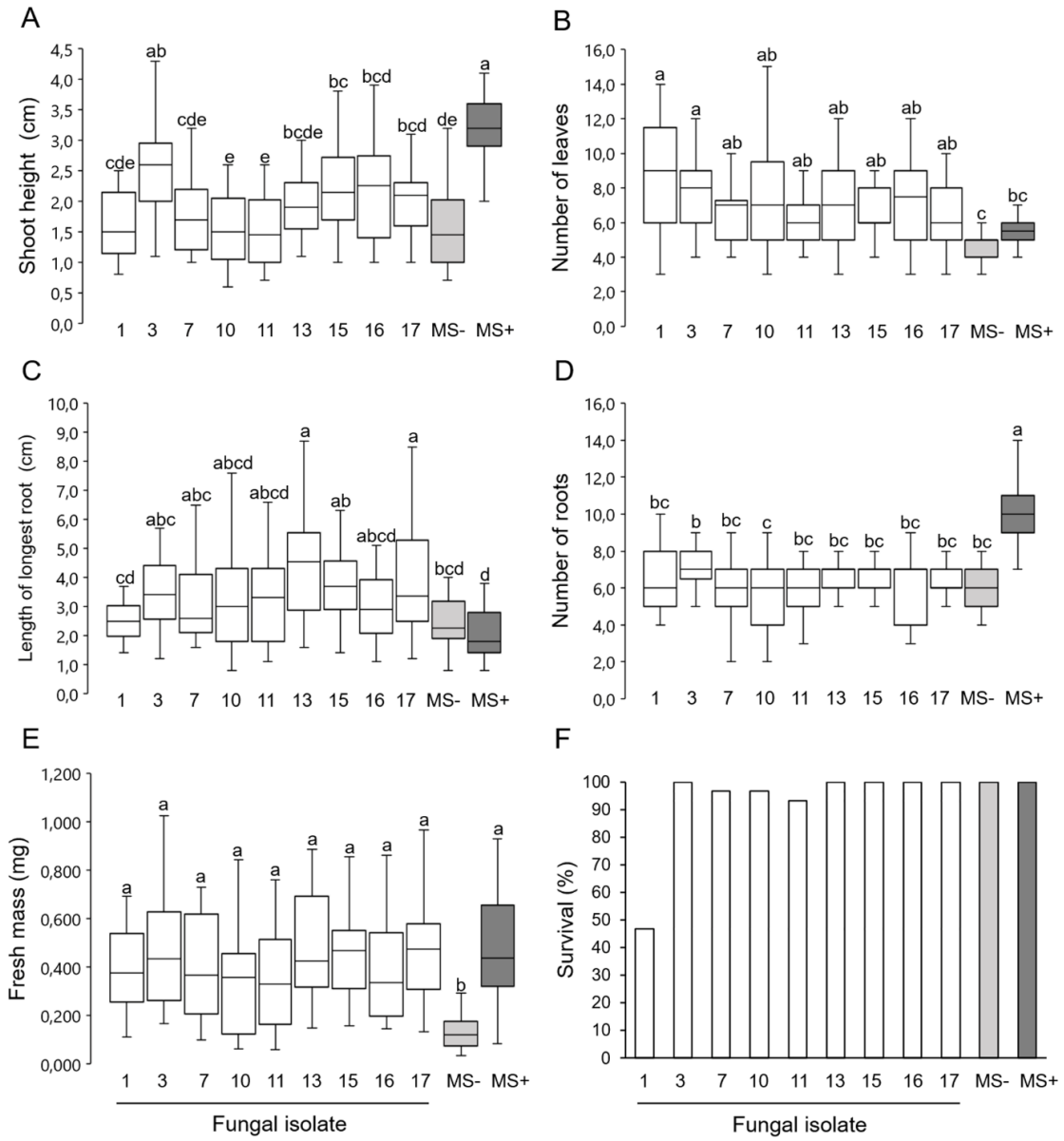


Figure 4. Box plot of (A) shoot height (cm); (B) number of leaves; (C) length of longest root (cm); (D) number of roots; (E) fresh mass (mg); (F) survival (%) of *Cattleya intermedia* seedlings propagated *in vitro* with *Tulasnella* isolates or with MS medium without (-) and with (+) activated charcoal. Font: Endres Júnior et al. (2023).

**Discussion**

Mycorrhizal fungi were successfully isolated from the roots of mature individuals of *C. intermedia* in nature and the isolates that were efficient *in vitro* seed germination and plant development showed characteristics usually assigned to the anamorphic genus *Epulorhiza* (Moore, 1987; Currah, Zettler & Mcinnis, 1997; Pereira et al., 2003; Nogueira et al., 2005). Studies demonstrated that *Epulorhiza* is one of the most important genera of fungi related to Brazilian orchids and is represented by *E. epiphytica*, *E. repens*, and *T. amonilioides* (current name) (Almeida, Van den Berg & Góes-Neto, 2014; Pereira et al., 2003; Pereira et al., 2011, 2015). The production of monilioid cells was not observed in any of the isolates growing in CMA medium, which is related to one of the main characteristics of *T. amoniloides*: the absence of these structures in pure culture (Almeida, Van den Berg & Góes-Neto, 2014).

*Tulasnella amonilioides* was initially isolated from epiphytic and rupicolous specimens of *Encyclia dichroma* (Lindl.) Schltr., *E. ghillanyi* Pabst, and *Brassavola tuberculata* Hook., orchids native of the Northeast Region of Brazil (Almeida, Van den Berg & Góes-Neto, 2014). At the time, the authors described it as *E. amonilioides*, based on morphological characters in pure culture and also by phylogenetic analysis. *Tulasnella* belongs to Tulasnellales and is the teleomorph of *Epulorhiza*.

The relationship between both described fungi taxa may be confirmed by molecular phylogenetic analyses, because these genera, initially described separately by morphological characters, constitute a monophyletic group with a high degree of genetic similarity (Kristiansen, Rasmussen & Rasmussen, 2001). Based on this, and since the International Code of Nomenclature (ICN) recommendations are used in these cases (McNeill et al., 2012), as was done in important studies concerning recommendations regarding competing genera of Ascomycota, the name *Tulasnella* was used in the present study (Stadler et al., 2013; Rossman et al., 2016), as it had already been proposed by Fujimori et al. (2019).

According to Almeida, Van den Berg & Góes-Neto (2014), the sequences of *T. amonilioides* were found to be identical to each other, and the authors verified a similarity of 99-100% for ITS rDNA and mtSLU sequences between *T. amonilioides* and T. *irregularis* (Tulasnellaceae, Cantharellales), suggesting that the hither to described *E. amonilioides* would be the anamorph of *T. irregularis*. *Tullasnella irregularis* was initially isolated and described as teleomorph from the roots of *Dendrobium dicuphum* F. Muell. (Warcup & Talbot, 1980). In our analyses, the *T. amonilioides* specimens, including those from Almeida, Van den Berg & Góes-Neto (2014), were retrieved in clades separate from *T. irregularis*, which was represented by the recently provided ex-type ITS sequence NR_160166 (Vu et al., 2019). Furthermore, DEJ01 was recovered as sister to *T. irregularis* and not even the present study could provide sufficient data to assign DEJ01 to the mentioned species. DEJ01 performed differently from *T. amonilioides* isolates in propagation tests, which reinforces their dissimilarity.

The nine isolates of mycorrhizal fungi obtained in the present study were capable of inducing seed germination of *C. intermedia* (testa rupture). Although the orchid seeds germinated in oatmeal agar medium even without fungal inoculation, the germination percentage was very low, as observed for *Epipactis flava* Seidenf. (rheophytic) (Suwannarach et al., 2021). Some studies have described germination failure without fungi inoculation (Guimarães et al., 2013; Pereira et al., 2015). For some orchid species the seed embryo may intumesce and increase in size, causing the testa rupture (Peterson, Uetake & Zelmer, 1998; Pereira et al., 2011; Duran-López et al., 2019), or even the production of rhizoids (Stewart & Kane, 2007; Sathiyadash et al., 2014; Duran-López et al., 2019) in the absence of mycorrhizal fungi on OMA medium. This development is related to water imbibition and, although the orchids possess hydrophobic testa (Stewart & Kane, 2006), the mycorrhizal fungal infection was not necessary for water absorption and the start of *C. intermedia* germination.

*Tulasnella* isolates are efficient *in vitro* orchid seed germination and plant development (Zettler, Poulter & McDonald, 2007; Sathiyadash et al., 2014; Pereira et al., 2015; Zettler & Dvorak, 2021). The germination percentages for most *T. amonilioides* isolates were equal to those with MS medium, corroborating the results of Guimarães et al. (2013), who compared the germination of *Cyrtopodium glutiniferum* Raddi (rupicolous) with asymbiotic germination using MS and Knudson C media. Jiang et al. (2015) observed similar germination percentages when *Anoectochilus formosanus* Hayata (terrestrial) seeds were grown in OMA medium inoculated with a group of mycorrhizal fungi, in an MS medium with half the concentration of nutrients and with a modified Hyponex medium. Orchid germination depends on the species and the origin and quality of the seeds, as well as the genera, species, and isolates of fungus (Otero, Bayman & Ackerman, 2005; Pereira et al., 2011).

Germination percentage of orchids may be highly variable, even from total failure (0%) to almost complete success (100%) (Otero, Bayman & Ackerman, 2005; Porras-Alfarro & Bayman, 2007; Zettler, Burkhead & Marshall, 1999; Zettler et al., 2013; Duran-López et al., 2019). Porras-Alfarro & Bayman (2007) studied the germination of the orchids of the genus *Vanilla* Mill. (hemiepiphytic) and found that seeds germinated with *Ceratobasidium* inoculation in a cellulose medium as a carbon source but failed to germinate when the medium was inoculated with *Tulasnella* and modified Knudson media. *Tolumnia variegata* (Sw.) Braem (epiphytic – Oncidiinae, Epidendroidae) in symbiotic cultivation had higher germination percentages and faster growth than plants of the same species propagated using commercial Knudson C medium (Otero & Bayman, 2009). In the same study, however, the authors observed equal germination rates when for symbiotic and asymbiotic culture of *Epidendrum ramosum* Jacq. (epiphytic and terrestrial - Laeliinae, Epidendroideae), *Lepanthes rupestris* Stimson (rupicolous – Pleurothallidinae, Epidendroideae) and *Psychilis monensis* Sauleda (epiphytic - Laeliinae, Epidendroideae). These species germinated with fungi isolated from *T. variegata*, which grows on different substrates and belongs to a different subtribe than the above species. *Tulasnella amonilioides* is also able to germinate seeds of *Cattleya sincorana* (Schltr.)

Van den Berg, which, as *C. intermedia* (present study), *E. dichroma*, *E. ghillanyi* and *B. tuberculata* (species from which this mycobiont were originally isolated; Almeida, Van den Berg & Góes-Neto, 2014), belongs to subtribe Laeliinae, subfamily Epidendroideae.

The findings of the present study corroborate those of Pereira et al. (2009, 2011) in that intra-specific variation among fungal isolates from the same plant population may exist and that this may interfere with mycorrhizal associations. Germination velocity is also highly variable, but, in general, symbiotic culture provides faster seed germination and plant development compared to asymbiotic culture for species of terrestrial habit (Guimarães et al., 2013; Jiang et al., 2015; Pereira et al., 2015). For *C. intermedia*, inoculation with the *T. amonilioides* DEJ03 isolate was seen to induce faster seed germination and plant development when compared to MS culture. One of the highest percentages of individuals in stage 5 at the 13th week (44.3%) was recorded with this isolate, while asymbiotic MS medium plants took four additional weeks to reach this stage, with only 2.6% of individuals in stage 5. The growth index is higher according to the speed at which plants pass through the stages of development, as long as the germination rates are the same. Considering these factors, the germination percentage and growth index for DEJ03 were not as good as that of the other isolates. *Tulasnella amonilioides* (except DEJ03) isolates had proportionately equal growth indexes when comparing them among themselves and to the MS treatment, as found by Nontachaiyapoom, Sasirat & Manoch (2011) for *Dendrobium draconis* Rchb. f. The authors discussed that the species' metabolism may be well adapted to responding to an exogenous and simple source of carbon, such as sucrose. This may explain the good development of *C. intermedia* in asymbiotic medium supplemented with sucrose.

Most of the studies that propagate orchids by symbiotic technique only focus on the initial stages of germination and plant development, in which the most advanced stage of a plant's ontogeny is seedling with the presence of one or two leaves and with or without true roots. In the present study, however, the continuity of plant propagation processes allowed us to observe the later stages of development of *C. intermedia* and the differences between seedling morphometric variables between symbiotic and asymbiotic culture. Seedlings propagated in OMA with inoculation of *T. amonilioides* isolates showed high survival rates (90–100%), and the fresh mass of these individuals did not differ from plants propagated with MS medium. No visual signs of nutritional deficiency were observed in the seedlings of most cultures, although they were transferred to OMA medium not supplemented with mineral salts. Zettler & Dvorak (2021) reported that *Spiranthes cernua* (L.) Rich. (terrestrial) showed chlorosis in the apical leaves, indicating nitrogen deficiency when the seedlings were grown in OMA. *Cattleya intermedia* seedlings were grown with inoculation of *Tulasnella* sp. DEJ01 had the lowest survival rate (46.7%). These plants died after suffering fast and intense chlorosis and presenting tissue necrosis, which was probably caused by the action of this fungal isolate on *C. intermedia* seedlings under *in vitro* conditions. Despite what was observed, *Tulasnella* is naturally composed of saprophytic species, which usually do not cause diseases in propagated plants, unlike *Ceratorhiza* and *Rhizoctonia* (see discussion in Pereira et al., 2009).

Except for seedlings grown with DEJ03, inoculated plants had lower shoot height, and all symbiotic treatments had a lower number of roots compared to asymbiotic propagation. *Cattleya intermedia* is reported to have greater growth of the aerial part when seedlings are propagated with a complete concentration of MS medium added at 60 g $L^{-1}$, while the number of leaves is less affected by the nutrient concentration of the medium (Sasamori et al., 2015). Lower shoot growth and root production in inoculated OMA may occur because this is a nutrient-poor medium compared to MS (Guimarães et al., 2013). Orchid seedlings grown in MS medium without the addition of activated charcoal had the lowest development for all morphometric variables in comparison to individuals grown in the same medium with this additive. Activated charcoal adsorbs phenolic substances, which are released by the plants into the media, and may act as a stimulant for rhizoid and root production. It also reduces or even prevents seedling browning and may improve the vegetative aspect of the plants (Fridborg et al., 1978; Pan & Van Staden, 1998; Van Waes, 1987; George & Ravishankar, 1997; Paul, Kumaria & Tandon, 2012; Kim et al., 2019). The higher number of leaves obtained in symbiotic culture may be explained by the production of new shoots by *C. intermedia* individuals since these plants were in a more advanced stage of development than plants growing in MS medium. The longer roots observed in seedlings propagated symbiotically allow for greater biomass of cortical parenchyma for fungal infection.

All the root segments of the wild orchid had intact and degraded pelotons inside cortical cells. The presence of degraded pelotons suggests that even adult *C. intermedia* plants support their

development with nutrients of mycotrophic origin (Cameron, Leake & Read, 2006; Cameron et al., 2007, 2008). The wild *C. intermedia* population assessed here showed conditions for maintaining reproduction since the plants naturally produced fruits, indicating pollinator occurrence, and their mycobionts were active in seed germination, which is suggested as limiting the long-term recruitment of orchids (Sharma et al., 2003; Rasmussen et al., 2015; Reiter et al., 2016).

## Conclusion

We have successfully isolated and identified mycorrhizal fungi from plant roots of a wild *C. intermedia* population. The phylogenetic analysis revealed that eight of the nine fungal isolates that were efficient in *C. intermedia* propagation were identified as *Tulasnella amonilioides*. Propagation tests in the present study indicate that *T. amonilioides*, besides being probably used by *C. intermedia* for nutrition in adulthood, is capable of inducing seed germination and plant development of host orchid propagules, enhancing the *in vitro* propagation. Thus, *C. intermedia* plants propagated symbiotically with *T. amonilioides* can be used in translocation initiatives, acting as inoculum sources for the establishment of new individuals, especially in environments that lack adequate mycorrhizal fungi for plant germination and growth.

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