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Seed germination and development of orchid seedlings (*Cyrtopodium saintlegerianum*) with fungi

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Abstract

Fungi of Rhizoctonia complex are mycorrhizal of orchids and may to germinate yours seeds and development the seedlings. In this context, our objective was to select a fungal isolate to promote seed germination and seedling development of *Cyrtopodium saintlegerianum*. Pelotons were found in the roots and three mycorrhizal fungi were isolated. We tested mycorrhizal isolates obtained from *C. saintlegerianum* roots and six mycorrhizal fungi from other orchids as well three pathogenic isolates (of rice and bean) to germinate the seeds in oatmeal-agar medium. Seeds not inoculated were used as control. The isolates En07 (*Waitea circinata*), Cs10 (*Tulasnella* sp.) and Ro88 (*Rhizoctonia oryzae*) were efficient to promote seed germination, but only En07 differing statistically of the control. The non-specific isolate En07 promoted germination in 81% of seeds and the specific isolate (Cs10) promoted 60%, evidencing the non-specificity mycorrhizal association in this orchid during germination. Axenic seedlings were inoculated with four mycorrhizal fungi (non-inoculated seedlings - control). After six months, the isolates En07 and Cs10 were efficient in the interaction with the seedlings, but did not differ to the control. Therefore, our results suggested that fungi of the Rhizoctonia complex can be used in the germination and seedling development of *C. saintlegerianum*.

Key words: mycorrhizal association, non-specific fungi, Orchidaceae, plant pathogen, Rhizoctonia complex.

Resumo

Fungos do complexo Rhizoctonia são micorrízicos de orquídeas e podem germinar suas sementes e desenvolver as plântulas. Neste contexto, nosso objetivo foi selecionar um isolado fúngico para promover a germinação e o desenvolvimento de plântulas de *Cyrtopodium saintlegerianum*. Foram encontrados pelotons nas raízes desta orquídea e foram identificados três fungos micorrízicos. Nós testamos os isolados obtidos das raízes de *C. saintlegerianum* e seis outros isolados micorrízicos assim como três isolados patogênicos (de arroz e feijão) para germinar as sementes em meio de aveia-agar. Sementes não inoculadas foram usadas como controle. Os isolados En07 (*Waitea circinata*), Cs10 (*Tulasnella* sp.) e Ro88 (*Rhizoctonia oryzae*) foram eficientes para promover a germinação, mas somente o En07diferiu estatisticamente do controle. O isolado não específico En07 promoveu 81% de germinação e o isolado específico (Cs10) promoveu 60%, evidenciando a associação micorrízica não específica durante a germinação. Plântulas axênicas foram inoculadas com quatro fungos micorrízicos (plântulas não inoculadas - controle). Após seis meses, os isolados En07 e Cs10 foram eficientes na interação com as plântulas, mas não diferiram do controle. Portanto, nossos resultados sugerem que fungos do complexo Rhizoctonia podem ser usados na germinação e no desenvolvimento de plântulas de *C. saintlegerianum*.

Palavras chave: associação micorrízica, fungos não específicos, Orchidaceae, patógenos de plantas, complexo Rhizoctonia.

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Introduction

Mycorrhizal associations are essential to the life cycle of orchids in natural habitat. The interaction begins during seed germination when the mycorrhizal fungi infect basal cells in embryo. The hyphal coils formed into these cells are digested by the orchid to obtain carbon and nutrients necessary for its developmental initial phase. The seed produces a heterotrophic structure called the protocorm, which then forms the seedling. The seedling produces its first root and fungi can colonize its cortical cells. From this phase, symbiosis with mycorrhizal fungi facilitates the acquisition of nutrients from the substrate (Peterson *et al.* 2004; Rasmussen & Rasmussen 2009; Dearnaley *et al.* 2012).

Orchids Mycorrhizal Fungi (OMF) verified in Brazilian orchids belong to the Rhizoctonia complex and were identified as *Ceratobasidium* D.P. Rogers, *Thanathephorus* Donk and *Tulasnella* Schroet teleomorphic genera (Nogueira *et al*. 2005; Pereira *et al.* 2011, 2015; Silva *et al.* 2016). And studies have shown that mycorrhizal isolates promote *in vitro* seed germination and seedling development better than axenic commercial media for orchid propagation (Pereira *et al.* 2011; Guimarães *et al.* 2013; Jiang *et al.* 2015). In this way, the mycorrhizal fungi inoculation has been highlighted as a promising strategy to improve orchid seedling production (Cribb *et al.* 2003).

Due to differences in specificity observed during mycorrhizal orchid interactions, fungal isolation has been required to select the suitable isolate to promote seed germination and seedling development (Dearnaley *et al.* 2012). Some orchids have a narrow specificity with some fungal genera. During seed germination experiments, *Coppensia doniana* (Batem. *ex* W. Baxter) Campacci and *Oncidium flexuosum* Sims demonstrated preference for the mycorrhizal fungi *Ceratorhiza* sp., anamorphic form of *Ceratobasidium* genera (Pereira *et al*. 2005). In contrast, *Epidendrum secundum* Jacq. and *Cyrtopodium glutiniferum* Raddi preferred fungi *Epulorhiza* sp., mycorrhizal anamorphic form of *Tulasnella* genera (Pereira *et al.* 2009, 2015). Understanding such specificities is valuable for selection of the symbiont that assure propagation and commercialization of healthy orchid seedlings.

Some mycorrhizal fungi also associate with non-host orchids. For example, the seeds of *Tolumnia variegata* (Sw.) Braem associated with the mycorrhizal isolate of *Ionopsis utricularioides* (Sw.) Lindl. (Otero *et al.* 2004); seeds of *Epidendrum nocturnum* Jacq. interacted with *Spiranthes brevialabris* Lind. mycorrhizal fungi (Zettler *et al.* 2007); and seeds of *Spathoglottis plicata* Blume had better germination with two mycorrhizal isolates of *Dendrobium anosmum* Lind. and *Paphiopedilum sukhakulii* Schoser & Senghas (Aewsakul *et al.* 2013). The performance of non-specific mycorrhizal fungi may be evaluated to select the suitable fungi for propagating of some orchids (Zettler *et al.* 2007).

Fifty terrestrial and epiphytic species of *Cyrtopodium* Rchb. f. have been reported from South America, thirty of which occur in area the Cerrado (Batista & Bianchetti 2005). Some species of the genus *Cyrtopodium* have been widely explored as sources for raw material for small medical industries and ornamental gardens (Barreto & Parente 2006; Dutra *et al.* 2009; Vogel & Macedo 2011; Pereira *et al.* 2015). *Cyrtopodium saintlegerianum* Rchb. f. occurs as an epiphyte on species of palm in the Brazilian Cerrado (Batista & Bianchetti 2006; Romero-Gonzáles *et al.* 2008; BFG 2018), and has been propagated in media axenic containing phytoregulators (Rodrigues *et al.* 2015; Silva *et al.* 2017). This species has been used in the ornamentation of gardens as well as to prepare dermatological plasters.

Little information has been reported about mycorrhizal association in these genera orchids. Recently, seeds and seedlings of *C. glutiniferum* exhibited satisfactory development when inoculated with mycorrhizal fungi of the genus *Tulasnella* (Guimarães *et al*. 2013; Pereira *et al*. 2015). However, more studies are required to determine the presence of mycorrhizal fungi *in situ*, their isolation and to evaluate fungi potential during *in vitro* symbiotic seed germination. In view of this aspect, the objective in present study was to select a suitable fungal to promote seed germination and seedling development of *C. saintlegerianum*. To achieve this aim, we first confirm root mycorrhizal colonization of *C. saintlegerianum* throw root anatomical analysis. Its mycorrhizal fungi were isolated and identified morphologically. The seed germination test was performed co-inoculating *C. saintlegerianum* seeds with different isolates: its own mycorrhizal fungi, others mycorrhizal isolates and some pathogenic Rhizoctonia-like fungi. Isolates that promoted the development of the embryo as well as other specific isolates were inoculated in axenic seedlings of *C. saintlegerianum* to test their potential to support the *ex vitro* development.

Material and Methods

Capsule and root collection

Capsules and roots of *C. saintlegerianum* (Fig. 1a) were collected during March 2010 to August 2011 from three different plants growing on three palms in pasture areas in the Brazilian Cerrado (16º07'66.6''S and 50º10'04.4''W). The biological material was transported to the Laboratório de Genética de Microrganismos (LGM) at the Universidade Federal de Goiás (UFG) Brazil. Some root fragments were fixed in FAA70 (Formaldeyde - Acetic acid - Alcohol 70%) (Johansen 1940) for two days and stored in ethanol solution (70%) until anatomical characterization of mycorrhizae. Others fragments were reserved to mycorrhizal fungi isolation. The capsules were stored into flask containing silica gel and kept at 4 °C until germination experiments.

Mycorrhizae microscopy characterization, fungal isolation and identification

Root fragments of *C. saintlegerianum* were sectioned by freehand for optical microscopy (OM) observation. The sections were cleared and subjected to 1% aqueous safranin and 0.3% astra blue (Krauss & Arduin 1997) for cell roots and fungal structures coloring. Root sections were prepared to Scanning Eletronic Microscope (SEM) observation according to Silva *et al.* (2016) in Laboratório Multiusuário de Microscopia de Alta Resolução - LabMic, Physics Institute, UFG.

The mycorrhizal fungi were isolated according to Gonçalves *et al.* (2014) using the PDA medium (Potato Dextrose Agar, composed of 200 g of potato, 20 g of dextrose, 20 g of agar and 1 L of water - Otero *et al.* 2004). Fungal isolates were cultivated on PDA plates under continuous fluorescent light for five days at room temperature (26±2 °C). Isolates with morphological characteristics of OMF (Currah & Zelmer 1992) were maintained in a growth chamber at 26±2 °C with a 16 h photoperiod.

The identification of OMF were performed using features described by Currah & Zelmer (1992), Nogueira *et al.* (2005), Pereira *et al.* (2005) and Silva *et al.* (2016). The cultural characteristics evaluated were colony diameter, growth tax, number of nuclei per cell, hyphal diameter, width and length of monilioid cells and polyphenol oxidase (PPO) production. The cultural characteristics (color, aerial mycelium, mycelium shape and size, and colony diameter) were evaluated after 72 and 336 h on PDA or CMA medium (Corn Meal Agar, composed of 15 g agar and 1 L broth obtained from cooking 30 g of corn meal).

The number of nuclei per cell and hyphal diameter were evaluated according to Meinhardt *et al.* (2001). The images of hyphae were captured using a Leica DMI6000 optical microscope (OM) with an epifluorescence accessory and processed in Leica IM50 editor. The width and length of monilioid cells of each isolate were assessed in fungal colonies cultured in CMA for two months and were measured from images taken under BelPhotonics microscope cells using the BelAnalyzer MicroImage software.

The fungal PPO production was evaluated in Petri plates containing YEA medium (Yeast Extract Agar, composed of 15 g Yeast Extract 15 g, 15 g Agar 15 g in water 1 L) with addition of tannic acid (5 g), according to Zelmer & Currah (1995). Petri plates without tannic acid were used as a control. The production of PPO was detected by the presence of an amber-colored halo around the colonies after five days.

In vitro symbiotic germination and *ex vitro* development

Seeds collected from a 12-month mature capsule were sterilized as described for Silva *et al.* (2016). After capsule opening, 50% of the seeds were used to test the viability of the embryo with triphenyltetrazolium chloride (TTC) and 50% were used for symbiotic cultivation. A TTC solution was used to evaluate seed viability adopting modifications to orchid seeds (Vujanovic *et al.* 2000). Ten sample (with approximately 0.001 g of seed) were transferred into microtubes containing 2 mL of 1% TTC solution and kept in a water bath at 40 °C in continuous darkness to prevent TTC precipitation. The embryos were observed for the capture of images under light microscopy (BelView software).

We tested mycorrhizal isolates obtained from *C. saintlegerianum* roots and six mycorrhizal fungi from other orchids (fungal isolates belonging to the LGM library). Three pathogenic isolates, taxonomically related to some mycorrhizal fungi (two of *Rhizoctonia oryzae* Ryker & Gooch and one of *Rhizoctonia solani* Kühn, pathogenics of rice and bean) were supplied by the Collection of Functional Microorganisms of Embrapa Arroz e Feijão (CNPAF) - Brazil (Tab. 1). All isolates selected were grown in plates with oatmeal-agar medium (OMA - Zettler *et al.* 2007; Steinfort *et al.* 2010).

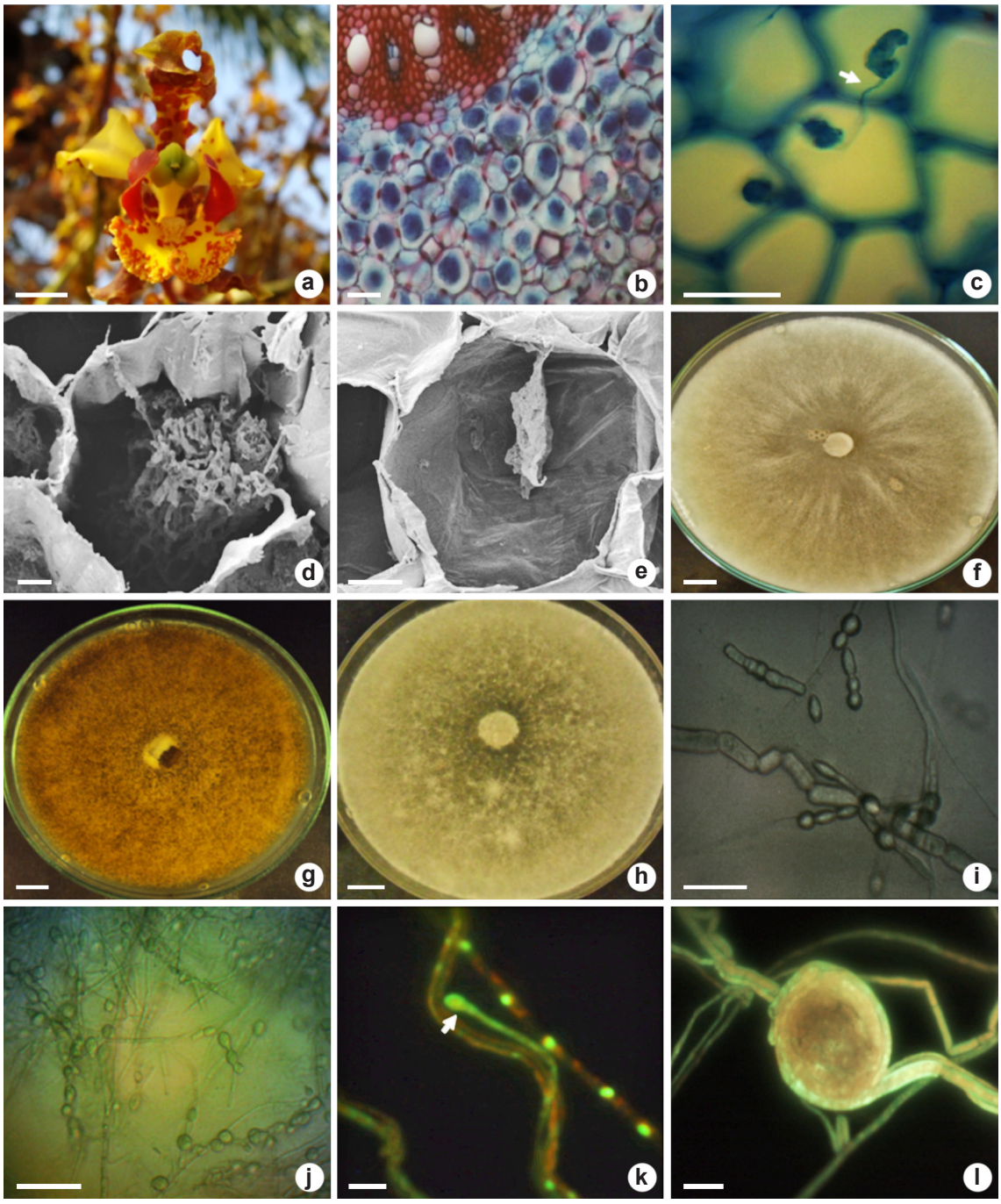

Figure 1 – Flower (in the natural habitat), mycorrhizal colonization and fungal identification (in laboratory) of *Cyrtopodium saintlegerianum* – a. flower with capsule formation; b. overview of the parenchymatous cortex roots colonized by pelotons (intact and degraded); c. pelotons in neighboring cortical cells connected by hypha (arrow); d. intact peloton inside cortical cell; e. peloton in degradation; f. isolate Cs02 after 336 hours in PDA; g. isolate Cs10 after 336 hours in PDA showing brown-colored mycelium; h. isolate Cs21 in PDA; i. chain of monilioid cells from isolate Cs10; j. chain of monilioid cells (Cs21); k. young monilioid binucleate cell of Cs02 (arrow); l. peloton formed of Cs10 in microculture (PDA). Bars = 1 cm (a,f-h), 100 µm (b), 50 µm (c,i-k), 20 µm (e), 10 µm (d,l).

A 9-mm mycelial disc was taken from the edge of each isolate colony and placed in plates with OMA and containing approximately 150 seeds. Plates containing seeds without fungi were maintained as controls. The experimental design was completely randomized with ten treatments and eight replicates (Tab. 1). The plates were incubated in a germination chamber at 26±2 °C and with a 16 h photoperiod. The data obtained from the seed viability test were normalized by square root transformation, whereas the germination data were transformed using arcsin. To compare each treatment we performed an analysis of variance and *a posteriori* Tukey test (at 5% probability) using the software R v 2.11.0 (Díaz & Álvarez 2009; Steinfort *et al.* 2010).

Germination was assessed every two weeks, under a light microscope coupled to a digital camera, using the parameters: 0 - no germination, 1 - swelled embryo and rupture of testa (germination), 2 - continued embryo enlargement and production of rhizoid (Fig. 2) adapted of Stewart & Zettler (2002). The final evaluation occurred nine months after sowing. Seeds were observed under an optical microscope to assess the presence of pelotons, which were stained according to Chutima *et al.* (2010). Seeds at different stages of germination were collected for SEM observation. Seed preparation was performed as suggested by Chou & Chang (2004) and visualization was done in the same mode as the root fragments.

For *ex vitro* mycorrhization assessment, asymbiotic seedlings with thirteen months were obtained from *in vitro* seed germination using MS medium (Silva *et al.* 2017). The mycorrhizal fungi were *Tulasnella* sp. isolates (Cs02, Cs10 and Cs21) obtained from *C. saintlegerianum* and *Waitea circinata* Warcup & Talbot (En07) from *E. nocturnum* (Tab. 1). Seedlings of *C. saintlegerianum* were inoculated with 0.2 g of mycelium of each isolate. The seedlings were grown in axenic substrate (*Sphagnum* sp.) in a complete randomized experimental design with five treatments and five replicates. Number of shoots, stem diameter (0.5 cm above the base), stem vigor in region of pseudobulb formation (1 cm above the base) and survival of the seedlings were evaluated after six months. To compare each treatment (isolate and control) we performed an analysis of variance and *a posteriori* Tukey test (at 5% probability) using the software R v 2.11.0 (Steinfort *et al.* 2010).

Results

Mycorrhizal colonization, fungal morphological characterization and identification

The pelotons were stained dark blue due to the presence of chitin in the hyphae (Fig. 1b-c). Pelotons were mostly intact with the hyphae occupying the cortical cells of the roots (Fig. 1b). Connective hyphae between pelotons within neighboring cells were found (Fig. 1c). Intact (Fig. 1d) and degraded (Fig. 1e) pelotons within cortical cells roots were observed.

Table 1 – Treatments (fungi isolates and controle – without fungal) used in the germination of *Cyrtopodium saintlegerianum*.

Code	Fungal identification	Host plant	Interaction
Control	-	-	
En07	*Waitea circinata*	*Epidendrum nocturnum* (orchid)	Mutualist
Cs02	*Tulasnella* sp.	*Cyrtopodium saintlegerianum* (orchid)	Mutualist
Cs10	*Tulasnella* sp.	*Cyrtopodium saintlegerianum* (orchid)	Mutualist
Cs21	*Tulasnella* sp.	*Cyrtopodium saintlegerianum* (orchid)	Mutualist
Cv10	*Waitea* sp.	*Cyrtopodium vernum* (orchid)	Mutualist
Cv17	*Tulasnella* sp.	*Cyrtopodium vernum* (orchid)	Mutualist
Ro88	*Rhizoctonia oryzae*	*Oryza sativa* (rice)	Pathogen
Ro89	*Rhizoctonia oryzae*	*Oryza sativa* (rice)	Pathogen
Rh21180	*Rhizoctonia solani*	*Phaseolus vulgaris* (bean)	Pathogen

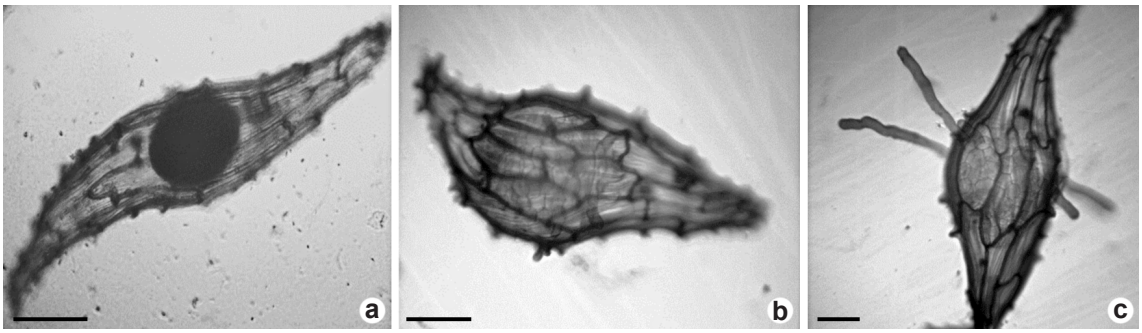

Figure 2 – Development stages used to determine in vitro germination of *Cyrtopodium saintlegerianum* – a. 0: no germination (viable embryo); b. 1: swelled embryo – rupture of testa (germination); c. 2: continued embryo enlargement – production of rhizoids. Bars = 50 μm (a,b,c).

From *C. saintlegerianum* roots were obtained three isolates (Cs02, Cs10 and Cs21 - Fig. 1f-h) with Rhizoctonia characteristics (Currah & Zelmer 1992). The isolate Cs02 presented faster mycelial growth than Cs10 and Cs21 in BDA medium. The mycelium of isolates Cs02 and Cs21 was white while Cs10 is brown (Fig. 1f-h). Only in CMA medium, these isolates produced round monilioid cells with chains containing up to five cells (Fig. 1i-j). The isolates presented two nuclei per cell and we registered two nuclei in one new monilioid cell of isolate Cs02 (Fig. 1k). After five days of growth on PDA microculture, the isolate Cs10 formed pelotons or hyphae bundles (Fig. 1l). None of the isolates (Cs02, Cs10 and Cs21) formed an amber-colored halo, indicating absence of PPO production. Based on morphological characterization, these isolates were identified as *Tulasnella* sp. (*Epulorhiza* sp., anamorphic phase).

In vitro germination and *ex vitro* development

Embryos of *C. saintlegerianum* seeds were stained at 3 h and the viability test demonstrated that 77% of the seeds were viable as indicated by their dark red color (Fig. 3a). The isolate non-specific En07 highlights from the other fungi providing seed tegument rupture and rhizoid formation (Stage 2) after two months (Fig. 3b). In the control (without fungus), there was no rhizoid formation (Fig. 3c). After nine months, the treatment with the En07 isolate provided 81% of germinated seeds (Stages 1 and 2). However, it was not statistically different from the isolates Cs10 (*C. saintlegerianum* specific) and Ro88 (pathogenic to rice), which presented 60 and 52% germinated seeds, respectively. The treatment with En07, CS10 and Ro88 showed a larger number of germinated seeds and differed statistically from the control (Tab. 2).

Seeds inoculated with En07 showed many rhizoids after nine months (Fig. 3d). Hyphae of this isolate penetrate the rhizoids and formed pelotons (Fig. 3f). Seeds inoculated with isolate Cs10 too showed rhizoids and embryo swelling (Fig. 3e). The isolates Cs02 (23%) and Cs21 (21%) were specific for *C. saintlegerianum*, but presented germination percentages lower than control (27%). The other isolates (Cv10, Cv17, Ro89 and Rh21180) were not efficient in germinating orchid seeds (Tab. 2).

In SEM we observed cracks in the coat of seeds inoculated with isolate En07, indicating seed coat rupture in consequence of embryo swelling (Fig. 3g-h). The seeds from the control showed intumescence, but did not germinate because there was no differentiation in the embryo or tegument rupture (Fig. 3i).

In the *ex vitro* symbiotic development, the isolates En07 and Cs10 (Fig. 3j-k) promoted thicker and vigorous stems in axenic seedlings of *C. saintlegerianum* after six months, but did not differ statistically from the control (Fig. 3l; Tab. 3). All isolates promoted shoot formation, but only En07 treatment presented a shoot number significantly higher than the control. Survival percentage of seedlings in association with fungi isolates was not differed from the control (Tab. 3).

Discussion

This is the first report of symbiotic germination and seedling development of *C. saintlegerianum*. Our study showed that embryos without fungi just exhibited intumescence, without rhizoids formation.

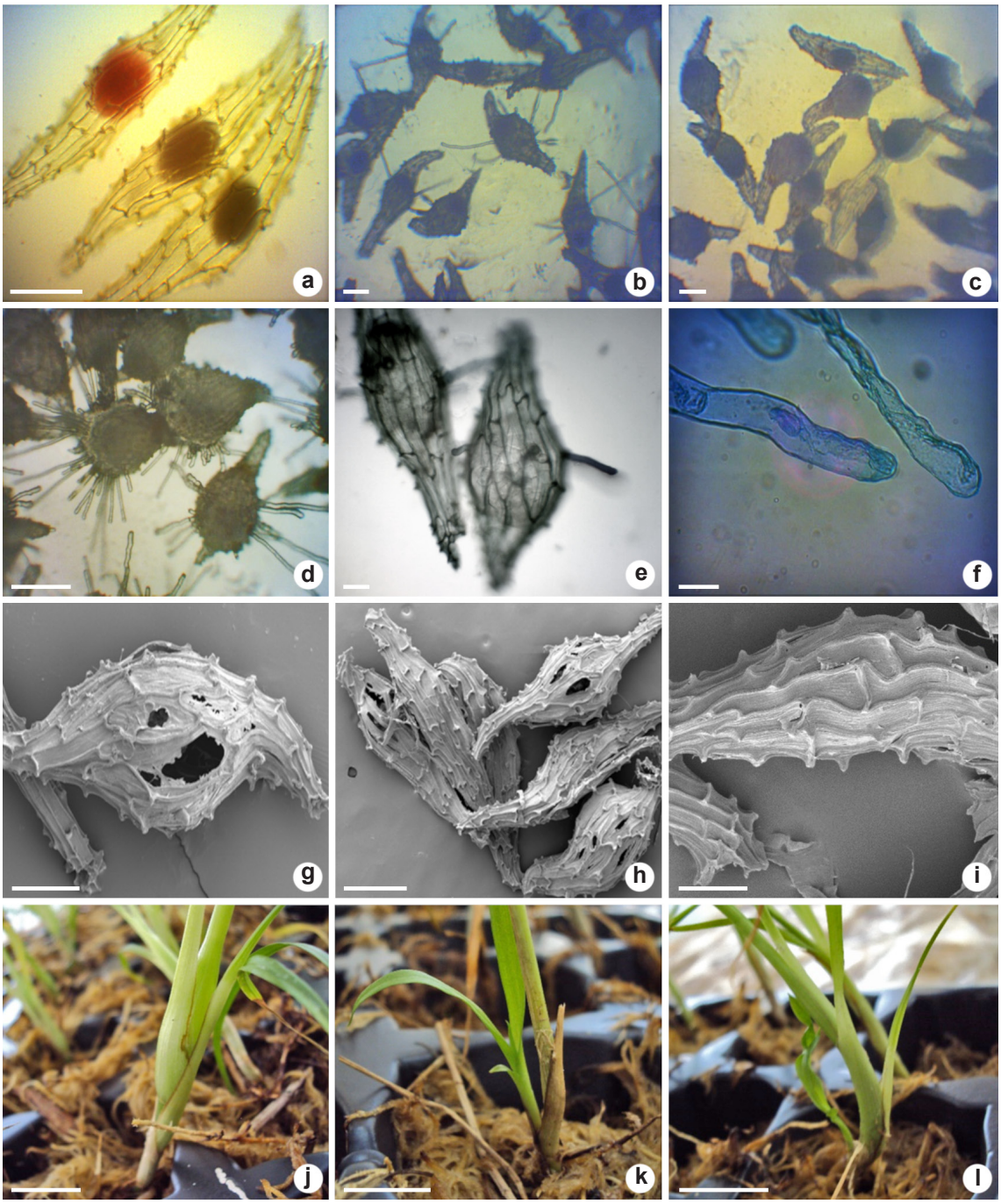

Figure 3 – *Cyrtopodium saintlegerianum* seeds and seedlings – a. viable (v) and non-viable (nv) embryos of seeds submitted to tetrazolium test (TTC); b. seed inoculated with isolate En07 (*Waitea circinata* of *Epidendrum nocturnum*) with rhizoids after two months; c. seed without fungi (control) were swollen after nine months of growth in oatmeal-agar media; d. seed germinated after nine months with En07 (showed many rhizoid – Stage 2); e. seeds showed rhizoids with isolate Cs10 (*Tulasnella* sp.); f. rhizoid emerging from the embryo and peloton inside (seed with En07); g. swollen seed showing a crack in the seed coat, suggesting that isolate En07 colonized the embryonic cells (Stage 2); h. non-germinated seeds (0) and swollen seeds (1) derived from the association with isolate En07; i. seeds with swollen embryos, but non-germinated (control – Stage 0); j. seedling six months after of ex vitro mycorrhization with isolate En07 (non-specific); k. seedling with isolate Cs10 (specific); l. seedling without fungal (control). Bars = 1 cm (j-l), 200 µm (b-d,h), 100 µm (g,i), 50 µm (a,e), 10 µm (f).

Table 2 – *In vitro* germination of *Cyrtopodium saintlegerianum* seeds after inoculation with fungal isolates (after nine months of incubation).

Isolate	Number of seeds				% Germination	
	Total	Stage 0	Stage 1	Stage 2		
Control	244	180	64	0	27*±7.4**	bc
En07	489	93	326	70	81±7.8	a
Cs02	391	298	93	0	23± 7.9	c
Cs10	555	221	310	24	60±12.6	ab
Cs21	401	314	80	7	21±5.8	c
Cv10	508	405	95	8	20±6.1	c
Cv17	489	291	188	10	40±14.7	bc
Ro88	502	240	258	4	52±4.6	abc
Ro89	548	332	193	22	38±15.7	bc
Rh21180	510	289	210	9	43±9.7	bc

*Means followed by the same lowercase letters in the same row did not differ from each other according to the Tukey's test ($P < 0.05$). ** Standard error.

Mycorrhizal fungi *Tulasnella* sp. and *W. circinata* promoted embryo development up to produce protocorm with rhizoids and ruptured coat, which confirm dependence of *C. saintlegerianum* during seed germination. Additionally, *W. circinata* isolate support better seedling development. Indeed, *W. circinata* is a potential isolate to be applied during symbiotic cultivation of *C. saintlegerianum*.

We observed a large number of intact and degraded pelotons in parenchyma cells of the root cortex in adult plants of *C. saintlegerianum* (Fig. 1b-e). These observations confirm the maintenance of the mycorrhizal association at adult phase. Thus, the presence of pelotons indicates the orchid needs fungal interaction to acquire nutrients. In addition, the pelotons degradation may be associated with the flowering period when roots were collected, in which the plant has a higher nutritional demand.

The morphological characteristics and no production of PPO confirm identification of *C. saintlegerianum* mycorrhizal isolates as *Tulasnella* sp. (Currah & Zelmer 1992; Zelmer & Currah 1995; Athipunyakon *et al.* 2004). We observed morphological differences among our *Tulasnella* (Fig. 1f-l), which propel us to select the three *Tulasnella* isolates to *in vitro* seed germination experiment. These isolates presented different results during seed germination and *ex vitro* seedling cultivation, corroborating with Pereira *et al.* (2009; 2011), who observed that morphologically different *Tulasnella* can present divergent results in seed germination experiments.

Some species interact with mycorrhizal fungi just during seed germination, although other species maintain the interaction during the adult phase (Peterson *et al.* 2004; Zettler *et al.* 2007; Rasmussen & Rasmussen 2009). Zettler *et al.* (2007) reported symbiosis maintenance between *Tulasnella* fungi and orchid *S. brevilabris* from embryo stage until adult phase. In the same way, Látalová & Baláz (2010) and Gonçalves *et al.* (2014) demonstrated interaction between orchid and mycorrhizal fungus from protocorm until adult stage. Silva *et al.* (2016) observed that *W. circinata* increases the vigor of *Epidendrum nocturnum* seedlings *in vitro* and a potential interaction was observed with seeds and seedlings (*in vitro* and *ex vitro*, respectively) of *C. saintlegerianum* too. Thus, our isolates can support the mycotrophism of this plant during *in vitro* and *ex vitro* propagation, even in natural habitat.

Earlier investigations have shown that *Tulasnella* sp. is associated with many tropical orchids as *C. vernun*, *C. glutiniferum, E. secundum*,

Table 3 – The number of shoots, stem diameter, vigor and survival of *Cyrtopodium saintlegerianum* seedlings six months after *ex vitro* mycorrhization with four isolates (Control: not fungal inoculation).

Code	Fungal identification	Shoot (number)	Stem vigor* (cm)	Stem diameter * (cm)	Survival (%)
Cs02	*Tulasnella* sp.	0.52 ab**	0.32 c	2.12 b	56 a
Cs10	*Tulasnella* sp.	0.46 ab	0.46 abc	2.37 ab	62 a
Cs21	*Tulasnella* sp.	0.48 ab	0.40 bc	2.24 b	64 a
En07	*Waitea circinata*	0.62 a	0.68 a	3.34 a	80 a
Control	-	0.30 b	0.64 ab	2.70 ab	76 a

*Stem vigor in region of pseudobulb formation (1 cm above the base) and stem diameter (0.5 cm above the base); **Means followed by the same lowercase letters in the same row did not differ from each other according to the Tukey's test ($P < 0.05$).

Epidendrum dendrobioides Thunb. e *Sophronitis milleri* (Blumenschein *ex* Pabst) C. Berg & M. W. (Nogueira *et al.* 2005; Pereira *et al.* 2005, 2009, 2015; Gonçalves *et al.* 2014). In this study, *W. circinata* (En07 isolate from *E. nocturnum*) was more efficient to support symbiotic development of *C. saintlegerianum,* associate in nature with *Tulasnella*. En07 presented germination percentage similar to seed viability, supported protocorm developed until Stage 2 (Tab. 2) and presented best results during seedling development (Tab. 3). In light of this, screening non-natural and natural mycorrhizal fungi is an important strategy to select a suitable isolate to orchid symbiotic cultivation. Some orchids present better development in association with their own OMFs. It is probably in consequence of narrow mycorrhizal specificity (Valadares *et al.* 2010; Silva *et al.* 2016). Other orchids respond better to OMFs of other plants, suggesting broad mycorrhizal specificity (Otero *et al.* 2004), regarding with present study.

The ability of *R. oryzae* and *R. solani* isolates (Tab. 2), pathogenic fungi of rice and bean plants, to promote *in vitro* seed germination of *C. saintlegerianum* have highlighted. Masuhara *et al.* (1993) had no success in seed germination of *Spiranthes sinensis* var. *amoena* with pathogenic isolates of *R. solani* and *R. oryzae*. However, Masuhara & Katsuya (1994) suggested that *Rhizoctonia* spp. pathogens of rice (*Oryza sativa*) would also germinate orchid seeds. It is an evidence that orchids suppress pathogenic potential of these fungi and use them as nutrient source during embryo and seedling development.

The En07 and CS10 improved seedlings vigor, shoot number and stem diameter of *C. saintlegerianum* seedling (Tab. 3; Fig. 2j-l), but through the evaluations carried out it was not possible to verify statistical difference. These improvements are indispensable to plant longevity and establishment during acclimatization. Hence, in future approaches on the increases from seedlings association with fungi, more refined methods of measurement will be needed. Benefits of mycorrhization during orchid seedling establishment have been reported to *C. glutiniferum* (Guimarães *et al.* 2013), *Phalaenopsis* sp. (Moreno *et al.* 2000; Wu *et al.* 2011) e *Spathoglottis plicata* Blume (Aewsakul *et al*. 2013). Additionally, mycorrhizal associations can also suppress biotic agents, such as plant pathogens (Peterson *et al*. 2004; Rasmussen & Rasmussen 2009). OMFs induced resistance in rice plants to pathogenic soil fungal (Mosquera-Espinosa *et al.* 2013) and our isolate En07 (*W. circinata*) demonstrated potential as biocontrol agent against *Magnaphorte oryzae*, a rice blast pathogen (Carvalho *et al.* 2015).

Our results support the use of mycorrhizal fungi in germination and development of *C. saintlegerianum*. This orchid has little mycorrhizal specificity, facilitating its interaction with fungi from other plants. Some *Rhizoctonia* isolates may supporte seed germination, plant vigor, greater longevity and resistance to environmental factors. Thereby, we advocate the use of fungi during *C. saintlegerianum* propagation and suggest testing the inoculation of these in other orchid seeds and seedlings. Future investigations are necessary in order to better understanding the orchid-fungal interactions as well as the evaluation of the increment of the application of the fungi in axenic seedlings.

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