Advanced Studies in Biology, Vol. 5, 2013, no. 5, 215 - 221
HIKARI Ltd, www.m-hikari.com
http://dx.doi.org/10.12988/asb.2013.326

Anatomical Characterisation of Mycorrhizal Fungi in Neotropical Orchids

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Abstract

The mycorrhizas are fungi associations considered to be a requirement for the survival of orchids that live naturally in ecosystems, since this group of plants depends on the fungi to germinate, as well as for their establishment. The knowledge of mycorrhizical fungi biodiversity of Brazilian orchids and characteristics of this interaction are of great importance for further reintroduction programs, conservation and handling of these vegetal species. Thus, in this work, we present the anatomical characterisation of mycorrhizas in Neotropical orchids by using a simple staining method. Moreover, unusual hyphae colonisation was also observed.

Keywords: anatomy, orchid mycorrhiza, “campos rupestres”

Two types of orchid mycorrhiza have been recognised: tolipophagic, which are found in most species, and ptiophagic, which are only observed in highly mycotrophic tropical orchids (Rasmussen 1995). In tolipophagic mycorrhiza, the compatible fungal hyphae grow vigorously within the parenchyma cells and form a structure called the peloton (hyphal coil). The hyphae may have branches and anastomosis within the cells (Hadley et al. 1971), and when fully developed, the peloton can occupy almost the entire cell volume; this coiling and branching provide an increased contact area between the symbionts (Hadley 1982). The fungal cytoplasm contains many mitochondria, ribosomes and other organelles (Hadley et al. 1971), and consequently, these hyphae appear electron-dense when analysed by electron microscopy (Peterson and Currah 1990; Uetake et al. 1992) and strongly stained by usual techniques for optical microscopy (Hadley et al. 1971; Peterson and Currah 1990; Uetake et al. 1992). In protocorms, a parenchymal structure that derives from the seed and originates the seedling, it is common to observe cells with various stages of peloton formation and degeneration (Rasmussen 1990; Peterson and Currah 1990).

The control of hyphal invasion in an orchid's tissue is due the production of phytoalexins, which are found in the roots and rhizomes, the production of which is induced by the fungal invasion and colonisation of orchid roots tissues (Gehlert and Kindl 1991; Reinecke and Kindl 1994). Studies about orchid mycorrhizal interactions in Brazil are scarce and, so far, focused on the morphological or molecular identification of mycobiont species (Pereira et al. 2001; Nogueira et al. 2005; Pereira et al. 2003ab; Pereira et al. 2005ab,) and the induction of *in vitro* germination in seeds (Pereira et al. 2005c). Information about the anatomical aspects of such interactions are restricted, and in most cases, concerned to species from temperate zones. Thus, the aim of this study was to evaluate the anatomical characteristics of mycorrhizae in Neotropical orchids of Rocky Fields vegetation (*Campos Rupestres*) in Ouro Preto, Minas Gerais, Brazil, by using a simple staining method.

The experiments were performed in the General Microbiology Lab. of the Science Biological Department of Federal University of Ouro Preto. We selected seven different genera of Neotropical orchids, easily found in Ouro Preto/MG/Brazil (Tab. 1). Healthy root samples were aseptically collected (fragments of ~2 cm) and fixed for 24 hours in FAA (50% ethanol, 10% formalin, 5% acetic acid). Subsequently, samples were stained as follows: root fragments were cleared overnight in a 10% KOH solution; after washing, fragments were immersed in 2% HCl for 5 min., then samples were mounted on Jung Tissue Freezing Medium® solution and transversely sectioned at -25°C (25-40 μm) in cryostat Leica CM 1850® and stained in 0.05% w/v trypan blue in lactoglycerol (1:1:1 lactic acid, glycerol and water), before being incubated in a water bath at 70ºC for 30 min. The stained sections were mounted on permanent slides with polyvinyl alcohol medium (PVA) for conventional and polarized light microscopic observation.

The methodology used for the anatomical characterisation of mycorrhizae was very effective for fungal colonisation evaluation in orchid roots. The species used in this study, as well as their respective root anatomical analyses, are presented in Table 1. The cortex colonisation rate is negatively correlated with hyphae degradation (Table 1). *Epidendrum secundum,* which is widely distributed in the Neotropics (Pinheiro and Barros 2008), had the highest rates of fungal colonisation in the root cortex and low hyphae degradation, for example (Table 1).

The distribution of hyphae on the velamen of this species roots was recorded (Fig. 1), as well as the infection waypoint by the root exodermis (Fig. 2). All root fragments were sampled from healthy plants. However, we found unusual micorrhyzae hyphae invasion in the root stele, and the waypoint by the root endoderm was also identified (Fig. 3). Several groups of orchid mycorrhizal fungi, such as *Rhizoctonia* sp., are recognised as pathogenic fungi (Ogoshi, 1987), and we believe that host plant can lose control of the colonisation process performed by the fungi under specific conditions; however, further studies are necessary to confirm this statement.

In *Oncidium blanchetii,* the host cell's nuclei and nucleolus were strongly stained by trypan blue (Fig. 4, arrow), indicating intense metabolic activity. This fact was also previously described (Williamson, 1970), where increased DNA synthesis was induced by *Tulasnella calospora* in colonised cells of *Spathoglottis plicata*. It was also possible to observe the crossing points between adjacent cells of hyphae (Fig. 4, arrow with asterisk), indicating the route of colonisation of cortex cells. The hyphae mass and degraded pelotons are separated from the cytoplasm of host's cells by a perifungal membrane, which is an extension of the host cell plasma membrane, and an interfacial matrix (Hadley 1982; Peterson and Currah 1990; Richardson et al. 1992; Uetake et al. 1992; Peterson et al. 1996; Genre and Bonfante, 2010). A distinctive feature presented by the membrane of the host is the high activity of the adenylate cyclase enzyme, whereas in the perifungal membrane this activity is absent (Peterson et al. 1996).

The anatomical aspect of mycorrhizal associations in roots of *Prescotia stachyoides* shows the colonisation only at a level where the roots were covered by sediments (*in natura*) (Fig. 4). The amount of digestive enzymes is much higher in infected tissues than in non-infected, and the presence of peroxidases, glutamate dehydrogenases, esterases and malate dehydrogenase acting on peloton lysis were verified (Senthilkumar et al. 2000). Probably, the control of fungal invasion by phytoalexins, as well as the action of enzymes as mentioned above, are responsible for the observed pattern in this terrestrial species, since in areas where inocula is not always present, there is inhibition of fungal colonisation.

Our results clearly support the usefulness of this technique for anatomical studies regarding mycorrhizae orchid associations. Ecological aspects, as well as the role of these interactions in the distribution and occurrence of species could be addressed through using anatomical techniques. Furthermore, we expect the resolution of issues regarding stele invasion and the crossing of mycorrhizal hyphae by root endodermis, which has never been described before.

Acknowledgements. We would like to thank Instituto Estadual de Florestas (IEF / MG) for providing the sampling licence, Eduardo dos Santos Cruz and Taciane Teodoro for their helpful support during this study, Dr Maria Catarina Megumi Kasuya (Microbiology Department/UFV) and Gleide Avelar (Morphology Department/UFMG) for their assistance, and Dr Hildeberto Caldas de Sousa (DEBIO/UFOP) for providing the infrastructure of his laboratory during the work. CNPq (National Council for Scientific and Technological Development/Brazil) funded this work.

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Table 1. Orchid species and their anatomical root characteristics

Specie	Cortex colonization rate (%)	Hyphae degradation	Velamen hyphae	CaOx crystals	Root stele
Bifrenaria tyrianthina (Loudon) Rchb. f.	70	Moderate	+	-	Polyarc
Elleanthus brasiliensis Rchb. f.	10	High	+	-	Polyarc
Epidendrum secundum Jacq.	95	Low	+	-	Polyarc
Habenaria sp.	5	High	+	+	Polyarc
Oncidium blanchetii Rchb. f.	80	Low	+	+	Polyarc
Prescotia stachyoides Lindl.	15	Moderate	+	-	Polyarc
Cattleya cinnabarina (Bateman ex Lindl.) Van den Berg	80	Low	+	-	Polyarc

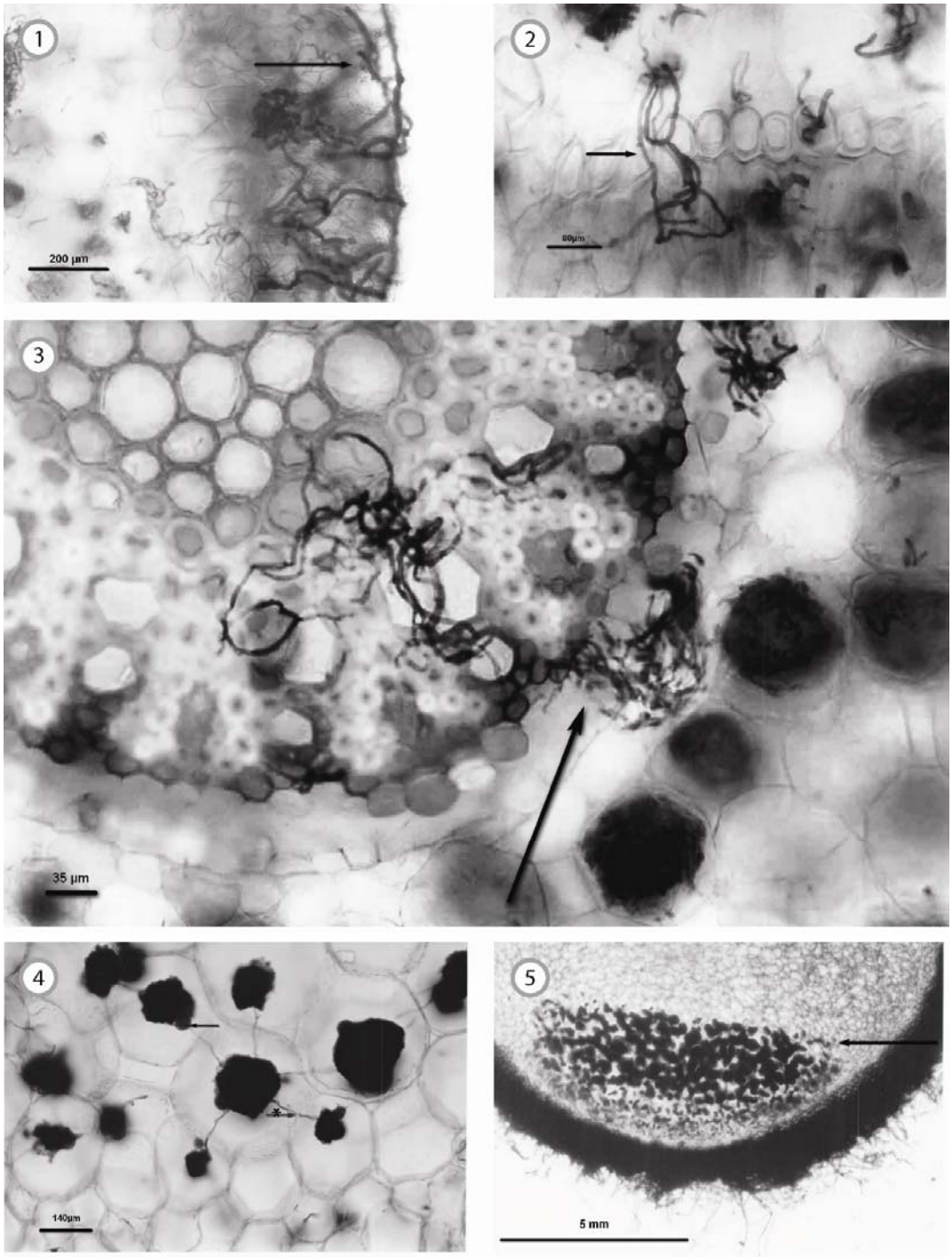

Figure 1-5. 1. Mycorrhizal colonisation of the velamen in *E. secundum* root (arrow). 2. Infection waypoint, by the root exodermis of *E. secundum* root (arrow). 3. Invasion of the stele by mycorrhizal fungi in *E. secundum*. The arrow indicates the crossing point of hyphae, by root endodermis. 4. Mycorrhizal colonization in the root cortex of *O. blanchetii*. Host cell nucleus (arrow), and hyphae crossing points between adjacent cells (arrow with asterisk). 5. Mycorrhizal fungi colonisation in root of *P. stachyoides*. Arrow indicates the level where the roots were covered by sediments (*in natura*).

Received: February 12, 2013