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Cyrtopodium paludicolum germination with two *Tulasnella* isolates

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ABSTRACT

Symbiosis between orchid seeds and mycorrhizal fungi has been reported to be a determining factor in the success of germination and protocorm development in vitro. The aim of this study was to isolate and identify by molecular analysis the mycorrhizal fungus associated with *Cyrtopodium paludicolum*, and to evaluate its efficiency in facilitating seed germination and development. Germination experiments were carried out using a fungus isolated from *C. paludicolum* (CH01) and *Epidendrum secundum* (M65), which has been successfully used a number of times in symbiotic germination. The experiments were conducted in a completely randomized design with treatments of CH01, M65 as well as under asymbiotic conditions. The mycobiont CH01 was successfully isolated from *Cyrtopodium paludicolum* and identified as *Tulasnella* sp. Treatments with both fungi reached a higher germination percentage than under asymbiotic conditions, indicating no specificity in the relationship between *Cyrtopodium paludicolum* and the fungi. The results presented have the potential to advance research into the propagation and conservation of *C. paludicolum*, a native of the Cerrado biome.

Keywords: Cerrado, ITS-DNAr, mycorrhiza, Orchidaceae, symbiosis

Introduction

Orchids of the *Cyrtopodium* genus are renowned for the beauty of their structures at both the vegetative and reproductive stages, due to their large pseudobulbs, exuberant foliage and long stalks with their displays of richly colored flowers, which offer significant potential for commercial exploitation. However, according to Batista & Bianchetti (2006) and Rodrigues *et al.* (2015), despite this potential for commercial floriculture and the therapeutic value of their pseudobulbs, many *Cyrtopodium* species are unknown in the commercial market, or lack efficient propagation protocols.

Cyrtopodium paludicolum belongs to the Epidendroideae subfamily, the Cymbidieae tribe and the Catasetinae subtribe (Chase *et al.* 2003). It is a terrestrial species and is found only in land areas subject to frequent flooding, in the midwestern and southeastern regions of Brazil. The pseudobulbs are long and can reach up to 40 cm. The flower stalk may reach up to 2 m in height, having a simple inflorescence (rarely branched), with yellow colored flowers, measuring about 3.8 cm in diameter. The flowering season is from December to April (Menezes 2000).

Cyrtopodium paludicolum has small seeds and, according to Arditti & Ghani (2000), orchid seeds are the smallest in the plant kingdom. Furthermore, despite the large number of seeds produced, seed germination is extremely low (less than 1 %) in orchids, because their embryos lack access to nutrient reserves, and successful germination of seeds *in vivo* is required for fungal stimulus (mycotrophy). This symbiosis

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is characterized mainly by the formation of complex hyphal coils (pelotons) within host plant cells, i.e., the cortical parenchyma cells of the root or embryo (Dearnaley 2007).

Symbiotic seed germination techniques represent an efficient way of promoting orchid–fungus association under *in vitro* conditions and of studying *in vitro* orchid mycobiont specificity (Zettler 1997; Stewart & Kane 2006). Culture media usually employed in the asymbiotic germination of orchid seeds under *in vitro* conditions are composed of agar, macro and micronutrients, vitamins, sugar. Occasionally, plant growth regulators can be added. Symbiotic germination media usually contain agar and any other ingredient with a complex carbohydrate source of fungus nutrition such as oatmeal (Guimarães *et al.* 2013; Jiang *et al.* 2015), potato (Pereira *et al.* 2014), and corn meal (Zettler *et al.* 2011).

Cyrtopodium paludicolum has aroused the interest of collectors and informal marketers of plants, resulting in intense gathering of this species, which increases the risk of extinction for this orchid. It is not an endangered species, and has been classified in the "Least Concern" category in The Red Book of Brazilian Flora (Martinelli & Moraes 2013). The threatening processes facing orchids are primarily anthropogenic, most often resulting directly from habitat destruction, modification and fragmentation, and in a number of regions, over-collection (Swarts & Dixon 2009).

The present study aimed to isolate and identify mycorrhizal fungus associated with *C. paludicolum* roots. We also aimed to promote seed germination using two *Tulasnella* spp. isolates, and compare the efficiency of an endophyte from *C. paludicolum* with an efficient mycorrhizal fungus in symbiotic germination.

Materials and methods

Plant material

Two adult plants of *C. paludicolum* Hoehne LC with mature capsules were selected and collected for this study. Sampling was carried out at the Catapani farm situated in the municipality of Costa Rica, in the state of Mato Grosso do Sul, Brazil (SISBIO permit 22570-2). Plants were cultivated in plastic pots, using Plantmax® - with HT as substrate, and kept in a greenhouse under daily irrigation. For species identification procedures, vouchers were prepared and deposited in the herbarium of the Instituto de Botânica, São Paulo, in the state of Sao Paulo, Brazil.

The mature capsules resulting from natural pollination were collected, disinfected and opened for removal of the seeds, which were mixed and deposited in test tubes containing silica gel and then stored in a refrigerator at 4 °C for later use in both symbiotic and asymbiotic seed germination.

The roots were collected from donor plants and immediately transferred to a laboratory for isolation procedures.

Isolation

Isolation procedures were implemented following Otero *et al.* (2002) and Valadares *et al.* (2012). Young, fresh and healthy roots representing a part of the active root system were cut into 3 cm segments and placed into Petri dishes with moistened filter paper and were taken to the laboratory where they were washed in tap water, and again in distilled water. These fragments were superficially disinfected by washing in ethanol (70 %, v/v) for 1 min, and 6 min in sodium hypochlorite solution (0.5 % active chlorine), followed by fivefold washings with sterilized water, and shaken by hand. Superficial disinfecting of root segments was carried out in a laminar flow chamber and thin transversal sections were excised using a scalpel under stereomicroscopy. Five small groups of the colonized root cortical cells were removed by hypodermic needle. The fragments containing pelotons were transferred to sterile Petri dishes containing 50 mL of potato dextrose agar (PDA) medium, pH 5.6, and were kept in an incubator – BOD (0/24 h day/night photoperiod, 28 ± 2 °C). The cortical cell fragments were examined daily for the presence of fungal growth, and hyphal tips were excised and transferred to a fresh PDA for morphological identification, DNA extraction for molecular analysis, and symbiotic seed germination. The isolate acquired has been cultured in PDA petri dishes and maintained in BOD (night photoperiod, 28 °C) at the Laboratório de Fisiologia Vegetal, Universidade Federal do Vale do São Francisco (CCA/UNIVASF).

DNA extraction and rDNA sequencing

Petri plates with samples of the mycorrhizal fungus CH01 isolate from *C. paludicolum* were sent to the Laboratório de Associações Micorrízicas at the Universidade Federal de Viçosa for submission to molecular identification procedures. Initially, the fungal mycelium was lyophilized and total DNA extraction was performed with NucleoSpin®Soil Kit (Macherey-Nagel) in accordance with the manufacturer's instructions. For the evaluation of DNA integrity, an agarose gel (0.8 %) was used and quantification was determined by spectrophotometry. The DNA extracted was submitted to PCR amplification using ITS1-ITS4 primers (White *et al.* 1990) to amplify the ITS1-5.8S-ITS2 region of the fungus ribosomal DNA (rDNA), and the reaction conditions described by Gardes & Bruns (1993). PCR products were purified using Exo-Sap (USB Corporation, Cleveland, Ohio), as recommended by the manufacturer. Both strands (forward and reverse) were sequenced by the Macrogen Inc. Company (South Korea). Amplified ITS sequences were assembled and edited using the Sequencher software program version 4.5 (GeneCodes, Ann Arbor, USA). Subsequently, the sequences were aligned and for application of the Mega software program version 4.0 (Tamura *et al.* 2007). Random representative sequences were selected and

searched with the BLASTn algorithm (Altschul *et al.* 1997) to select sequences from the NCBI database (GenBank, http://www.ncbi.nlm.nih.gov).

Symbiotic and asymbiotic seed germination

To evaluate the germination percentage and protocorm development, seeds previously stored for 15 days were submitted to a disinfection procedure. They were immersed in a sodium hypochlorite solution (0.5 % active chlorine) for 10 minutes followed by threefold washing in distilled and autoclaved water.

For the germination test, the disinfected seeds were kept in suspension in 50 mL of sterile water. Four hundred microliters were scattered on a filter paper disc (Germitest®, 6 cm diameter), placed in the center of a Petri dish containing 20 mL of a modified oat-agar culture medium (Dixon 1987) with 4 g L^{-1} oatmeal and 10 g L^{-1} agar (HiMedia®), and added to 2 g L^{-1} activated charcoal with the pH adjusted to 5.6. The filter paper disk served as a receptor of the seeds, which isolated the seeds from initial excess moisture in the culture medium. For symbiotic germination, each plate was inoculated with a 5mm diameter disc of fungal inoculum, one fungal mycobiont per plate (CH01 or M65), and a total of six replicate Petri dishes per mycobiont. Six uninoculated plates served as the control, i.e., asymbiotic treatment. The mycoboint M65 (Pereira *et al.* 2009) was a *Tulasnella* sp. from the orchid mycorrhizal fungi collection of the Laboratório de Associações Micorrízicas (BIOAGRO / UFV). M65 was subcultured once and maintained in BOD at room temperature before use in the germination experiment, showing satisfactory growth after the subculture step.

Petri dishes were sealed with PVC film (Dispafilm do Brazil Ltda) and then placed in a growth room (16/08h day/night photoperiod, 27 ± 2 ºC). Irradiance, supplied by cool white fluorescent bulbs, was measured at 40 μmol m^{-2} s^{-1}. The seed stage development was carried out 30 d after seed sowing using a stereomicroscope. Seed germination and development were assessed on a scale of 0–2 modified from Bektas *et al.* (2013) as follows: stage 0: seeds with viable and unswollen embryo, intact seed coat, no germination; stage 1: seeds with swollen embryo but without seed coat rupture, no germination; stage 2: swollen embryo with seed coat rupture, rhizoid presence or absence, germination. The germination percentage was calculated by dividing the number of seeds at stage 2 by the total number of seeds multiplied by 100. The growth index of the seeds was calculated using the following formula (Otero *et al.* 2005): $GI = (N_1+N_2.2) / (N_0+N_1+N_2)$, with GI as the growth index; and N_0 as the number of seeds at stage 0, N_1 the number of seeds at stage 1, and N_2 the number of seeds at stage 2.

The data collected were submitted to a one-way ANOVA with an F-test and a comparison of averages by Tukey test at the 5% significance level, using the Sisvar program (Ferreira 2011). Germination data were arcsine transformed to normalize variations before statistical analysis.

Microscopic analyses

For light microscopic examination, fungal hyphae growing on PDA, and root cross sections from adult orchid and cross sections in protocorm obtained from symbiotic germination (CH01) were individually mounted on a microscopic slide, covered with a cover slip and examined under a light microscope (Nova Optical Systems) coupled with a digital camera (Sony Cyber-shot). Sections were cut by hand and tissues were stained with Alcian blue and safranin in combination in all cases.

For the Scanning Electron Microscope (SEM) the protocorms were subjected to an ethanol dehydration process (from 5 to 95% v/v, 1 h each) after which manual cross sections were cut by hand using a razor blade. All cuts were kept in a kiln (35 °C for 24 h). The histological sections were positioned on stubs prior to gold sputtering in a Sputter Coater (Balzer FDU010). Following coating with a thin layer of gold (about 20 nm), the specimens were observed and micrographed using a TEM at 15 kV.

Results and discussion

Isolation and identification of mycorrhizal fungus

The presence of peloton was detected in the root cortex cells collected from vegetative adult plants of transversally sectioned *C. paludicolum* (Fig. 1A), confirming the symbiotic status of *C. paludicolum*. After the isolation process, only one endophyte, named CH01, was recovered from pelotons extracted from the root cortical parenchyma cells of *C. paludicolum* (Fig. 1B). On PDA, the colony appeared white and cottony with radial growth, aerial thin hyphal growth (Fig. 1B), and a vegetative hyphae septate right-angle branching pattern of hyphae with constriction at branching prints, as well as monilioid cells which were thin walled and barrel shaped, with nearly spherical branched chains (Fig. 1C).

Molecular analysis by ITS region characterization of the isolated mycorrhizal fungus from *C. paludicolum* showed that the isolate CH01 had a high homology level (94 %) with the *Tulasnella* genus, and it was registered as code KP973894 (Tab. 1). The sequence obtained from the isolate clustered with known *Rhizoctonia* sequences was retrieved from the Genbank. *Tulasnella* consists of filamentous fungi containing saprophytic species. Additionally, mycorrhizal fungi in orchid are one of the teleomorphic phases of the form-genus *Rhizoctonia*, an important symbiont genus in orchids (Mosquera-Espinosa *et al.* 2010). Integration of morphological characterization and ITS analysis have been used as more accurate identifiers of fungi (Cruz *et al.* 2011; Valadares *et al.* 2012).

Rhizoctonia-like fungi are difficult to classify due to the scarcity of sexual sporulation necessary to a definition of teleomorphic genera as *Ceratobasidium*, *Thanatephorus*,

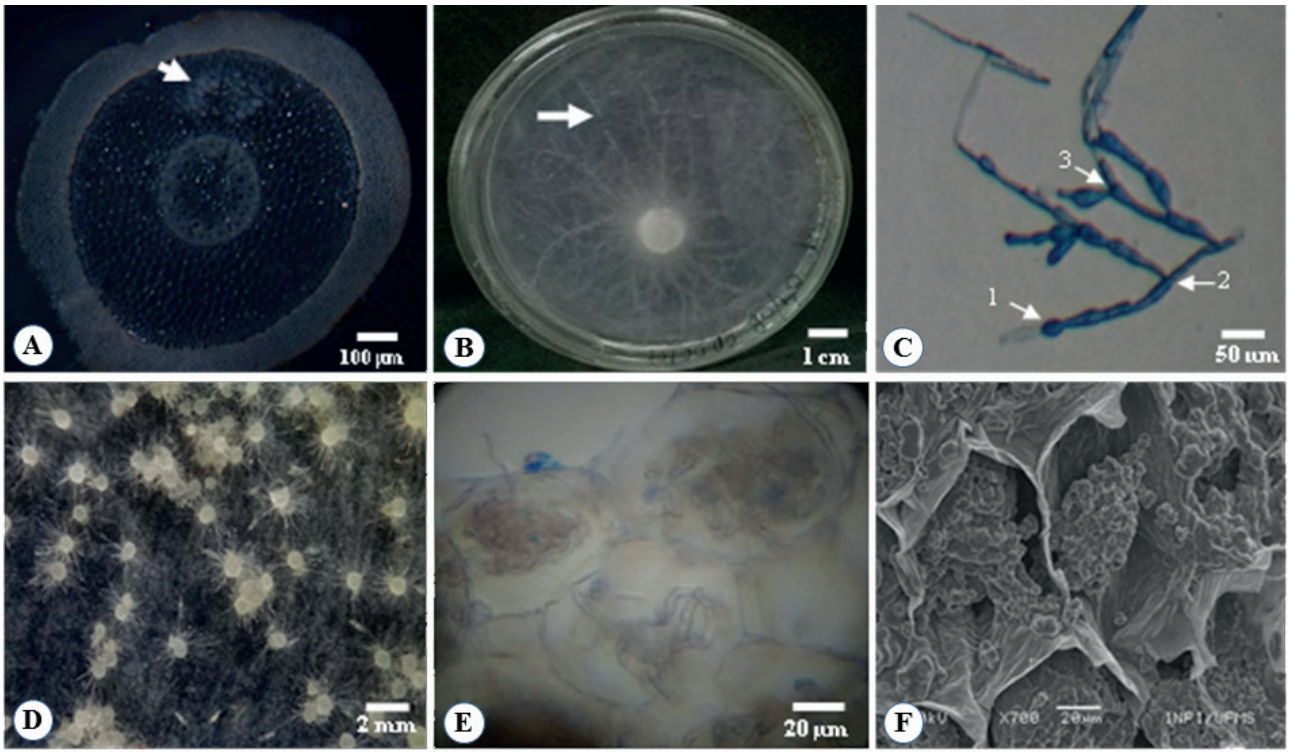

Figure 1. Transversal root section of *Cyrtopodium paludicolum* showing endophyte (arrow) presence in cortical parenchyma cells (**A**); CH01 fungus isolated on PDA medium with well development hyphae (**B**); Fungal hyphae with arrows indicating the presence of the monilioid cells (1), a right-angle branching (2), a constriction at the branch point and a septum in the branch hypha near its point of origin (3); (**C**); symbiotically raised protocorms (stage 2) with CH01 (**D**); optical (**E**) and scanning electronic (**F**) microscopies images showing colonized cortical parenchyma cells from *C. paludicolum* protocorms cultivated in symbiotic medium with CH01 isolate.

Tulasnella or *Sebacina*, and the morphological similarity of anamorphic genera to *Ceratorhiza*, *Epulorhiza*, *Moniliopsis* and *Rhizoctonia* in this artificial group (Currah *et al.* 1997; Roberts 1999). Consequently, molecular methods have become the standard means of assigning orchid fungi to groups within the *Rhizoctonia* alliance (Taylor *et al.* 2003; Shefferson *et al.* 2005), since these molecular methods enable a finer distinction of the strains compared to cytomorphological and ultrastructural characterizations (Dearnaley 2007).

Tulasnella has been found to be mycorrhizal in other orchid species, such as *Spathoglottis affinis* and *Eulophia spectabilis* (Chutima *et al.* 2011), *Cymbidium goeringii* and *C. faberi* (Yu *et al.* 2015). In Brazil, many isolated fungi from orchids belong to this respective anamorphic, the *Epulorhiza* genus, obtained from *Epidendrum dendrobioides* and *Sophronitis milleri* (Nogueira *et al.* 2005), *Epidendrum secundum* (Pereira *et al.* 2009; 2014; Nogueira *et al.* 2014), *Acianthera limae* and *Polystachya concreta* (Nogueira *et al.* 2014) and *Cyrtopodium vernum* (Gonçalves *et al.* 2014).

After 30 days, seed germination in the symbiotic process resulted in three different development stages (0, 1, 2), but only stage 2 was characterized as germinated seeds. Light and scanning electronic microscopical observations (Fig. 1E, F, respectively) showed that fungal pelotons were present in nearly all cross-sections of sampled protocorms obtained by seed selection at stage 2. These observations confirmed that the *C. paludicolum* protocorms were colonized by the CH01 isolate during the symbiotic germination process. Pelotons were distributed throughout the cortex, with no difference between protocorm cortical layers (Fig. 1E, F). TEM observations also confirmed the known mycorrhizal-protocorm interaction, showing degenerating hyphae attached to collapsed pelotons in SEM images (Fig. 1F). Suárez *et al.* (2006) also described the presence of degenerated hyphae, though in an active root cortical cell of *Stelis concinna*.

The simple presence of fungi in orchid roots does not necessarily indicate functional association. These fungi will need to be isolated and grown in orchid seedlings before they can be designated as mycorrhizal partners (Dearnaley 2007). Additionally, Bonnardeaux *et al.* (2007) affirmed that symbiotic germination remains the only practical method available for confirming compatibility between orchids and fungi. They found that symbiotic germination tests, in combination with accurate fungal identification, provided more information about the specificity of orchid fungi relationships than either method alone would have provided.

Table 1. Phylogenetic affiliation of operational taxonomic units (OTUs) based on fungal ITS1-ITS4 primers to amplify the ITS1-5.8S-ITS2 region of the fungus ribosomal DNA (rDNA).

Sequence length (pb)	Filogenetic relations		
	Family	GenBank accession number	Sequence identity (%)
575	Tulasnellaceae	KP973894	94%

Germination tests

Both the M65 and CH01 fungi examined in this study significantly increased the seed germination percentages and growth index of the *C. paludicolum* protocorms compared to the asymbiotic treatment (Tab. 2), irrespective of their origin. These results indicate that symbiotic inoculation with either mycorrhizal fungi CH01 or M65 was beneficial to the germination, but additional experiments will be necessary to confirm if these benefits also extend to protocorm development of *C. paludicolum*.

The fact that the germination percentage of both symbiotic treatments (CH01 and M65) are similar, leads to the inference that there is no specific relationship between *C. paludicolum* and the two symbionts. Consequently, the M65 endophyte, isolated from other orchid species, can be considered compatible with *C. paludicolum*. A compatibility relationship is usual in orchid symbiosis and similar results were obtained by Chutima *et al.* (2011), in an experiment on the symbiotic germination of *Pecteilis susannae* seeds, using different mycorrhizal fungi, including three obtained from their own orchid. These authors observed that the average percentage germination and development obtained by using their own isolated species were no different from the averages obtained by using another isolate from *Eulophia spectabilis*. Additionally, Pereira *et al.* (2015) suggested that *Cyrtopodium glutiniferum* has a preference for strains of *Tulasnella* and that fungus digestion is essential to protocorm development. In contrast, these authors observed that the isolates of *Ceratorhiza* and *Rhizoctonia* did not promote germination in *Cyrtopodium glutiniferum* seeds.

An understanding of the specificity in orchid mycorrhizal association is essential to the establishment of conservation strategies based on symbiotic propagation (Zettler 1997; Fracchia *et al.* 2014). Once orchids have been symbiotically propagated they have a higher survival rate when they are transplanted *ex vitro* (Ramsay & Dixon 2003). A lot of research needs to be done to unravel the underlying mechanism of mycorrhizal associations in orchids which may then help to prevent their decline and extinction in nature (Aggarwal *et al.* 2012).

Finally, the results of this research may contribute to the production of plants most likely to adapt to the natural environment which can be used in conservation programs through reintroduction into the Cerrado biome, subject to continuous degradation as a consequence of the impact of human activity. As part of our future intentions related to our research, we will investigate seedling acclimatization, the time required to further plant growth, and an evaluation of the survival rate in nature. These actions are necessary to ensure the success of future conservation programs of *Cyrtopodium paludicolum* and other native orchid species in the Cerrado.

Table 2. Seed germination and growth index (GI) of the *Cyrtopodium paludicolum* protocorms, 30 days after *in vitro* cultivation on 16/0 photoperiod in growth room ambient, in the absence (control) or in the presence of the fungi *Tulasnella* CH01 or M65.

Mycorrhiza treatment	Germination (%)[1]	GI
Control	34.44 b	0.91
CH01	52.89 a	1.16
M65	48.04 a	1.09

[1] Values followed by the same letter are not significantly different according to Tukey's multiple range test (P=0.05).

Conclusions

An endophytic fungus belonging to the genus *Tulasnella* was isolated from *C. paludicolum* roots. *Cyrtopodium paludicolum* seeds are not specific to the fungus obtained, CH01. Symbiotic germination is beneficial compared to asymbiotic germination. CH01 showed similar contributions to seed germination compared to isolated M65, a fungus with proven success in seed germination.

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