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Arbuscular mycorrhizal associations and dark septate endophytes in Yacon (*Smallanthus sonchifolius*) and a wild relative (*Smallanthus macroscyphus*)

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Summary: Mycorrhizal associations in *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson (Asteraceae), Yacon, an ancient Andean crop and *Smallanthus macroscyphus* (Baker ex Martius) A. Grau, wild yacon, a close wild relative are described for the first time. Yacon fibrous roots growing under field conditions have high levels of colonization by arbuscular mycorrhizal fungi (86 %). Other fungi colonizing roots included dark septate endophytes (45 %) and unidentified fungi that are probably saprophytic (25 %) were observed. Only 9% of the samples analyzed were not colonized by any type of fungi. *Glomus, Acaulospora*, *Scutellospora*, *Gigaspora* and *Pacispora* were the main genera of arbuscular mycorrhiza identified. A similar high degree of mycorrhizal colonization was observed in *Smallanthus macroscyphus*, in natural populations associated with *Juglans australis* native forest. The high level of mycorrhizal colonization, the low number of fine absorbing roots and the large roots diameters observed, suggest that both *Smallanthus* species are likely dependent on mycorrhiza.

Key words: *Smallanthus sonchifolius, Smallanthus macroscyphus*, mycorrhizas, arbuscular mycorrhizas, dark septate endophytes.

Resumen: Micorrizas arbusculares y endófitos septados oscuros en yacón (*Smallanthus sonchifolius*) y un pariente silvestre (*Smallanthus macroscyphus*). Se describen por primera vez las asociaciones simbióticas micorrícicas en *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson (Asteraceae), yacón, un cultivo andino precolombino, y en el yacón del campo, *Smallanthus macroscyphus* (Baker ex Martius) A. Grau, especie silvestre estrechamente emparentada. Las raíces fibrosas de yacón creciendo en un cultivo presentaron un alto nivel de colonización por hongos micorrícicos arbusculares (86 %). Además se observaron endófitos septados oscuros colonizando las raíces (45 %) y otros hongos no identificados, probablemente saprofíticos (25 %). Solo el 9% de las muestras analizadas no presentaba colonización. Los hongos micorrícicos arbusculares identificados correspondieron mayormente a los géneros *Glomus, Acaulospora*, *Scutellospora*, *Gigaspora* y *Pacispora.* Ejemplares de poblaciones naturales de *Smallanthus macroscyphus* creciendo asociados a *Juglans australis* presentaron un nivel de colonización semejante. El alto nivel de colonización y las pocas raíces fibrosas observadas sugiere que ambas especies se comportan como micótrofas dependientes.

Palabras clave: *Smallanthus sonchifolius, Smallanthus macroscyphus*, micorrizas, micorrizas arbusculares, endófitos septados oscuros.

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Introduction

Plant roots interact with a wide variety of rhizospheric microorganisms, including bacteria and fungi, which greatly affect plant growth and productivity (Barea & Jeffries, 1995; Akiyama & Hayashi, 2002). Arbuscular mycorrhizae (AM) are the most widespread form of symbiotic associations known to occur between the roots of more than 80 % of higher plants and a group of fungi that belongs mainly to order Glomerales, phylum Glomeromycota (Schüßler *et al*., 2001; Koide & Mosse, 2004). In addition to the widely studied AM fungi, increased attention has recently been given to a ubiquitous group of miscellaneous fungi designated as dark septate endophytes (DSE) characterized by melanized septate hyphae and microsclerotia (Peterson *et al.,* 2004). These fungi are frequent root colonizers of a broad range of plants in temperate and tropical habitats (Jumpponen & Trappe, 1998) including mycorhizal macrophytes (Marins *et al.,* 2009) and may function as mutualistic fungi (Jumpponen, 2001; Barrow & Osuna, 2002).

Yacon, *Smallanthus sonchifolius* (Poepp. & Endl.) H. Robinson (Asteraceae), is an ancient Andean crop (cultivated from Colombia to NW Argentina) used for centuries by the native inhabitants as food (Fig.1 A-B). Yacon tuberous roots are rich in oligofructans (Goto *et al.,* 1995; Lachman *et al.,* 2003) and antioxidants (Simonovska *et al.,* 2003; Yan *et al.,* 1999) and are potentially useful as prebiotics, natural sweetener, functional food, and dietary supplements (Seminario *et al.,* 2003; Genta *et al.,* 2005; Genta *et al.,* 2009). In the last two decades the use of yacon leaves to treat diabetes has increased. Infusions made from dry yacon leaves produce a remarkable hypoglycemic effect on normal and diabetic rats (Aybar *et al.,* 2001). Yacon popularity has increased and it is cultivated and sold in markets of Peru, Brasil, NW Argentina, Japan, China, Corea, New Zealand, and some European countries.

Smallanthus macroscyphus (Baker ex Martius) A. Grau, is a wild species found in deciduous forests and riverbanks in Southern Bolivia and NW Argentina and is taxonomically related to yacon (Grau & Rea, 1997) (Fig.1 C-D).

Yacon and *S. macroscyphus* have a large aerial biomass of leaves and stems (Kortzart Gonzales, 2009) and subterranean organs consisting of rhizomes with large adventitious tuberous roots and few fibrous absorbent roots (Machado *et al*., 2004; Coll Aráoz *et al.*, 2008). Plant species with root systems of this nature are frequently dependent on mycorrhizal fungi for adequate mineral and water uptake (Varma, 1999; Jakobsen *et al.*, 2003). Our main objective was to determine the presence of mycorrhizal fungal symbiotic interactions as well as spore populations in rhizosphere soils in yacon under cultivated conditions and *S. macroscyphus* in its natural habitat. A second objective was to screen roots for other fungal endophytes.

Materials and Methods

Sample collection

Soil samples, roots and rhizomes were collected from ten individuals of yacon randomly selected cultivated at Horco Molle University Center (600 masl, 27ºS, 65ºW, Tucumán, Argentina) in April 2007 at the flowering stage. Ten individuals of *S. macroscyphus* in flowering stage growing in a *Juglans australis* dominated forest were collected in February of 2008 in Rearte, Trancas, Tucumán, Argentina. Voucher specimens of both species were deposited in the herbarium of "Fundación Miguel Lillo", San Miguel de Tucumán, Tucumán, Argentina LIL Mercado and Ponessa s/ nº LIL 607175, 607375. Roots and rhizomes from both species were treated individually and were carefully cleaned and preserved in FAA (formalin: glacial acetic acid: 70% ethanol, 5:5:90) until processing. Three samples of rhizosphere soil (1 Kg each) were collected from both localities (Horco Molle and Rearte). From each sample 100 g were dried at 65ºC for three days until constant weight and passed through a 2 mm sieve and used for physical chemical analyses. The rest of the soil samples were used for isolation of fungal spores.

Soil properties of the study sites

The soils at the study sites were classified as Ustifluvens (Mon & Suayter, 1972; Toselli *et al*., 1975), slightly acid and very rich in available nutrients and organic matter, particularly Rearte (Table 1).

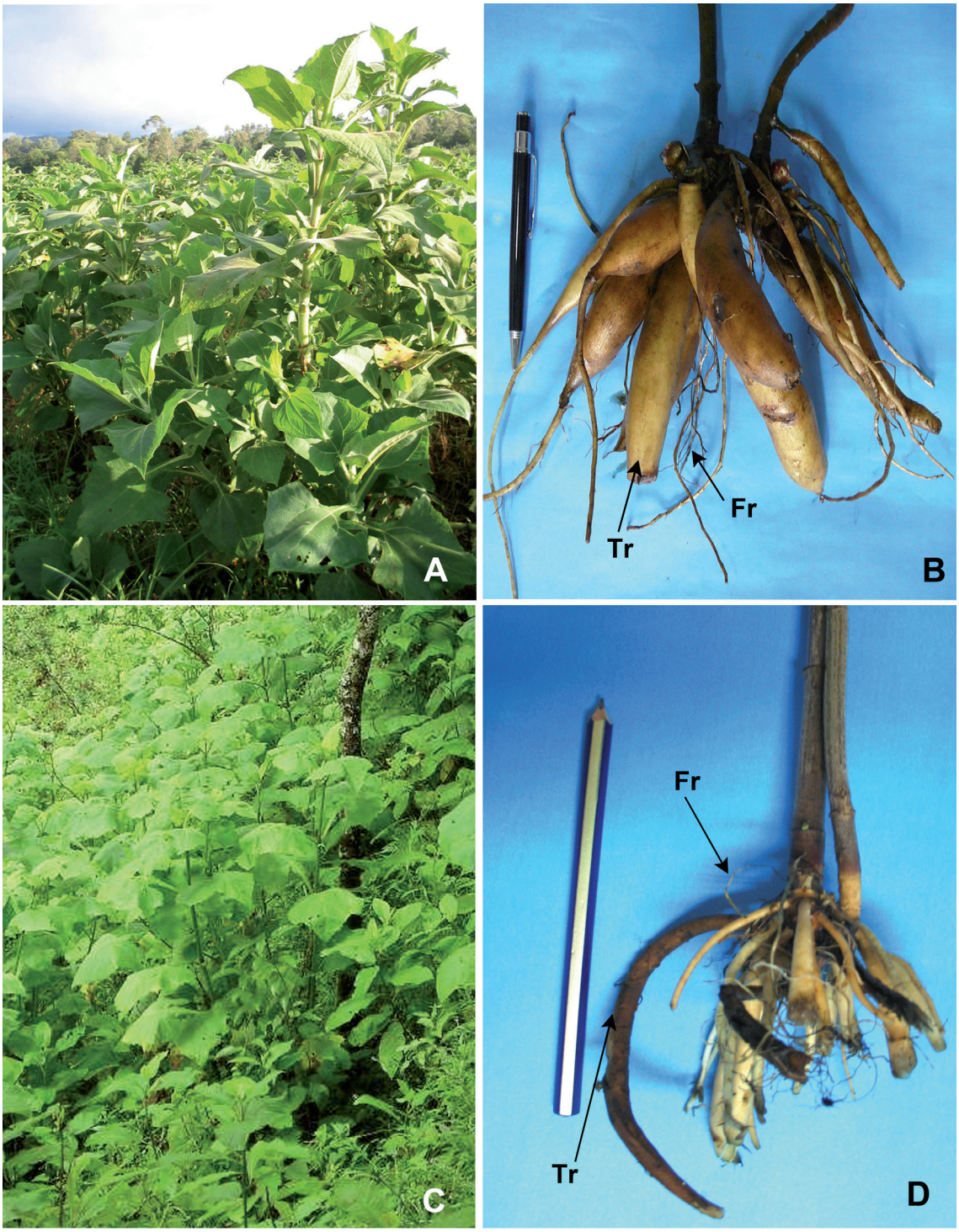

Fig.1. General aspect, panels **A** and **C** and detail of tuberous (Tr) and fibrous (Fr) roots, panels **B** and **D**..of *S. sonchifolius*.. *S. macroscyphus* respectively.

Table 1: Physical chemical analysis of soils in two regions of the Yungas. Tucuman, Argentina. 2008.

Soil sample	Granulometry			Texture	pH[1]	Organic mater[2] %	N[3] %	P[4] ppm	K[5] me.100g^{-1}
	S %	s %	C %						
Horco Molle (*S. sonchifolius*)	58.1	33.1	8.8	Sandy loam	5.6	5.33	0.31	62.5	0.86
Rearte (*S. macroscyphus*)	71.9	24.2	3.9	Sandy loam	6.6	12.23	0.63	46.8	1.57

References: S, sand; s, silt; C, clay; (1) potentiometry ratio soil: water 1:2.5; (2) Walkey-Black method; (3) total nitrogen Kjeldahl method; (4) available phosphor Bray-Kurtz method; (5) Morgan method.

Assessment of roots and rhizomes for colonization

Due to the limited availability of fibrous roots in each plant, particularly *S. macroscyphus* (Coll Aráoz *et al.*, 2008), a composite sample was prepared from the ten individuals of each specie to estimate AM and DSE colonization. Fibrous roots fixed in FAA were cleared in 10% KOH solution at 80 °C, acidified with HCl 5 % and stained with Gueguén (lactic acid: 100 g, Trypan blue: 0,1 g, Sudan IV Or: 0,1 g, and Iodine tincture: 10-30 droplets) (Verna & Herrero, 1952). Roots that remained dark after clearing were bleached with an alkaline solution (169 ml distilled water: 3 mL NH_3: 20 mL H_2O_2) before staining. Four replicates each consisting of twenty five stained fibrous root 1 cm long were examined for each species. Root segments were mounted on microscope slides in lactoglycerol and examined for fungal structures. A root segment was considered as AM positive if it showed extraradical mycelium, intraradical hyphae, arbuscules or vesicles and DSE positive if pigmented dematiaceous septate hyphae or microsclerotia were observed. Other unidentified fungal structures where classified as "other". Percent of root colonization by AM fungi or DSE was estimated according to the magnified intersect method (McGonigle *et al*., 1990).

The rhizomes of the ten individuals of both species were cleared and bleached in an identical manner as the roots and analyzed separately. After clearing they were free-hand sectioned, stained with Gueguen, mounted in lactoglycerol and examined with a compound light Karl Zeiss Axiostar plus microscope with a Canon compact digital camera, model PowerShot A 620 IS M52 (O) 12.1 Megapixel, for assessment of fungal colonization.

Isolation and identification of spore population

Three dry soil samples from each species weighing 100 g were analyzed and considered as three replications. The spore population was assessed by the wet sieving and decanting method (Gerdeman & Nicolson, 1963) followed by centrifugation on a 80 % sucrose gradient (Sieverding, 1991). All the spores were isolated from the supernatant and extracted with a brush into a watch glass with a small quantity of water. Intact fungal spores were mounted in polyvinyl alcohol-lactoglycerol with Melzer's reagent and were identified using INVAM keys (http://www.invam.caf.wvu.edu) and Schenck & Perez (1990) by Dra. Marta Cabello, Instituto Spegazzini, Facultad de Ciencias Naturales y Museo, La Plata, Argentina. Because of the low abundance of certain morphotypes, species identification was performed only with spores in good condition (no sign of degradation or parasitism) and some could only be identified to genus.

Results

A high level of mycorrhizal colonization was observed with simultaneous occurrence of AM fungi and DSE in most cases. AM associations were observed in 75 % of the samples of *S. macroscyphus* and 82 % of the samples of *S. sonchifolius*. DSE colonized more than 40 % of the samples in both species. The extent of unidentified fungal structures colonization, probably saprophytic fungi, ranged between 5 % for *S. macroscyphus* and 25 % for *S. sonchifolius*. Only 9 % to 11% of the samples analyzed were not colonized by any type of fungal structures (Fig. 2).

In both species AM associations were typified

as *Arum*-type (Fig. 3 A, B) characterized by root entry with formation of an appressorium (Fig. 3 G), intercellular hyphal growth in the root cortex, with short lateral branches into cortical cells forming arbuscules (Fig.3 A, B,C), and vesicles filled with several types of lipid droplets (Fig. 3 D, E). On the other hand DSE were frequently characterized by brown, narrow, septate, runner hyphae commonly occurring on the root surface and typically running parallel to the long axis of roots (Fig. 3A, G). Individual hyphae sometimes grew along the furrows between adjacent epidermal cells and colonized roots intercellularly. Penetration through root hairs was not observed. Once in the epidermis, hyphae grew from cell to cell within the epidermis parallel to the main axis of the host root, causing no distortion of host root. At regions the hyphae penetrated the cortical cells filling each cell with microsclerotia (Fig. 3 F). The root stele was not colonized in any of the roots that had DSE fungal associations and there was no evidence of damage to host root tissues arising from fungal colonization.

The rhizome samples analyzed were not colonized by any fungal structure.

In the rhizospheric soil samples of both species, five AM fungal genera were identified on the basis of spore morphology and Melzer's reaction: *Glomus, Acaulospora*, *Scutellospora, Gigaspora* and *Pacispora*. In *S. sonchifolius* rhizospheric soil these included, four morphotypes belonging to *Acaulospora* (*A. scrobiculata, A. elegans, A. denticulata* and *Acaulospora* sp.)*,* two *Glomus* (*G. clarum* and *Glomus* sp.) two *Scutellospora* (*S. pellucida* and *Scutellospora* sp.), two *Gigaspora* and one *Pacispora* morphotypes. In *S. macroscyphus* rhizospheric soil three *Acaulospora* (*A. scrobiculata, A. foveata* and *A. bireticulata*), five *Glomus* (*G. clarum*, *G. etunicatum*, *G. mosseae*, *Glomus* sp.[1] and *Glomus* sp.[2]), two *Scutellospora*, one *Gigaspora* and one *Pacispora* morphotype were observed.

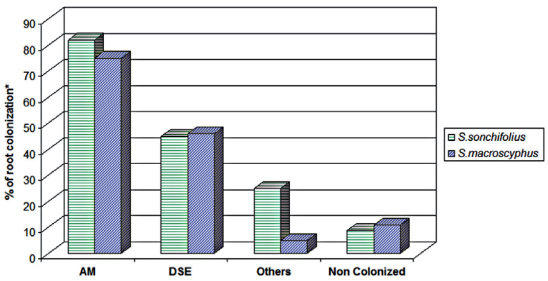

Fig. 2. Ratio of different types of root-associated-fungi colonizing *Smallanthus sonchifolius* and *S. macrocyphus* fibrous roots. References: Percent of root colonization by AM fungi, DSE or other unidentified fungi (Others) was estimated according to the intersect method of McGonigle *et al.*, 1990. *Detection of AM, DSE and other unidentified fungi is expressed as percentages for each fungus in relation to the total number of samples observed (n=100).

Discussion

Smallanthus sonchifolius and *S. macroscyphus* show high productivity and development under cultivation and in its natural habitat respectively (Grau & Rea, 1997; Seminario *et al.,* 2003; Kortzart Gonzales, 2009). Natural mycorrhizal symbiotic associations may enhance plant growth and production through improved access to mineral and water resources, among others beneficial effects (Finlay, 2004).

Simultaneous occurrence of AM fungi and DSE was observed in both species. Simultaneous colonization by AM and DSE was previously reported for other medicinal and aromatic plants (Jupponen & Trappe, 1998; Muthukumar *et al.,* 2006). DSE are common plant colonizers in temperate and tropical habitats (Jumpponem & Trappe, 1998), while *Arum*-type AM seems to be abundant among herbaceous plants such as yacon and *Paris*-type AM seems to be more frequently found among trees (Muthukumar *et al.*, 2006).

According to Brundrett (2004, 2009), the high level of mycorrhizal colonization associated with a poorly developed absorbing root system with large roots diameters and a large biomass conformed by tuberous roots, leaves and aerial stems, suggests a mycotrophic relationship dependent on mycorrhiza. All this characteristic of both *Smallanthus* species (Machado *et al.,* 2004; Coll Araoz *et al.,* 2008; Kortzart Gonzales, 2009).

Rhizomes of obligate mycorrhizal plant species such as orchids and *Psilotum* can be colonized by AM (Brundett, 2002). These associations were not observed in any of the *Smallanthus* species under study.

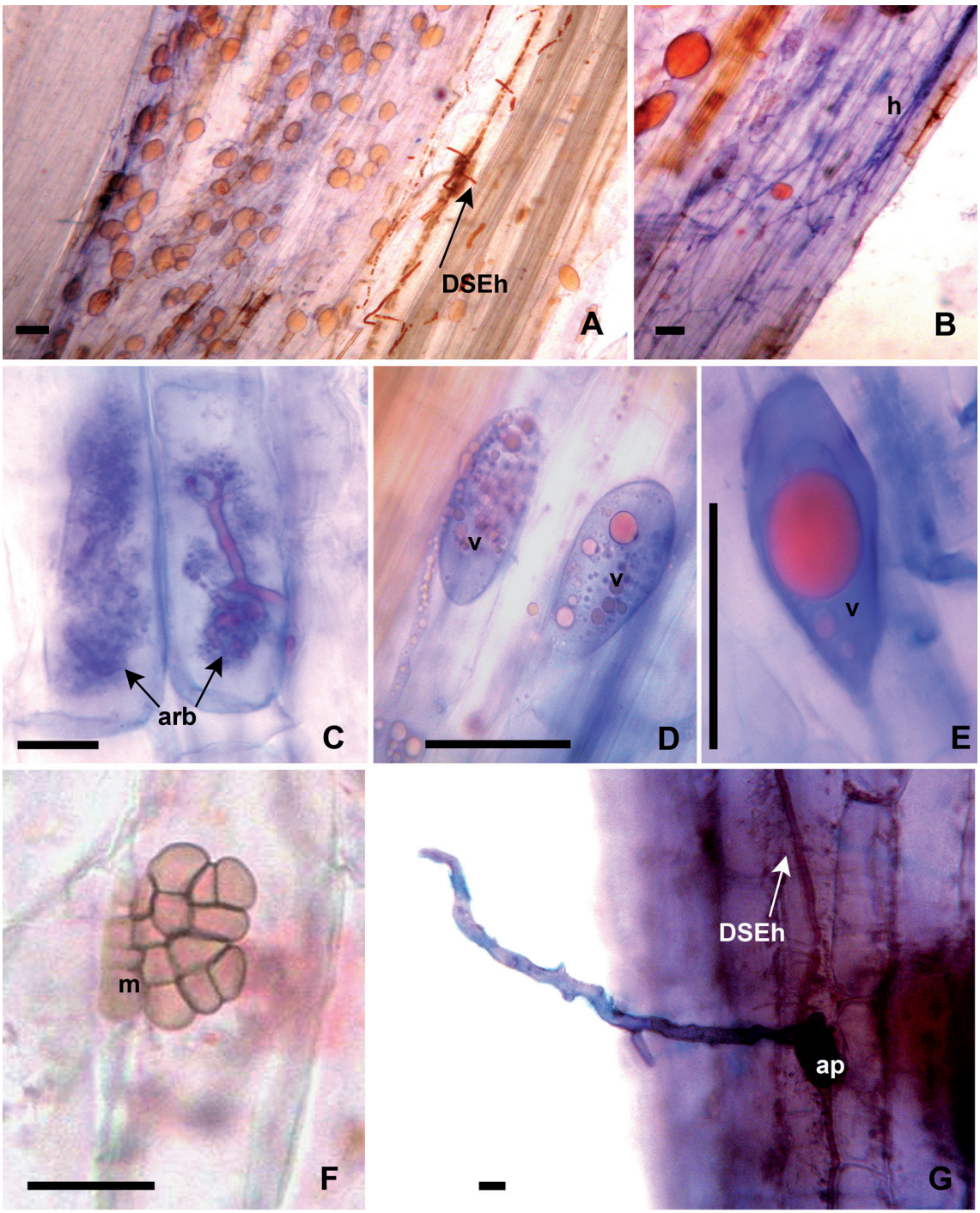

Fig. 3. Light micrographs. **A** and **B:** Arbuscular mycorrhizal *Arum*-type hyphae (h), vesicles (v) and dark septate endophytic intraradical septate hyphae (DSEh) stained with Gueguén. Scale bar = 100 µm. **C:** AM arbuscule (arb). **D** and **E:** AM vesicle (v) with fatty acids stained in red. **F:** DSE Microsclerotia (m). **G:** Appressorium (ap) and DSE hyphae (DSEh) on the root surface of *Smallanthus sonchifolius*. Scale bar = 50 µm.

Further studies are ongoing to characterize the AM and DSE involved in this mycorrhizal association in order to investigate the effect of nutrition mediated by these fungi in the production of secondary metabolites and determine their potential promoting growth in both *Smallanthus* species.

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