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Short communication

Morpho-anatomical and molecular characterization of a native mycorrhizal *Amanita* species associated with *Guapira opposita* (*Nyctaginaceae*) in the brazilian Atlantic Forest

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ABSTRACT

In this work, we characterize naturally occurring mycorrhizae formed by *Amanita viscidolutea* on *Guapira opposita* in the Atlantic Forest in Brazil. We sequenced the rDNA ITS region from the mycorrhizae and basidiomata to identify both symbionts. *Amanita viscidolutea* mycorrhizae were up to 43 mm long, mostly simple, and unbranched to irregularly pinnate. The fungal mantle surface was velvety to slightly cottony and white to yellowish with silver patches. Hyphal strands were infrequently present. Although the fungal mantle consisted of clampless hyphae, emanating hyphae and hyphal strands had sparsely distributed clamp connections. A unique character of the mycorrhizae was the absence of a Hartig net.

Keywords: *Amanitaceae*, Hartig net, neotropical region, restinga, symbiosis.

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Amanita Pers. (*Amanitaceae*, *Agaricales*) is one of the most widely recognized groups of mushroom-forming fungi with a worldwide distribution (Bas, 1969). While a few *Amanita* species consistently grow as free-living saprotrophs (Wolfe, Tulloss, & Pringle, 2012; Tulloss et al., 2016), most species establish ectomycorrhizal symbioses with a wide range of hosts (e.g. Tedersoo & Brundrett, 2017) and play an important role in maintaining ecosystem dynamics (Gray & Kernaghan, 2020). In the tropics, *Amanita* species frequently occur in spatially restricted areas with monodominant or codominant ectomycorrhizal host trees, suggesting that many species have a small geographic range (Henkel et al., 2012; Mighell, Henkel, Koch, Goss, & Aime, 2019).

Around 35 species of *Amanita* have been described from South American lowland tropical forests, of which 25 have been recorded in native Brazilian forests, where the plant diversity is higher than the areas where other *Amanita* spp. have been found in the tropics. However, the mycorrhizal status of these species has been inferred by the occurrence of basidiomata near potential host plants rather than through observations of root tips (Roy et al., 2016). Only a few observations mentioned root tips, but the plants were not identified (Singer, Araujo, & Ivory, 1983). Despite the ecological importance of naturally occurring *Amanita* mycorrhizae, the morpho-anatomy of the mycorrhizae is still poorly understood.

Guapira Aubl. (*Nyctaginaceae*, *Caryophyllales*) has ca. 70 species (Chagas & Costa-Lima, 2020) recorded from Mexico and the West Indies to Brazil, Paraguay, Peru and Ecuador (Moyersoen, 2012). This genus and other members of *Nyctaginaceae* are known to be ectomycorrhizal hosts throughout the tropics (Chambers, Hitchcock, & Cairney, 2005; Haug, Weiss, Homeier, Oberwinkler, & Kottke, 2005; Tedersoo, May, & Smith, 2010; Hayward & Horton, 2012) and exhibit specific fungal associations (Tedersoo, Sadam, Zambrano, Valencia, & Bahram, 2010).

In this work, we describe the mycorrhizae formed by *Amanita viscidolutea* Menolli, Capelari & Baseia on *Guapira opposita* (Vell.) Reitz based on morpho-anatomical and molecular data. This *Amanita* species is rare (Neves & Furtado, 2020) and only occurs in restinga, an Atlantic Forest vegetation type along coastal Brazil that is threatened by habitat loss and fragmentation due to human population growth and expansion (SOS Mata Atlântica, 2020).

Collections were made in Jun 2019 in the restinga in Parque Natural Municipal das Dunas da Lagoa da Conceição in Florianópolis, Brazil (S 27.694028, W 48.506587; 90 m a.s.l.). We collected six soil samples, analyzed 33 roots, and observed fourteen *Amanita* mycorrhizae. This vegetation is associated with the Quaternary

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coastal sand deposits and rocky coastal habitats (Magnago, Martin, Schaefer, & Neri, 2012) and grows on sandy soil between the ocean and forest. Possible host plants that grow in restinga include species of *Fabaceae*, *Moraceae*, *Myrtaceae*, *Nyctaginaceae*, *Polygonaceae* and *Salicaceae* (Binfaré, 2016). Even though the original description of *A. viscidolutea* includes *Coccoloba* as a possible host, there are no individuals of *Coccoloba* that occur in the restinga that we studied (Binfaré, 2016; Menolli Jr., Capelari, & Baseia, 2009). Specimens of *A. viscidolutea* (Fig. 1A) were identified by comparing them with the morphology described in the literature (Menolli Jr. et al., 2009; Wartchow, Maia, & Cavalcanti, 2012) and by using DNA barcoding of the internal transcribed spacer of nuclear ribosomal RNA gene (nrDNA ITS) region. The specimens were collected next to *G. opposita* plants (Fig. 1B). After conducting morphological analyses, the basidiomata were dried at 40 °C for further preservation. The identification of the plant was made by a botanist and confirmed by DNA barcoding of ITS region (Cheng et al., 2015). Soil samples (approximately 20 cm^3 each) were collected below the basidiomata and left in water overnight. The roots were washed and carefully selected under a stereomicroscope. Roots that had a fungal mantle were morphotyped. From each root system with a mantle, several tips were transferred to 70% ethanol and stored at -20 ºC for the DNA analysis. Additionally, part of the root system with a mantle was fixed in 4% glutaraldehyde for the anatomical analysis. Voucher material of the basidiomata (ANMF 765 and ANMF 767), mycorrhizae (M70(A)R315, M70(B)R320, M70(C) R321, M70(D)R322, M70(E)R323, M70(F)R324, M70(G)R325, M70(H)R326, M70(I)R327, M70(J)R328, M70(K)R329, M70(L) R330 and M70(M)R332), and plants were deposited in the FLOR herbarium and fungarium and the permanent collection of the mycology laboratory (Micolab) at the Universidade Federal de Santa Catarina (UFSC), Florianópolis, Brazil.

Scalped fungal mantles from root tips were fixed on microscope slides and observed under a compound light microscope (Axioplan 2, Zeiss, Jena) at 1000 × total magnification, at the University of Cagliari, Italy. Images were taken using a digital camera (DFC230, Leica, Wetzlar) attached to the microscope. The anatomical analyses were conducted at the University of L'Aquila (UnivAq), Italy. For these, mycorrhizal tips were embedded in LR White resin (Multilab Supplies, Surrey, UK), sectioned at 2.5 μm thick with a ultramicrotome (Ultracut R, Leica), stained with 1% toluidine blue in 1% sodium borate buffer for 10–15 s at 60 ºC, observed with a microscope, and photographed using a digital camera. Ultrathin sections (60–80 nm) were made with a diamond knife, mounted on copper grids, and stained with UranyLess EM (Electron Microscopy Sciences, Hatfield, PA). The sections were examined using a transmission electron microscope (CM100, Philips, Eindhoven) operating at 80 kV and photographed with a digital camera (PHURONA, Emsis, Münster). The morphotype descriptions followed Agerer (1986, 1987–2012). Munsell Soil Color Charts (2000) were used as references to describe the colors of the mycorrhizae. The mycorrhizal descriptions were based on 6–10 root tips from six samples collected under *A. viscidolutea* basidiomata.

The molecular identification of the mycorrhizae was done at the Laboratório Multiusuário de Estudos de Biologia (LAMEB-UFSC), in Florianópolis, Brazil. Genomic DNA was extracted from the basidiomata using a DNA isolation kit (PowerPlant®Pro, MO BIO Laboratories Inc., Carlsbad, CA) and the manufacturer's protocol adapted for fungi. The nrDNA ITS region was amplified by PCR using the primers ITS1F and ITS4 (Gardes & Bruns, 1993) and the following cycling parameters: an initial denaturation at 94 ºC for 2 min; 40 cycles of 30 s at 94 ºC, 45 s at 55 ºC and 1 min at 72 ºC; and a final extension at 72 ºC for 7 min. A direct PCR approach was applied to amplify the ITS region from the root tips (voucher M70(H)R326) isolated from soil samples (Iotti & Zambonelli, 2006). The mycorrhizal tips were selected and directly amplified using the same primer pair (ITS1F/ITS4). Two microliters of 20 mg/mL BSA (Bovine Serum Albumine) solution was added to each reaction tube to prevent PCR inhibition. The parameters applied to the PCR cycles followed Leonardi et al. (2013). To identify the plant host, DNA was extracted from the mycorrhizal root tips using an isolation kit (see above), and the ITS region for the plant was amplified using the ITS-u1 and ITS-u4 primer pair (Cheng et al., 2015) and the following cycling parameters: an initial denaturation at 94 ºC for 4 min; 34 cycles of 30 s at 94 ºC, 40 s at 55 ºC and 1 min at 72 ºC; and a final extension at 72 ºC for 10 min. The DNA extracted from *G. opposita* leaves was used as a positive control. PCR products were purified using polyethylene glycol (Sambrook, Fritsch, & Maniatis, 1989). Sanger sequencing was performed with a BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Waltham, MA) at the company Myleus Biotecnologia, in Minas Gerais, Brazil, following the manufacturer's instructions and using the same primers cited above for the correspondent symbionts. When needed, the generated sequences and their respective chromatograms were manually edited with Geneious R9 (Kearse et al., 2012). The newly generated sequences obtained from *G. opposita* leaves (MW000475), *A. viscidolutea* basidiomata (MW000472; MW000473) and the roots with the mycorrhizal mantle (MW000471) were compared to those in GenBank using a Blastn search. To identify the mycorrhizae, ITS sequences were compared with *Amanita* sequences from GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) and RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007), using the Warcup database (Deshpande et al., 2016) in the latter.

The mycorrhizal system did not become compact with age. It was mostly scattered (Fig. 1C), simple, unramified to irregularly pinnate, (1.5–)6.5–12.5(–17.5) mm long, mostly loose and long, and the main axis was 0.2–0.3 mm diam. The mantle surface was velvety to slightly cottony, white to yellowish with silver patches (HUE 2.5Y 8/2, 8/4, 8/6) to yellow (HUE 2.5Y 7/8) in older parts (Fig. 1D), and the unramified ends were mostly bent, sometimes straight, and (1.4–)2.6–4.7(–7.4) mm long and 0.1–0.2 mm diam. Hyphal strands were infrequently present, rounded, not ramified, connected to the mantle at a restricted point, and white (HUE 2.5Y 8/1) but lighter than the mantle surface. Emanating hyphae were scarce and thread-like (Fig. 1E). Sclerotia were absent.

The fungal mantle was (17–)23–25(–28) μm thick and had thin-walled hyphae and no tannin cells. Additionally, it often had three discernible layers, i.e., outer, middle, and inner layers. The outer layer (Fig. 2A) was weakly developed in some places, plectenchymatous, loose, composed of ramified and anastomosing hyphae, and formed a weak ring-like pattern. The hyphae in this layer were 7.5–10 × 5.0–6.5 μm, hyaline, smooth, thin-walled, clampless, with a globular thickening at the septa often present, and frequently ramified into short, finger-like branches. The middle layer looked like a transition between plectenchymatous and pseudoparenchymatous organization, with hyphae crossing in the longitudinal and transverse directions. The hyphae in this layer were 3.5–7.5 × 3.0–5.0 μm and sometimes interwoven with the inner layer. The inner layer (Fig. 2B) was plectenchymatous and more compact than the outer layer. The hyphae in this layer were 7.5–17 × 2.5–4.0 μm, hyaline, smooth, thin-walled, clampless, sometimes filled with homogeneous yellowish-green content, rather irregularly arranged and often grow in longitudinal directions, with at least some hyphal segments irregular in shape and enlarged. The emanating hyphae (Fig. 2C) were sparse, hyaline, 80–126 μm long, (1.5–)2–2.5 μm

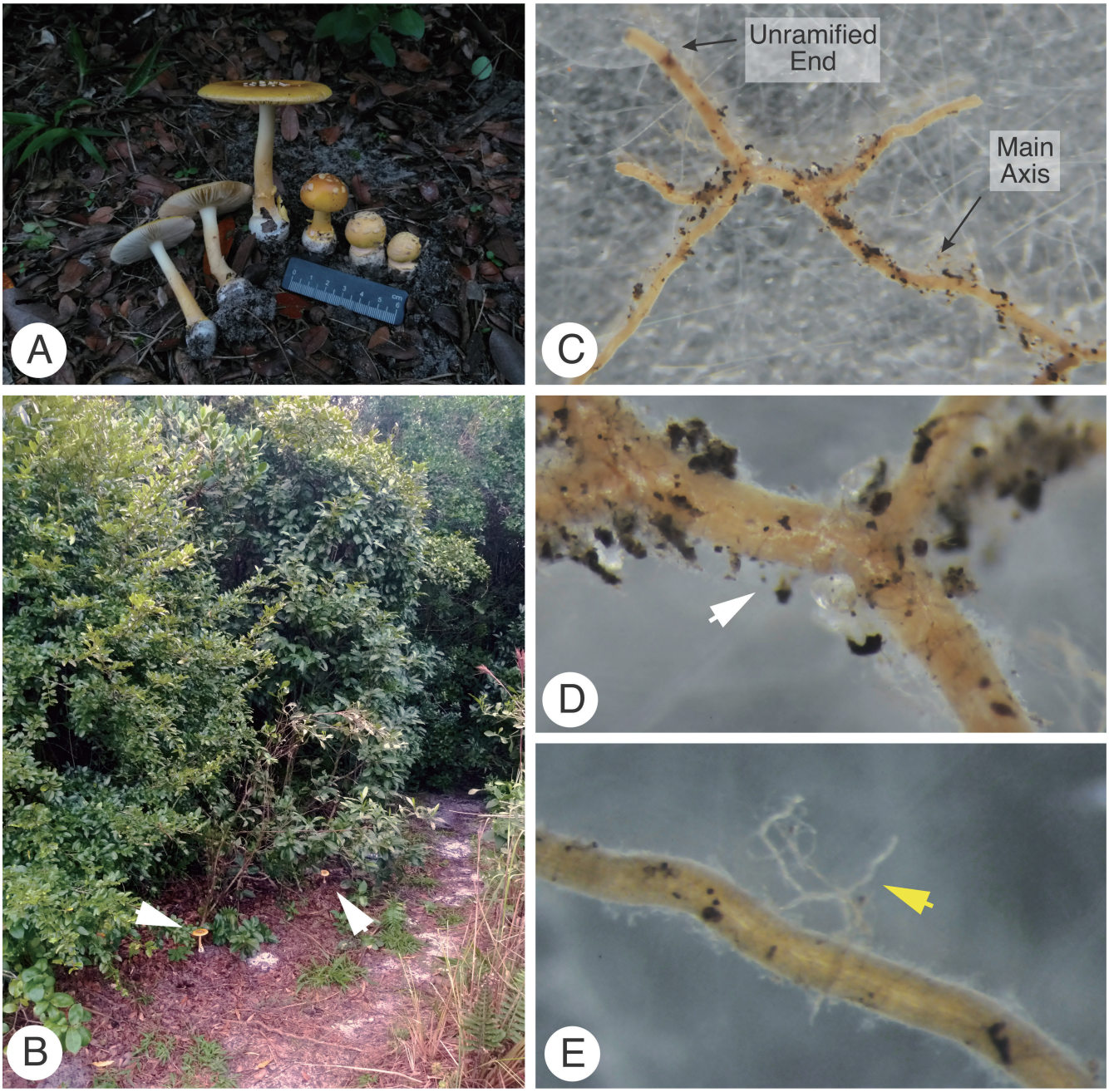

Fig. 1 – *Amanita viscidolutea* basidiomata in habit and mycorrhizae in *Guapira opposita*. A: Basidiomata (ANMF 765). B: Collection site and plant habitat. White arrows indicate basidiomata of *A. viscidolutea*. C: Mycorrhizal root system of *G. opposita* showing the irregularly pinnate branching. D: Mantle surface with silver patches (white arrow). E: Hyphal strands (yellow arrow) connected to the mantle at a restricted point.

diam, not ramified; clamp connections were present but infrequent. The cell walls of these hyphae were smooth, thin, sometimes filled with homogeneous yellow content and awl-shaped, with slightly acuminate apical ends. Hyphal strands (Fig. 2D) were rarely present, started from restricted points on the mantle surface, and 16–26 μm thick. The inner hyphae of these strands were highly differentiated with vessel-like structures in the center, 6–13(–16) μm diam, and thick-walled (up to 1 μm). The outer hyphae of these strands were loosely woven, hyaline, smooth, 2.8–4 μm thick, clamped and often slightly thick-walled (up to 0.5 μm); the hyphal segments were often filled with yellow content or had gelatinized walls. Cystidia were absent.

Electron microscopy (Fig. 3) revealed tight connections between the layered fungal mantle and root cortical cells. No fungal intercellular and intracellular penetration was observed at the root cortex. The cortical cells were in 2–3 rows, longitudinally rectangular, (30–)45–62(–67) × 10–15(–25) μm, and tangentially oriented.

ITS sequences of *A. viscidolutea* basidiomata matched those from the scalped fungal mantles on *G. opposita* roots with 100% similarity, confirming the identity of the mycorrhizae collected. Reference sequences of *A. viscidolutea* (MN123998, MN123990, MN124011, MN124010) previously generated by our group and deposited in GenBank as *Amanita* sp. (Scheibler, 2019) also matched the data of *A. viscidolutea* basidiomata in the present study. The plant roots were identified as *G. opposita* with 100% similarity to *G. opposita* leaves.

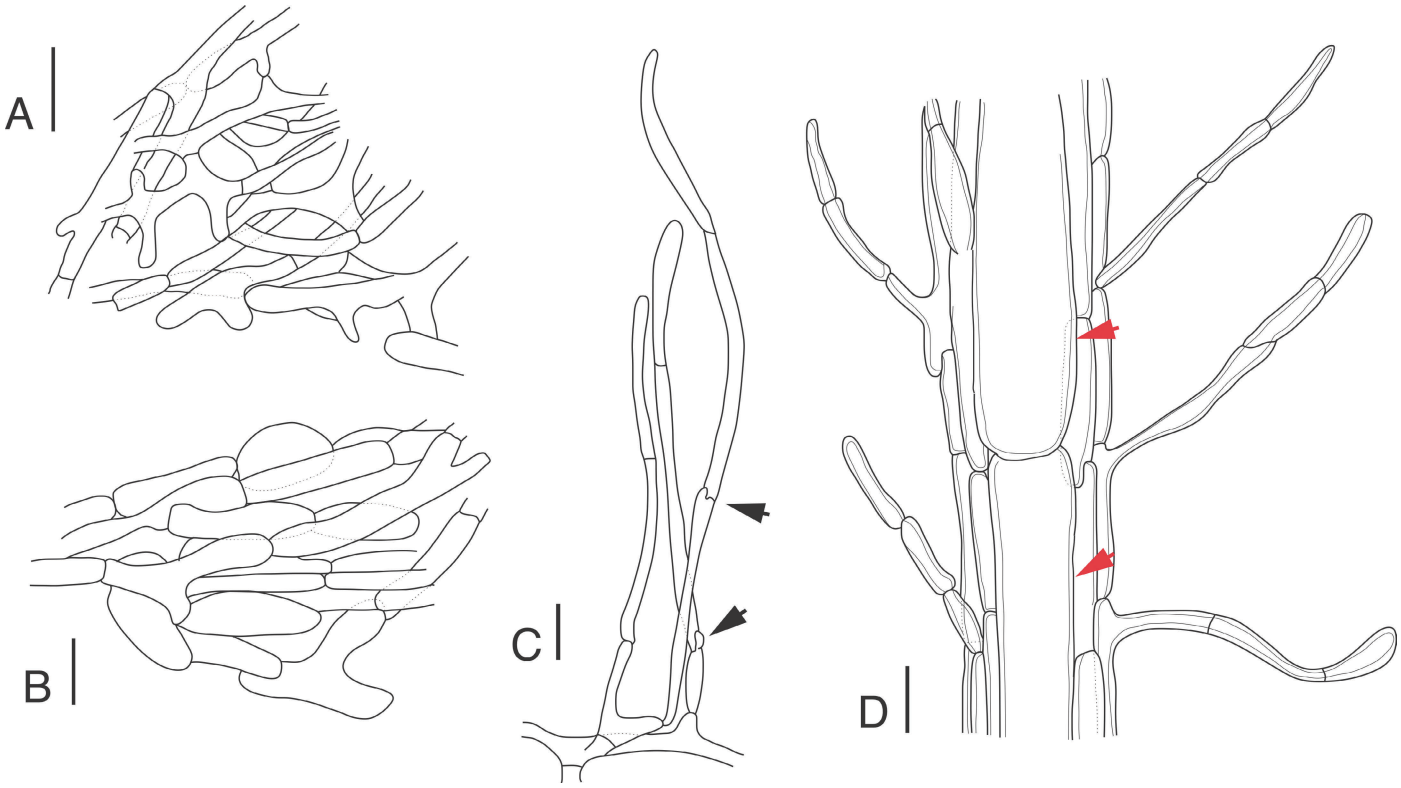

Fig. 2 – *Amanita viscidolutea* mycorrhizae (voucher M70(H)R326). A: Loosely plectenchymatous outer mantle layer. B: Plectenchymatous inner mantle layer. C: Emanating hyphae with clamp connections (black arrows). D: A hyphal strand with vessel-like central hyphae (red arrows). *Bars*: A–D 10 μm.

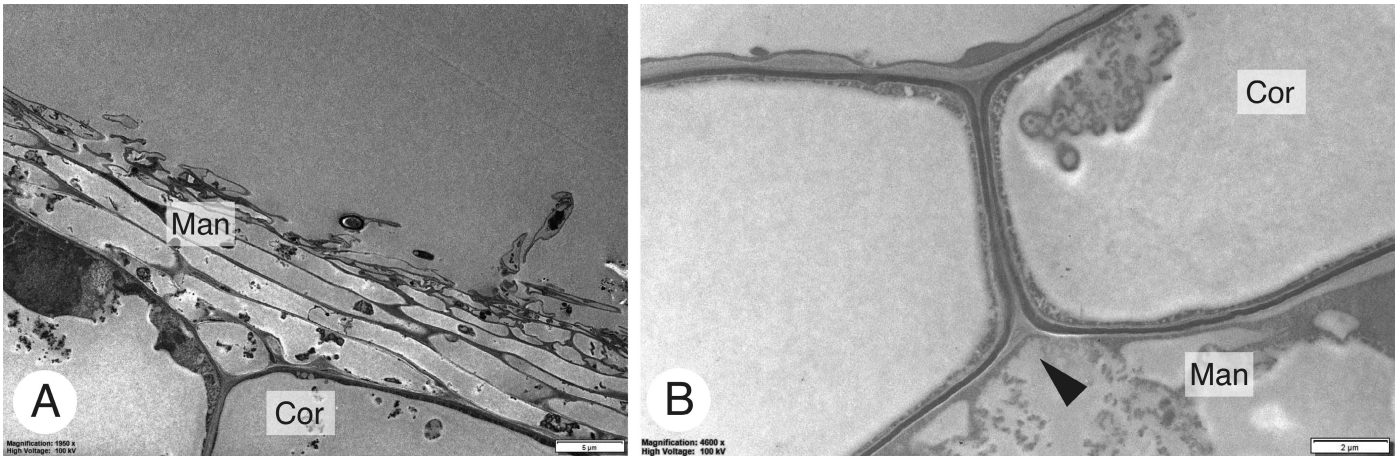

Fig. 3 – A: Longitudinal section of a mycorrhizae showing the fungus mantle covering the cortical cells of host plant root. B: Boundary between fungal mantle hyphae and cortical cells (black arrow). No epidermal cells are visible. Man: mantle; Cor: cortical cell. *Bars*: A 5 μm; B 2 μm.

The *A. viscidolutea* mycorrhiza had a thin mantle that was identical to general ectomycorrhizae of *Amanita* (Supplementary Table S1) but was unique because it lacked a Hartig net. In general, mantle hyphae of *Amanita* are clampless, but emanating hyphae and hyphal strands can have sparsely distributed clamp connections in *A. viscidolutea* and *A. muscaria* (Cuvelier, 1990). The mycorrhizal status of some *Guapira* species has been documented; however, no detailed descriptions of the mycorrhizae were given, and the fungus species were rarely identified (Alvarez-Manjarrez, Garibay-Orijel, & Smith, 2017). Our study is the first detailed morpho-anatomical characterization of a native *Amanita* mycorrhiza associated with the native plant host *G. opposita* in restinga forest in South America. *Nyctaginaceae* is known to establish ectomycorrhiza-like associations with a low number of fungal species in the neotropical region (Haug, Kottke, & Suárez, 2014; Corrales, Henkel, & Smith, 2018). The mycorrhizal status of this family is not homogeneous, i.e., several species have both arbuscular and ectomycorrhizal associations (Moyersoen, 1993; Teste, Jones, & Dickie, 2020), and other species are non-mycorrhizal (Tedersoo & Brundrett, 2017; Brundrett & Tedersoo, 2019). Mycorrhizal structures observed on *Pisonia* (*Nyctaginaceae*) have been defined as the "pisonioid" type, in which the Hartig net is poorly developed or not developed at all; instead, there are "transfer cells" in the root epidermis and cortex (Ashford & Allaway, 1982; Imhof, 2009). Haug et al. (2005) and Alvarez-Manjarrez et al. (2017) observed ectendomycorrhizae on *Guapira* associated with *Tomentella/Thelephora* and *Membrano-*

myces, in which intraradical hyphae and paraepidermal Hartig net hyphae were present on and between the root epidermal and cortical cells. However, the mycorrhiza of *A. viscidolutea* on *G. opposita* did not have an intraradical hyphal arrangement. This suggests that fungal penetration structures on the mycorrhizae vary in *Nyctaginaceae*. Intriguingly, besides in *Pisonia*, the absence of a Hartig net on mycorrhizae has been reported for *Achatocarpus gracilis* H. Walter (*Achatocarpaceae*, *Caryophyllales*) when associated with *Tremelloscypha* sp. and *Sebacina* sp. in a neotropical dry forest in Mexico (Alvarez-Manjarrez et al., 2017). Overall, this evidence suggests the significance of *Caryophyllales* as the hosts for "ectomycorrhizal fungi" in neotropical ecosystems. At this moment, it is hard to infer how *A. viscidolutea* fully exchanges nutrients with *G. opposita* without a Hartig net. It is possible that this is a new type of mycorrhiza that occurs in the neotropical region. Ecophysiological studies are necessary to better understand this symbiosis.

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Disclosure Statements

Competing interests

No potential conflict of interest was reported by the authors.

Availability of data and material

Details of the availability of the data and material used in this study can be found within the text. DNA sequences were submitted to the NCBI GenBank database.

Authors' contributions

Ariadne Nóbrega Marinho Furtado, Andrea C. Rinaldi and Maria Alice Neves conceived the study. Ariadne Nóbrega Marinho Furtado collected the data, performed the molecular and phylogenetic analyses and led the writing of the manuscript. Ariadne Nóbrega Marinho Furtado, Ornella Comandini and Marco Leonardi conducted the morpho-anatomical analyses of the mycorrhizae. All authors actively partook in writing this manuscript and gave final approval for publication.

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