



ARTIGO ORIGINAL

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Isolation and characterization of mycorrhizal fungi in *Cyrtopodium verum* Rchb. F. & Warm (Orchidaceae)

Isolamento e caracterização de micorrizas orquidoides em Cyrtopodium verum Rchb. F. & Warm (Orchidaceae)

ABSTRACT: Mycorrhizal fungi establish mutualistic associations with the majority of vascular plants. In orchids, mycorrhizal fungi favor the processes of seed germination, seedling establishment and adult plant development. Isolation and identification of fungi that occur in mycorrhizal associations with orchids can assist in the establishment of seedlings, the reintroduction of species in nature and large-scale production. In August 2008, roots from *Cyrtopodium verum* were collected in the Biological Reserve Professor José Ângelo Rizzo, a 144-ha remnant forest in the city of Mossamedes, GO. The predominant vegetation type in the area is cerrado rupestre, and species were found growing in rocky, dry and acidic soils with low fertility. The roots were used for the isolation of mycorrhizae and to obtain transverse and longitudinal histological cuts. Two genera of *Rhizoctonia*-like fungi were isolated and identified: *Epulorhiza* and *Rhizoctonia*, both frequently found in association with terrestrial and rupicolous orchids. Masses of hyphae, called *pelotons*, were observed in histological sections of the roots within the parenchymal cells of the cortex of the roots.

RESUMO: Fungos micorrízicos estabelecem associações mutualistas com a maioria das plantas vasculares. Em orquídeas, esses fungos podem favorecer o processo de germinação de sementes, o estabelecimento de plântulas e o desenvolvimento da planta adulta. Trabalhos de isolamento e identificação de fungos que ocorrem em associações micorrízicas com espécies de orquídeas podem auxiliar no estabelecimento de mudas, na reintrodução de espécies na natureza e na produção em grande escala. Os objetivos deste trabalho foram isolar, identificar e caracterizar micorrizas orquidoides associadas às raízes de *Cyrtopodium verum*, uma espécie nativa do Cerrado. Em agosto de 2008, foram coletadas raízes de *C. verum* na Reserva Biológica Professor José Ângelo Rizzo, um remanescente florestal de 144 ha, no município de Mossamedes-GO. A vegetação predominante na área é do tipo cerrado rupestre, no qual as espécies foram encontradas crescendo em solos rochosos, secos, ácidos e com baixa fertilidade. As raízes foram utilizadas para o isolamento das micorrizas e para a obtenção de cortes histológicos. Dois gêneros de fungos rizoctonioides foram isolados e identificados: *Epulorhiza* e *Rhizoctonia*, ambos encontrados, com frequência, em associação com orquídeas terrestres e rupícolas. Nos cortes histológicos das raízes, verificaram-se, nas células parenquimáticas do córtex, massas de hifas denominadas *pelotons*.

1 Introduction

Orchid mycorrhizae are very important in the life cycles of species of the Orchidaceae family, which must be colonized by symbiotic fungi for germination to occur, as the orchids possess very small seeds with little nutritional reserve (Pereira et al., 2003; Rasmussen; Rasmussen, 2009). During mycorrhizal colonization of the seeds, an intercellular structure called *peloton* is formed; this structure is composed of a mass of fungal hyphae. From the enzymatic digestion of this structure, the embryo begins its development with the formation of a tuberous structure (usually chlorophyllated) called the protocorm (Kraus et al., 2006), which is completely dependent on a symbiotic fungus for development (Pereira et al., 2003). Additionally, these fungi also aid in the development of the adult plant (Pereira et al., 2003), as they increase nutrient absorption and tolerance to biotic (phytopathogens) and/or abiotic stresses such as water, salinity and heavy metals (Rasmussen; Rasmussen, 2009; Trigiano et al., 2010).

Records that describe orchid mycorrhizae of the American continent are rare, particularly for epiphytic and rupicolous taxa (Richardson et al., 1993; Pereira et al., 2003). Additionally, many orchid species of the Cerrado (Brazilian tropical savanna) are under threat from human activity, whether due to predatory extractivism or habitat alteration (Ribeiro; Rodrigues, 2006).

Cyrtopodium verum Rchb. f. & Warm (Orchidaceae) is a terrestrial species that is native to Brazil and endemic to the Cerrado and Caatinga (semi-arid scrub forest) regions. The species occurs in arid environments, sandy soils and full sun. Another important characteristic of the species is its ornamental character, due to the beauty of its flowers and bulbs (Menezes, 2000). As *C. verum* is adapted to conditions of low moisture and high brightness, these characteristics render the species viable for both the domestication process and conservation and reintroduction programs (Stumpf et al., 2007). However, to assist in this process of domestication, an understanding of the mycorrhizal fungi associated with the species is necessary. The objectives of this study were to isolate, identify and characterize orchid mycorrhizae associated with the roots of *Cyrtopodium verum*, a species native to the Cerrado.

2 Materials and Methods

Plant material was collected in the Biological Reserve Professor José Ângelo Rizzo, located in the Serra Dourada State Park, in Mossâmedes - Goiás state (GO), Brazil (16° 04' 36.4" S, 50° 11' 16.9" W, 1006 m altitude), in August 2008 during the dry season. *C. verum* exhibits sympodial growth with lateral sprouting and with new pseudobulbs emerging at each growth cycle. Thus, the criterion for plant selection was the number of pseudobulbs. All selected plants should have more than three pseudobulbs, indicating a minimum growth period and adaptation to the environment. The roots of three *C. verum* plants growing directly on litholytic neosoils, which are characteristic of the cerrado rupestre (a rocky Cerrado vegetation), were selected, marked, and collected. The plants were collected from different areas of the Biological Reserve and at distances of approximately 400 m from one

another. Collected samples were sent to the Laboratory of Phytopathology (Embrapa Arroz e Feijão, Santo Antônio de Goiás, GO, Brazil) and to the Laboratory of Plant Anatomy (Universidade Federal de Goiás, Goiânia, GO, Brazil).

Soil sampling occurred in November 2008, and a composite sample of soil obtained from 10 sub-samples was collected from each of the three plant collection locations. Each sub-sample was taken at a distance of 20 cm from adult *C. verum* plants and at a depth ranging from 5 to 20 cm, as some locations did not always have deep soil. The samples were sent to the Laboratory of Soil and Leaf Analysis (Universidade Federal de Goiás, Goiânia, GO, Brazil) for physical and chemical characterization.

For the isolation of mycorrhizal fungi, five root fragments of approximately 10 cm in length were selected from each one of the three selected plants and washed in running water for 10 min. Next, fragments 2 to 3 cm in length were selected and superficially disinfected by immersion in 70% ethanol (v/v) for 1 min in a solution of commercial sodium hypochlorite at a ratio of 1:10 (0.25% active chlorine) for 5 min, followed by five successive washes in sterilized distilled water (Otero et al., 2002). The velamen was removed with a sterile scalpel, and the roots were ground in a porcelain mortar, previously sterilized at a temperature of 120 °C for 20 min (Pereira et al., 2003). The paste was spread on the surface of a Petri dish containing 10 mL of Potato Dextrose Agar (PDA) medium to promote the growth of fungal mycelium (Nogueira et al., 2005). The dishes with the fungi were incubated for 3 days at 28 °C and observed daily under a dissecting microscope. Fungi that grew in the PDA culture were isolated and transferred to tubes containing the same medium.

From the isolation of mycorrhizal fungi, the growth of each isolate was observed in PDA medium at 25 °C, characterizing the coloration, the mycelial character of the colony, the presence of aerial mycelium and the presence of a halo. The identification of mycorrhizae was based on morphological characteristics for this type of fungus, described by Currah and Zelmer (1992). Non-mycorrhizal fungi were also identified at the genus level, following Barnett and Hunter (1998). All isolates are currently stored at the Laboratory of Microorganism Genetics (Universidade Federal de Goiás, Goiânia, GO, Brazil).

To locate the root infection by mycorrhizae, roots were fixed in FAA₅₀ (Johansen, 1940) for 48 h and stored in 70% (v/v) alcohol until processing. Cross-sections and longitudinal sections were cut freehand and clarified in a commercial sodium hypochlorite solution (2.5% of active chlorine) for approximately 5 min. Subsequently, the sections were washed in distilled water and subjected to 1% aqueous safranin for 2 min and 0.3% astra blue for 30 s. For permanent slide mounting, the sections were dehydrated in a graded ethanol series, ending in an ethanol and xylol series with ratios of 3:1, 1:1, 1:3, up to pure xylol. Slides were mounted using clear glass varnish (Acrilex® - Acrilex Special Paints Corporation - São Bernardo do Campo, São Paulo, Brazil) (Paiva et al., 2006), and digital photomicrographs were taken on a Nikon Eclipse 80i microscope.

3 Results and Discussion

The soil analysis results were interpreted according to Sousa and Lobato (2004). These results indicate that *C. vernum* grows in acidic (pH between 4.0 and 4.2), sandy ($673 \pm 4.0 \text{ g kg}^{-1}$ of sand), highly leachable, nutrient-poor soils with low levels of organic matter ($31 \pm 0.5 \text{ g kg}^{-1}$) and low water retention. Studies on the interaction of plants, mycorrhizae and soil types are still scarce. However, McCormick et al. (2012) studied the relationship of germination and protocorm development of three species of terrestrial orchids under different soil compositions and in the presence or absence of mycorrhizae. The authors concluded that the establishment and development of orchids depends much more on the abundance and distribution of mycorrhizae in the soil than on the presence of nutrients in adequate concentrations. This result reinforces the importance of the fungal-plant association for the survival of orchids in infertile soils.

The following fungal genera were isolated and identified from the ground roots: *Fusarium* sp. (two isolates), *Aspergillus* sp. (one isolate), *Nigrospora* sp. (one isolate) and two isolates of mycorrhizal fungi: *Epulorhiza* sp. and *Rhizoctonia* sp.

The first mycorrhizal isolate was identified as *Epulorhiza* sp. because the mycelia appeared as a lumpy and sparse colony with brown coloration and became grayish in a few

days (Figure 1A). This colony exhibited vegetative mycelium consisting of septate hyphae, with some branching at a right angle, the absence of a clamp connection and the presence of monilioide cells (Figure 1B). The results confirm that this fungal genus is more frequent in terrestrial and rupicolous species (Nogueira et al., 2005; Pereira et al., 2009) and, together with the genus *Ceratorhiza*, is the genus most associated with native orchids of the tropics (Richardson et al., 1993; Nogueira et al., 2005; Pereira et al., 2005; Valadares et al., 2012).

The second mycorrhizal isolate was identified as *Rhizoctonia* because it exhibited a cottony mycelium with abundant aerial growth with regular margins and a greyish-white coloration (Figure 1C), hyphae with branching at a right angle and the formation of monilioide cells (Figure 1D). The mycorrhizal fungus *Rhizoctonia* sp. was also observed in association with the orchid species *Gomesa crispa* (Lindl.) Klotzsch ex RchB. f., *Campylocentrum organense* (RchB. f.) Rolfe and *Bulbophyllum* sp. (Pereira et al., 2005); with *Maxillaria acicularis* Herb. ex Lindl and *Sarcoglottis* sp. (Nogueira et al., 2005); and with *Coppensia doniana* (Batem. ex W. Baxter) Campacci (Valadares et al., 2012). However, while the genus type *Rhizoctonia* is one of the most common among the mycorrhizal fungi, it is also a polyphyletic group of endophytic and saprophytic pathogens (Trigiano et al., 2010).

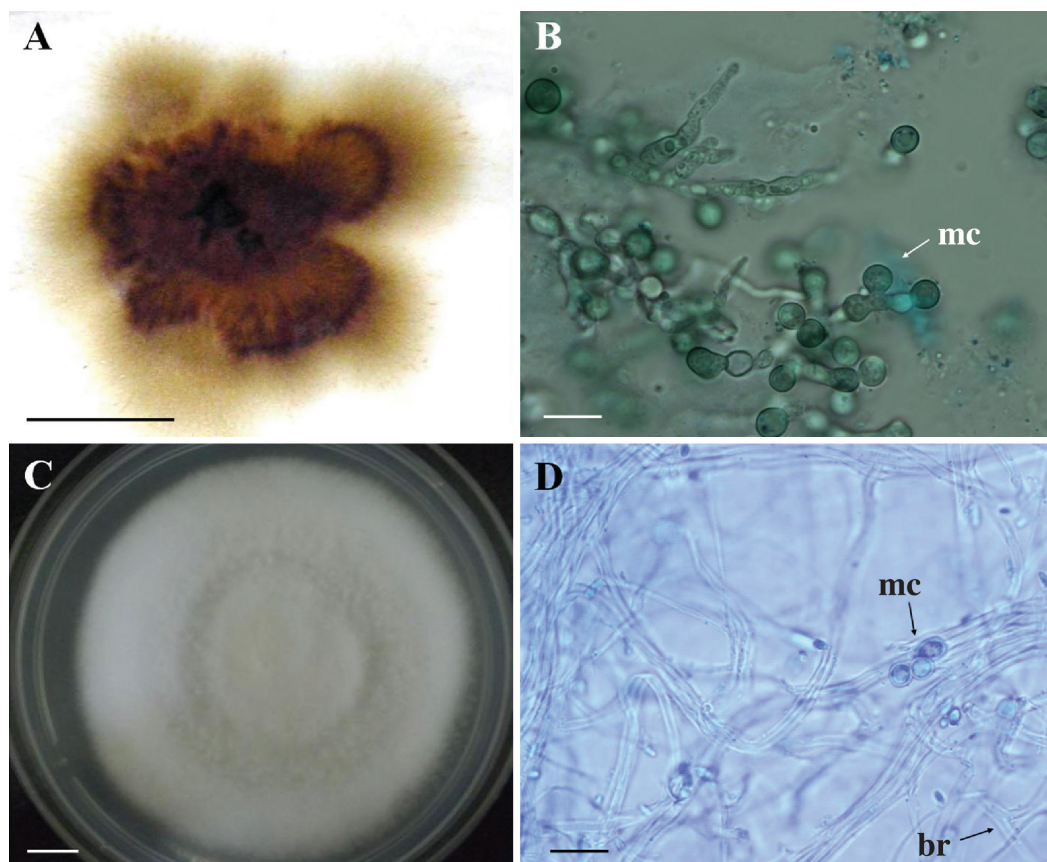


Figure 1. Mycorrhizal fungi isolated from the roots of adult plants of *Cyrtopodium vernum* growing directly on litholytic, acidic, sandy neosoils with high aluminum saturation, characteristic of cerrado rupestre, in the Biological Reserve Professor José Ângelo Rizzo, Mossâmedes - GO. (A) Characteristics of a colony of *Epulorhiza* sp. on PDA. (B) Mycelium of *Epulorhiza* sp. with monilioide cells. (C) Characteristics of a colony of *Rhizoctonia* sp. on PDA culture. (D) Mycelium of *Rhizoctonia* sp. with branching at a right angle. (mc) monilioide cells and (br) branching at a right angle. Bars: A and C = 1 cm; B and D = 10 μm .

The fact that the majority of orchid mycorrhizal fungi only develop in association with a living host plant makes its production in the laboratory and its utilization as an inoculant difficult. However, simple culture media have already produced good results for the maintenance and growth of species of *Epulorhiza* and *Rhizoctonia*. Inoculants of *Epulorhiza* sp. were used in the symbiotic germination and promotion of growth of *Cyrtopodium glutiniferum* Raddi (Guimarães et al., 2013), and inoculants of *Rhizoctonia*-like mycorrhizal fungi promoted greater survival of seedlings of *Cymbidium aloifolium* (L.) and *Cymbidium giganteum* Waal. Ex Lindl. cultivated *in vitro* (Hossain et al., 2013).

In the roots of *C. vernum*, the mycorrhizae occur in the form of *pelotons* in the cortical region (Figures 2 and 3). However, the *pelotons* were not uniformly distributed in this region in the majority of sections analyzed (Figure 2A). Moreover, it was possible to distinguish intact *pelotons* (Figures 2B, 2C, 2D) and degraded *pelotons* (Figures 3A, 3B).

The degradation stage of a *peloton* begins with its deformation, followed by a reduction in volume and the subsequent formation of a mass in which it is no longer possible to distinguish the hyphae but that remains in the interior of cells (Uetake et al., 1997). Connective hyphae were observed between both intact and degraded *pelotons* (Figure 3B). According to Rasmussen and Whigham (2002), remaining hyphal masses can act as inoculants for future

infections because the *pelotons* remain connected even after degradation and reduction in size.

Degraded *pelotons* were observed with more frequency when compared with intact *pelotons*. Graham and Dearnaley (2012) identified few cortex cells colonized by *Ceratobasidium* sp. forming *pelotons* in roots of the Australian orchid *Sarcochilus weinthalii* (F.M. Bailey) Dockrill. The authors also confirmed that the majority of these *pelotons* were in the process of degradation. Therefore, the confirmed pattern of colonization in roots of *C. vernum* differs from the more commonly observed pattern in root sections of terrestrial temperate orchids, which normally show a balance between degraded and intact *pelotons* in the interior of cortical cells (Zelmer et al., 1996). The confirmation of the presence of mycorrhizae in terrestrial orchids found in the cerrado rupestre is important information for both better understanding of this type of symbiotic relationship and the development of *in vitro* symbiotic propagation strategies and species conservation.

Based on the results of this research, advances in the knowledge of the non-pathogenic relationship between mycorrhizae and plants are expected, thus supporting a better understanding of plant-fungus interactions. However, it is worth mentioning that it is difficult to estimate how many species of fungus and new patterns of association may emerge from the analysis of roots of not only terrestrial, but also rupicolous, saxicolous, and epiphytic orchids, which grow in

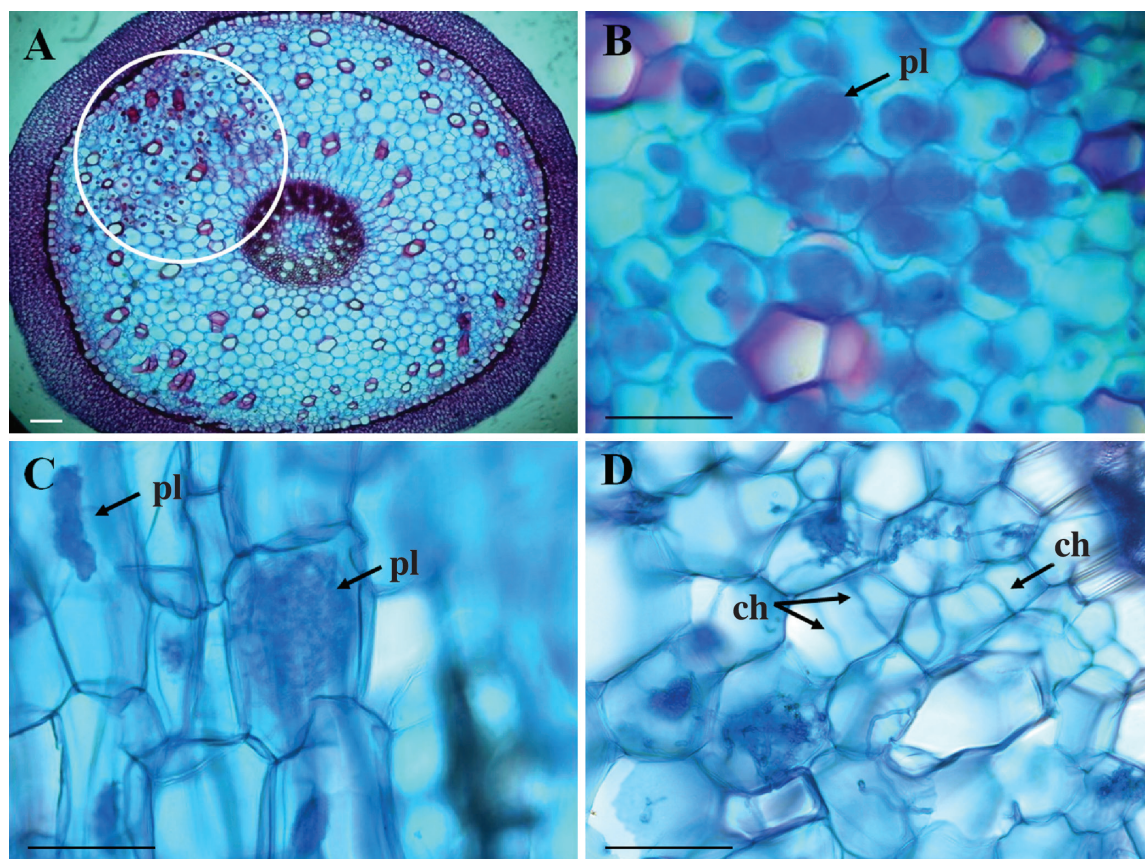


Figure 2. Cross-sections (A and B) and longitudinal sections (C and D) of the root of *Cyrtopodium vernum*. (A) Regions of the cortex with colonization by mycorrhizal fungi, identified by the formation of *pelotons*. (B and C) Detail of *pelotons* (pl) in the interior of cortical cells. (D) Connective hyphae (ch) between *pelotons*. Bars = 100 μ m.

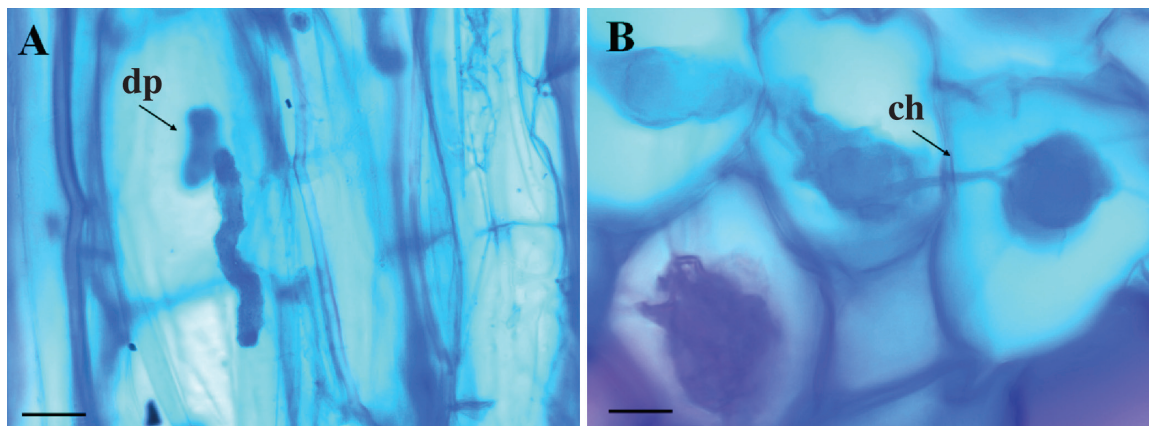


Figure 3. Degraded *pelotons* in cortical cells from a root of *Cyrtopodium vernum*. (A) Detail of a degraded *peloton* (dp). (B) Connective hyphae (ch) between the degraded *pelotons*. Bars = 10 µm.

such a diverse and little-studied environment as the Cerrado (Nogueira et al., 2005; Pereira et al., 2005). Furthermore, new and promising lines of research can be explored, such as investigating if these mycorrhizal fungi are antagonistic to phytopathogens, if they provoke defense responses in the host, or if they will promote the growth of other cultivated species.

4 Conclusions

Two isolates of mycorrhizal orchid fungi were identified in the roots of *Cyrtopodium vernum*: *Epulorhiza* sp. and *Rhizoctonia* sp. The formation of *pelotons* in the root sections was also identified and characterized. These results may support research aimed at *in vitro* symbiotic germination of seeds of the species, the development of seedlings in screened nurseries, and the biocontrol of phytopathogens.

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