

Ultrastructural study of ectomycorrhizas on *Pinus caribaea* Morelet. var. *hondurensis* Barr. & Golf. seedlings¹

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RESUMO – (Estudo ultraestrutural de ectomicorrizas em plântulas de *Pinus caribaea* Morelet. var. *hondurensis* Barr. & Golf.). A ultraestrutura das ectomicorrizas formadas por *Pinus caribaea* var. *hondurensis* inoculado com *Pisolithus tinctorius* (Pers.) Coker & Couch e *Thelephora terrestris* (Ehrenb.) Fr. foi analisada antes do transplante dessas mudas para o campo, com o intuito de verificar se o fungo estava estabelecido nas raízes. Os fungos ectomicorrízicos inoculados formaram um manto compacto e bem desenvolvido nas raízes laterais. Nas hifas desse manto foram observados vacúolos, núcleos e septos dolipóricos, enquanto que no citoplasma das hifas da rede de Hartig, que ficam adjacentes às células corticais, foram frequentemente observados vários núcleos, retículo endoplasmático e mitocôndrias polimórficas. Células corticais altamente vacuolizadas, contendo gotículas de material elétron-denso, apresentaram núcleo e algumas organelas na sua estreita região citoplasmática periférica. As ectomicorrizas de *P. caribaea* var. *hondurensis* apresentaram características ultraestruturais de uma associação compatível e fisiologicamente ativa.

Palavras-chave: ectomicorriza, *Pinus caribaea* var. *hondurensis*, ultraestrutura, *Pisolithus tinctorius*, *Thelephora terrestris*

ABSTRACT – (Ultrastructural study of ectomycorrhizas of *Pinus caribaea* Morelet. var. *hondurensis* Barr. & Golf. seedlings). The ultrastructure of ectomycorrhizas formed between *Pinus caribaea* var. *hondurensis* inoculated with *Pisolithus tinctorius* (Pers.) Coker & Couch and *Thelephora terrestris* (Ehrenb.) Fr. was analyzed just before the transplant of these seedlings to the field to ascertain if fungi are established in the roots. Ectomycorrhizal fungi formed a well-developed compact mantle in lateral roots. Vacuoles, nuclei and dolipore septa were observed in mantle hyphae and numerous nuclei, endoplasmatic reticulum and polymorphic mitochondria were frequently located in the cytoplasm of Hartig net hyphae adjacent to plant cortical cells. Highly vacuolated cortical cells contained droplets of electron-dense material, nucleus and some organelles were observed in a narrow region of peripheral cytoplasm. The ectomycorrhizas of *P. caribaea* var. *hondurensis* exhibited typical ultrastructural characteristics of a compatible and physiological active association.

Key words: ectomycorriza, *Pinus caribaea* var. *hondurensis*, ultrastructure, *Pisolithus tinctorius*, *Thelephora terrestris*

Introduction

Ectomycorrhiza is the symbiotic association between roots of some woody plants and fungus, which belong to the Basidiomycota, Ascomycota and Glomales (Zygomycota), characterized by the presence of hyphae between root cortical cells producing a netlike structure called the Hartig net (Sylvia *et al.* 1997). In this association carbon flows to the fungus, which absorb and translocate nutrients from the soil to the host plant (Sylvia *et al.* 1997). The ultrastructure of many ectomycorrhizal forest tree species has been widely studied since the early works of Foster & Marks (1966; 1967). Electron microscopy is a useful tool for cytochemical localization (Rincon *et al.* 2001) and imunolocalization (Tagu *et al.* 2001) allowing the researches to compare the ultrastructure of different types of ectomycorrhizas.

The importance of ectomycorrhizal fungi in forestry was first revealed when attempts to establish exotic pine forests were enhanced when transplanted seedlings were first colonized by ectomycorrhizal fungi (Daniel *et al.* 1982). It is now a common practice to inoculate nursery seedlings in reforestation programs. In the Southern plains of Anzoategui and Monagas States (Venezuela) in a large forestation area belong to CVG (Corporación Venezolana de Guayana) PROFORCA (Productos Forestales de Oriente Compania Anonima), seedlings of Caribbean pine (*Pinus caribaea* var. *hondurensis*) are inoculated with the ectomycorrhizal fungi *Pisolithus tinctorius* and *Thelephora terrestris* to improve the effectiveness on seedlings establishment. For more than 30 years this region of Venezuela has been planted with Caribbean pine and until December 2000, pine comprised an extension of 615,000ha, which represents the largest tropical area with a monospecific

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forestral plantation (Cedeno *et al.* 2001). The purpose of the present study is to examine the ultrastructure of *P. caribaea* var. *hondurensis* ectomycorrhizas just before the transplant of these seedlings to the field to ascertain if fungi are established in the roots.

Materials and methods

Biological material and location - Seedlings (160-180 days old) of *Pinus caribaea* Morelet. var. *hondurensis* Barr. & Golf., grown under natural conditions in pots containing autoclaved soil from a forest of the same *Pinus* variety, near the El Mery reforestation area, and inoculated with *Pisolithus tinctorius* (Pers.) Coker & Couch and *Thelephora terrestris* (Ehrenb.) Fr. were obtained from the CVG PROFORCA in El Mery (latitude 8°39'N, longitude 62°48'W, 60m high) Monagas State, Venezuela. Ectomycorrhizal roots of these seedlings were collected, washed in water and 1mm³ pieces were sectioned and fixed in Karnovsky modified solution (2.0% glutaraldehyde, 2.0% paraformaldehyde in 0.05M cacodylate buffer pH 7.2) at room temperature for at least 3 hours.

Electron microscopy - The root samples collected in El Mery were post-fixed at the Universidade Estadual Paulista, Rio Claro, SP, Brazil. For scanning electron microscopy (SEM) the samples were rinsed in cacodylate buffer, dehydrated in ethanol, and critical point dried in a Balzers CPD 030 instrument using CO₂ as the transition fluid. Dried specimens were sputter-coated with gold in a Balzers SCD 050 sputter coater, mounted on stubs and examined with a Jeol P-15 SEM. For transmission electron microscopy (TEM), samples were rinsed in cacodylate buffer, post-fixed for 1h in osmium tetroxide (2%) in 0.05M of the same buffer, dehydrated in a graded acetone series, and embedded in Spurr resin. Thick sections (3.0 to 5.0nm) were stained with 1.5% methylene blue and 1.5% azure II for light microscopy. Ultrathin sections were cut with a glass knife using a Porter-Blum MT2-B ultramicrotome, picked up on copper grids, stained for 10 min with ethanolic uranyl acetate and 5 min with aqueous lead citrate, and observed with a Zeiss EM900 and a Philips CM100 TEMs at 80kV.

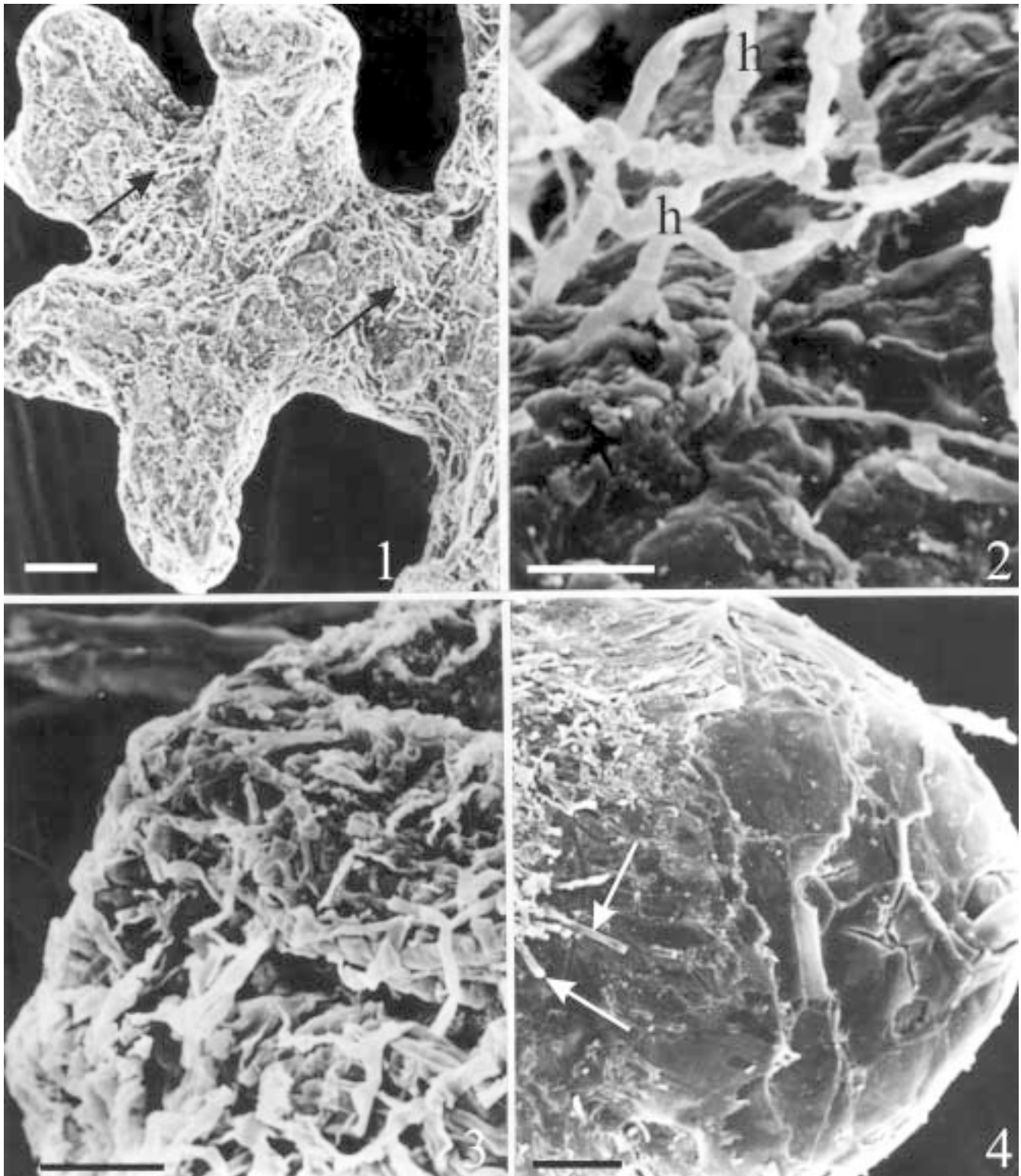
Different developmental regions of the ectomycorrhiza were identified using conventional light microscopy and ultrastructural observations were restricted to the active mycorrhiza infection zone. Approximately 34 root segments were analyzed by electron microscopy.

Results

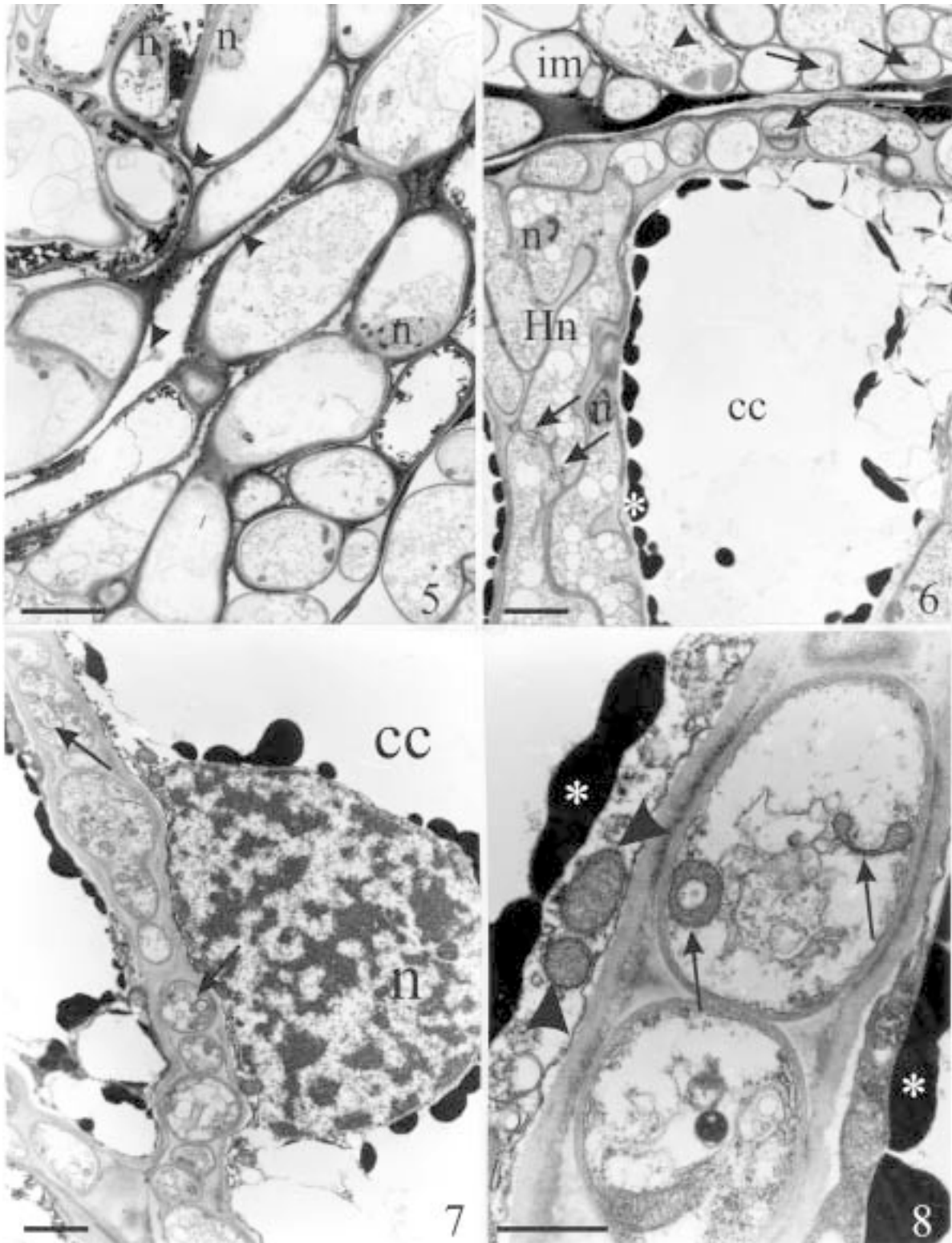
Apices of lateral roots branched dichotomously and occasionally acquired a coraloid appearance (Fig. 1). A compact well-developed mantle of closely-packed hyphae covered the mycorrhizal roots (Fig. 2). Branching extramatrical hyphae were present (Fig. 3), and in some roots hyphae were not evident on the root cap (Fig. 4). Observation of the outer mantle hyphae by TEM revealed a fibrillar extracellular material (Fig. 5). Fungal cytoplasm was vacuolated with sparsely distributed organelles (Fig. 5). Inner mantle and Hartig net hyphae were more cytoplasmic (Fig. 6), with numerous dolipore septa (Fig. 6; 13-14). In the mature Hartig net region hyphae penetrated between the 3th-4th layers of the cortical cells (data not shown) and formed a typical labyrinthine Hartig net (Fig. 6-7). The fungus penetrated the middle lamella, but there were no obvious signs of root cell wall degradation. Intercellular hyphae sometimes presented mitochondria with different morphologies (Fig. 7-8), and were multi-nucleated (Fig. 11-12), contained glycogen rosettes (Fig. 9), endoplasmatic reticulum and small vacuoles (Fig. 6-9; 11-12). Plant cortical cell cytoplasm contained a nuclei (Fig. 7, 11), mitochondria (Fig. 7-8), dictyosomes (Fig. 9-10), endoplasmatic reticulum (Fig. 7-12) and electron-dense material in the vacuole (Fig. 6-9; 11-12). Dictyosomes located close to the plant cell wall appeared to be producing vesicles (Fig. 9-10), and plant cell wall ingrowths were observed in some regions adjacent to the Hartig net (Fig. 11) near to cytoplasmic hyphae (Fig. 11-12). The septal walls both from Hartig net and the mantle hyphae are thinner than the walls of outgrowths and are continuous between the outer walls of the hyphae (Fig. 13). In higher magnification endoplasmatic reticulum was observed in association with the dolipore septum (Fig. 14).

Discussion

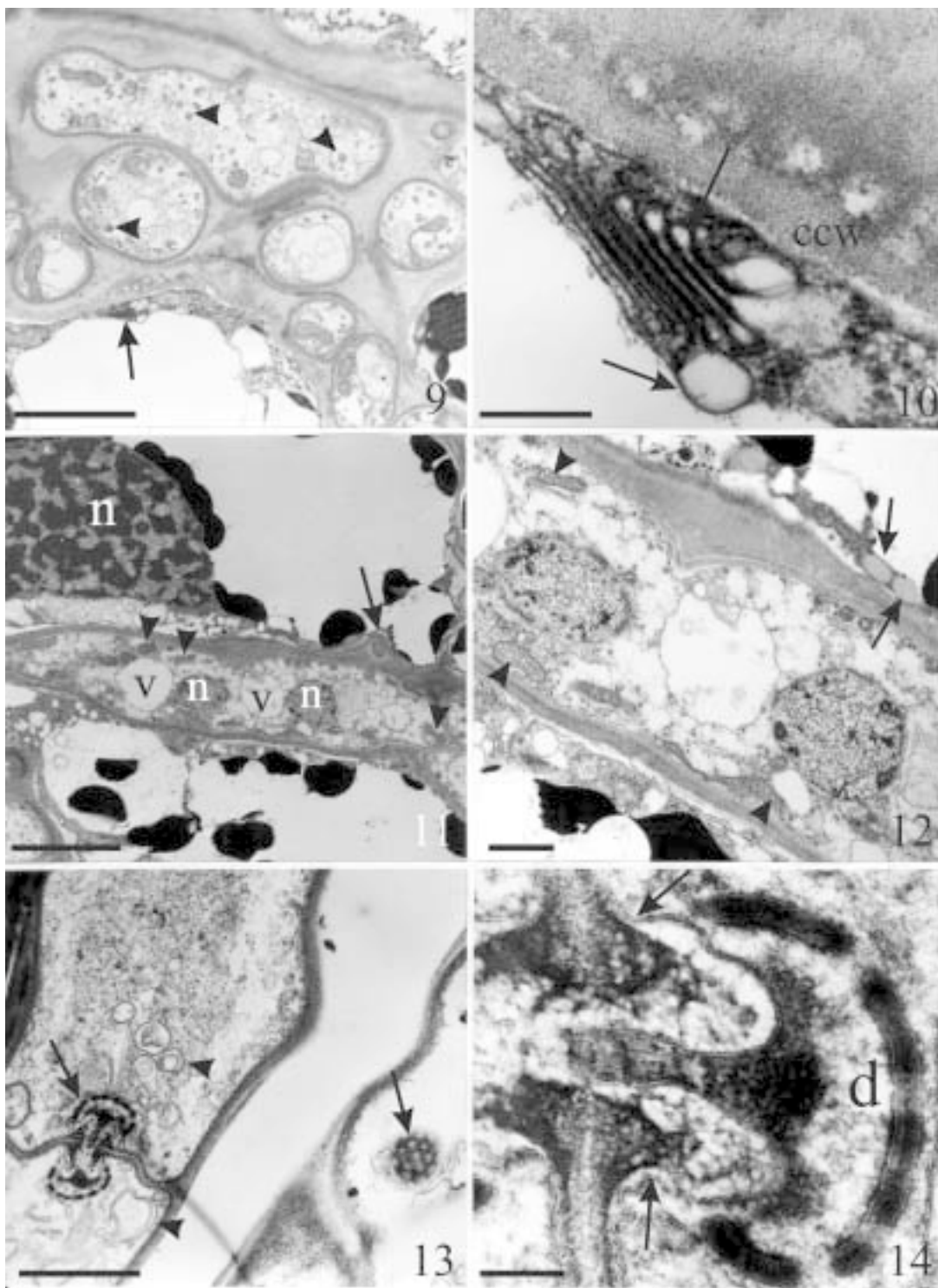
Mantle morphology is the result of the interaction between mycobiont and phycobiont genomes (Massicote *et al.* 1987) and the environment (Peterson & Bonfante 1994). According to Melville *et al.* (1988) the outer mantle morphology may depend on the rate at which additional hyphae accumulate and the extent to which the growth of outer hyphae is affected by proximity to the root. As in other ectomycorrhizae (Massicotte *et al.* 1986; Melville *et al.* 1988) the outer mantle hyphae are highly vacuolated and it is assumed that the root less affects these hyphae (Melville *et al.* 1988). Hyphae from



Figures 1-4. Scanning electron micrographs of *Pinus caribaea* var. *hondurensis* ectomycorrhizas. 1. A young twice dichotomized lateral root with a smooth-undulate mantle. Few hyphae can be individualized (arrows). Bar = 20 μ m. 2. Detail of inner smooth-undulate mantle with some hyphae (h) arising from it. Bar = 10 μ m. 3. Lateral root tip entirely surrounded by a compact mantle. Bar = 20 μ m. 4. Lateral root apex without evidences of mantle and with root cap. Some hyphae (arrows) can be seen in other part of root. Bar = 20 μ m.



Figures 5-8. Transmission electron micrographs of *Pinus caribaea* var. *hondurensis* ectomycorrhizas. 5. Vacuolated hyphae from the outer mantle with some nuclei (n). Fibrillar material (arrowheads) occur between these hyphae. Bar = 3 μ m. 6. Inner mantle (im) adjacent to Hartig net (Hn) and host cortical cells (cc). Glycogen grains (arrowheads) and dolipore septa (arrows) are observed in mantle and Hartig net hyphae. (Asterisk = phenolics). Bar = 3 μ m. 7. Cytoplasmic hyphae of Hartig net with mitochondria (arrows), which possess different morphologies. Nucleus (n) of cortical cell (cc) possesses hetero and euchromatic regions. Bar = 3 μ m. 8. Detail of other Hartig net region shows mitochondria (arrows) with different shapes in the hypha. Note that mitochondria of cortical cells (arrowheads) have their usual form. Asterisk = phenolics. Bar = 1 μ m.



Figures 9-14. Transmission electron micrographs of *Pinus caribaea* var. *hondurensis* ectomycorrhizas. 9. Hartig net hyphae with vacuoles (v) and glycogen grains (arrowheads) surrounded by middle lamella. Arrow indicates a dycytosome in cortical cell. Bar = 2 μ m. 10. Detail of dycytosome of Fig. 9. Note the vesicles (arrows) produced by this organelle. CCW = cortical cell wall. Bar = 0.4 μ m. 11. A large binucleate hypha with some vacuoles (v) and mitochondria (arrowheads). Adjacent to Hartig net interface can be seen a plant cell wall ingrowth (arrow). n = nucleus. Bar = 5 μ m. 12. Detail of large hyphae of Fig. 11. Cristae of mitochondria are conspicuous (arrowheads) and most part of nuclei are euchromatic. In cortical cell some droplets of lipids (arrows) can be observed. Bar = 1 μ m. 13. Mantle hyphae with a dolipore septum (arrows). Note that many profiles of endoplasmatic reticulum (arrowheads) are localized near from the dolipore septum region. Bar = 1 μ m. 14. Detail of dolipore (d) of Fig. 13. Arrows indicate profiles of endoplasmatic reticulum closely associated with the dolipore structure. Bar = 0.1 μ m.

the inner mantle of *P. caribaea* var. *hondurensis* are embedded in an extracellular material, which fills the interstices (Seviour *et al.* 1978). This material could be composed of polysaccharides and glycoproteins (Piché *et al.* 1983; Massicotte *et al.* 1987; 1990; Dexheimer *et al.* 1994) and might act as an adhesive (Piché *et al.* 1983; Alexander & Hogberg 1987). Adhesins (microbial ligands) that interact with host receptors (Jones 1994; Martin *et al.* 1999) are often found in fibrillar material around plant and fungal cells. Increased secretion of extracellular fibrillar polymers between hyphae and root surface could denote a compatible ectomycorrhizal association. Isolates of *P. tinctorius* with delayed symbiosis development do not secrete this fibrillar material (Lapeyrie *et al.* 1989; Lei *et al.* 1990). Our ultrastructural observations showed this mucilaginous bridge, which links hyphae and root cells, and a substance between the mantle hyphae in *P. caribaea* var. *hondurensis* mycorrhizas, which could denote a compatible mycorrhizal association.

Distribution of glycogen-like rosettes in the hyphae was consistent with that found by several authors (Foster & Marks 1966; 1967; Jordy *et al.* 1998). Glycogen found in the mantle and Hartig net hyphae could be a result of seasonal variation (Duddridge & Read 1984; Genet *et al.* 2000) and related to the physiological state of the association. Labyrinthic branching increases the apoplastic and symplastic exchange interface between the symbionts (Peterson & Bonfante 1994) and may have diagnostic value to identify compatible mycorrhizal associations, as postulated by Nylund & Unestan (1982) and Piché *et al.* (1983).

The presence of cellular constituents such as nuclei, mitochondria and endoplasmic reticulum, also indicate metabolic activity in the mantle and mainly in the Hartig net hyphae found in *P. caribaea* var. *hondurensis*. Cortical cells adjacent to the Hartig net in this studied ectomycorrhiza appear to be alive and physiologically active, since they possess nuclei, a peripheral lining of cytoplasm and organelles. Presence of these ultrastructural features in *P. caribaea* var. *hondurensis* roots may denote the health of ectomycorrhizal association. Wall ingrowths similar to those observed in *Alnus crispa* (Massicotte *et al.* 1986) and in *Dryas integrifolia* (Melville *et al.* 1988) ectomycorrhizas occurred in cortical cells adjacent to the Hartig net. These transfer cell-like wall ingrowths and plasma membrane could increase the surface area for nutrient exchange between root and fungus (Allaway *et al.* 1985).

The ultrastructural appearance and size of the dolipore septa, which is characteristic of Basidiomycota

(Moore & McAlear 1962), were very similar to that of *Pisolithus tinctorius* (Orlovich & Ashford 1994). When combined with other evidences such as the presence of dikariotic mycelia, and the formation of fruitbodies of *Pisolithus tinctorius* in the pots with seedlings of *P. caribaea* var. *hondurensis* suggest that the plant roots were colonized by this fungus in spite of co-inoculation with *Telephora terrestris* due to the non-aseptic conditions of our experiment.

In conclusion, this study demonstrated that the *P. caribaea* var. *hondurensis* inoculated seedlings exhibited the typical ultrastructural characteristics of a compatible and physiologically active ectomycorrhizal association, giving a general view of the ultrastructural condition of the seedlings that will be transplanted to the field.

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