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Morphological characterization of the mycorrhiza formed by *Helianthemum almeriense* Pau with *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire

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Abstract This work presents the first anatomical description of the mycorrhizal systems of *Helianthemum almeriense,* and of the structure and ultrastructure of the mycorrhizae formed by this plant species with the ascomycetes *Terfezia claveryi* and *Picoa lefebvrei*. Four different mycorrhizal systems are described, the club-shaped mycorrhiza being the most abundant. The type of mycorrhiza formed depended on the mycorrhiza culture conditions, but not on the fungal species. For both fungal species, *H. almeriense* formed an endomycorrhiza in natural field conditions, an ecto- and ectendomycorrhiza without a sheath in pot cultures, and an ectomycorrhiza with a characteristic sheath and Hartig net in in vitro cultures. This is the first report of a typical sheath in *Helianthemum*-desert truffle mycorrhizal associations. The results support the idea that culture conditions can induce changes in mycorrhiza morphology and that there is no clear barrier between the two main types of mycorrhiza organization in *Helianthemum* species. The ultrastructural study confirmed the regular presence of *T. claveryi* intracellular hyphae in direct contact with the host wall, a localization which seems to be a characteristic of the *T. claveryi* mycorrhiza organization. The *P. lefebvrei* mycorrhiza organization was characterized by intracellular hyphae with large amounts of electron-dense globules, probably with a lipidic content, and a warty ornamentation on the wall of the root external hyphae.

Keywords Ultrastructure · Desert truffle · *Helianthemum* · *Terfezia* · *Picoa*

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Introduction

The species of the genus *Terfezia*, together with other fungi such as *Picoa*, *Tirmania* or *Balsamia,* are called desert truffle or "turmas" and are found in many Mediterranean countries. *Terfezia claveryi* Chatin and *Picoa lefebvrei* (Pat.) Maire are two very frequent hypogeous ascomycetes in marl-gypsum soils of the semi-arid areas, which establish mycorrhizal symbiosis with several annual and perennial species of the *Helianthemum* genus (Honrubia et al. 1992). Desert truffles are of considerable interest for ecological, agroforestry and commercial purposes. Moreover, there is growing interest in introducing desert truffle cultivation into dry environments as a useful way of exploiting lands which until now have been regarded as unproductive (Morte et al. 2000).

Mycorrhizae synthesized by the *Helianthemum* genus were studied by Read et al. (1977) and Cano et al. (1991). According to these authors, the mycorrhizae were light to dark brown coloured. Two types of mycorrhizal root were observed: one was short, lateral and dark brown in colour, and the other was long, enlarged and light brown. Non-mycorrhizal roots were white. More recently, Kovacs and Jakucs (2001) exhaustively described "*Helianthemirhiza hirsuta*" ectomycorrhiza, from *H. ovatum* (Viv.) Dun., which is characterized by ochre to brown cottony, simple mycorrhizal systems with straight, slightly bent or tortuous ends.

Mycorrhizae of *H. ledifolium* (L.) Mill. and *H. salicifolium* (L.) Mill. with different *Terfezia* (*T. boudieri* Chatin and *T. claveryi*) and *Tirmania* species [*T. nivea* (Desf.) Trappe and *T. pinoyi* (Maire) Malençon] were described by Awameh et al. (1979) and Awameh and Alsheikh (1980). In all these syntheses, an endomycorrhiza was observed. Chevalier et al. (1984), on the other hand, observed that the mycorrhiza formed by *H. guttatum* (L.) Mill. and *Terfezia leptoderma* Tul. was endo-, ectendo- or ectomycorrhiza according to the fertility of the substrate (Dexheimer et al. 1985; Fortas and Chevalier 1992). However, Kowacs et al. (2002)

observed no such effect on the anatomical features of *H. ovatum* and *Robinia pseudoacacia* L. roots colonized by *Terfezia terfezioides* (Matt.) Trappe in in vitro conditions using different phosphate concentrations.

The present work gives the first detailed anatomical description of the mycorrhizal systems in *H. almeriense* Pau and the structure and ultrastructure of the mycorrhiza formed by this plant species with *T. claveryi* and *P. lefebvrei*.

Materials and methods

Material

Three types of samples were studied:

1. Roots from 2- to 3-year-old naturally colonized plants of *H. almeriense* collected from areas of natural desert truffle production (Zarzadilla de Totana, Lorca, Murcia, Spain). Sixty plants were studied for 1 year (15 per season).
2. Roots of *H. almeriense* plants inoculated separately with spores or mycelium of either *T. claveryi* and *P. lefebvrei* under greenhouse conditions in 300-ml pots of a mixture of soil:vermiculite 8:1 (v/v). The soil used was collected from the same areas as indicated in (1), mixed together, and autoclaved 3 times at 100°C on alternate days. Roots of fifteen 6-month-old plants were studied for each treatment.
3. Roots of micropropagated *H. almeriense* plants in vitro, inoculated with mycelium of *T. claveryi* (ref. TCV) or *P. lefebvrei* (ref. PJG) on MH medium (patent P9402430), as described by Morte and Honrubia (1994, 1995) and Gutiérrez (2001). Roots of 20 plants were studied 2 months after inoculation.

The phosphorus content of the soil, taken from areas of natural desert truffle production (Zarzadilla de Totana, Lorca, Murcia, Spain) and used for the synthesis of mycorrhiza in pots, was 28.7 ppm determined according to the MAPA method (1981). The phosphorus concentration of the MH medium was 42.5 ppm.

Mycorrhizal systems and fungal colonization assessment

Substrate particles were removed from the root systems of the three types of samples and were observed by stereomicroscope (Olympus SZH) to determine the pattern of the mycorrhizal system. Afterwards, fungal colonization was assessed on cleared and stained roots (Phillips and Hayman 1970). The percentage of fungal root colonization was estimated according to the gridline intersect method (Giovannetti and Mosse 1980) under the stereomicroscope.

Microscopy

One-millimetre-long pieces of mycorrhizal roots were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, for 3 h at 4°C. At the beginning of this process, a vacuum was created by means of a water vacuum pump (10–15 min) to facilitate the penetration of the fixative into the root tissues. The specimens were then rinsed 3 times in the cacodylate buffer (30 min each time) and postfixed in 2% osmium tetroxide in the same buffer, for 2.5 h at 4°C. After postfixation, samples were soaked in the buffer overnight and rinsed samples were poststained with uranyl acetate for 2 h at 4°C. Samples were dehydrated through an alcohol series (30–100%) to propylene oxide and embedded in Spurr resin (Spurr 1969). Samples were cut with a diamond knife ultramicrotome (Reichert Ultracut E). Semi-thin sections (0.5 μm) were stained with toluidine blue for light microscope (Olympus BHT) observation. Ultra thin sections (80 nm) were cut for electron transmission microscopy and stained with uranyl acetate for 5 min and lead citrate for 1 min (Reynolds 1963). The sections were examined using a Zeiss 10C electron microscope at 75 kV.

Results

Four different mycorrhizal systems were observed in *H. almeriense* from the three types of samples and with both fungal species:

1. Club-shaped mycorrhiza. This is an unbranched mycorrhiza, 6–8 μm in length. It had a whitish-ochre enlarged apex (0.6 μm) and a brownish to ochre constricted base of varying length (Fig. 1A). It was the most frequent type of mycorrhizal system, appearing in 45.7% of the studied plants.
2. Capitate mycorrhiza. This is an unbranched mycorrhiza, 7–10 μm in length. It had a slender base and an enlarged darker brown apex (0.4 μm) (Fig. 1B). This mycorrhizal system appeared in 25.7% of the studied plants.
3. Moniliform mycorrhiza. This mycorrhizal system is characterized by swellings at regular intervals. It was transparent to white in the early development stage, later turning to a buff colour (Fig. 1C). This mycorrhizal system was observed in 22.8% of the studied plants.
4. Branched mycorrhiza. This was the least common mycorrhizal system with a frequency of 5.8% of the studied plants. It had dichotomous or pseudodichotomous branches of a dark brownish-ochre colour (Fig. 1D).

In general, external mycelium was observed around all the mycorrhizal systems. This mycelium was loose, bright white and not organized into a true sheath except in the in vitro mycorrhiza of both fungal species, where a true sheath was observed (Fig. 2D).

The frequency of appearance of each mycorrhizal system did not change with the different seasons, nor with the mycorrhizal culture conditions or fungal species used. The four mycorrhizal systems presented the same type of development at the cellular level, which only changed with the synthesis conditions but not with the fungal species. In field conditions, root colonization was mainly intracellular (Fig. 2A), forming an endomycorrhiza with large, septate and moniliform hyphae. Colonization only concerned the cortical cells of the roots. Intercellular hyphae were rarely observed. In no case was a sheath observed around the root; only in some cases did isolated hyphae appear around the root.

In pot-synthesized mycorrhiza under greenhouse conditions, fungal colonization was both inter- and intracellular. Intercellular colonization gave an ectomycorrhizal Hartig net among the second to third elongated cell layer (Fig. 2B, C). No sheath was observed in any case, but in some cases isolated hyphae were observed around the

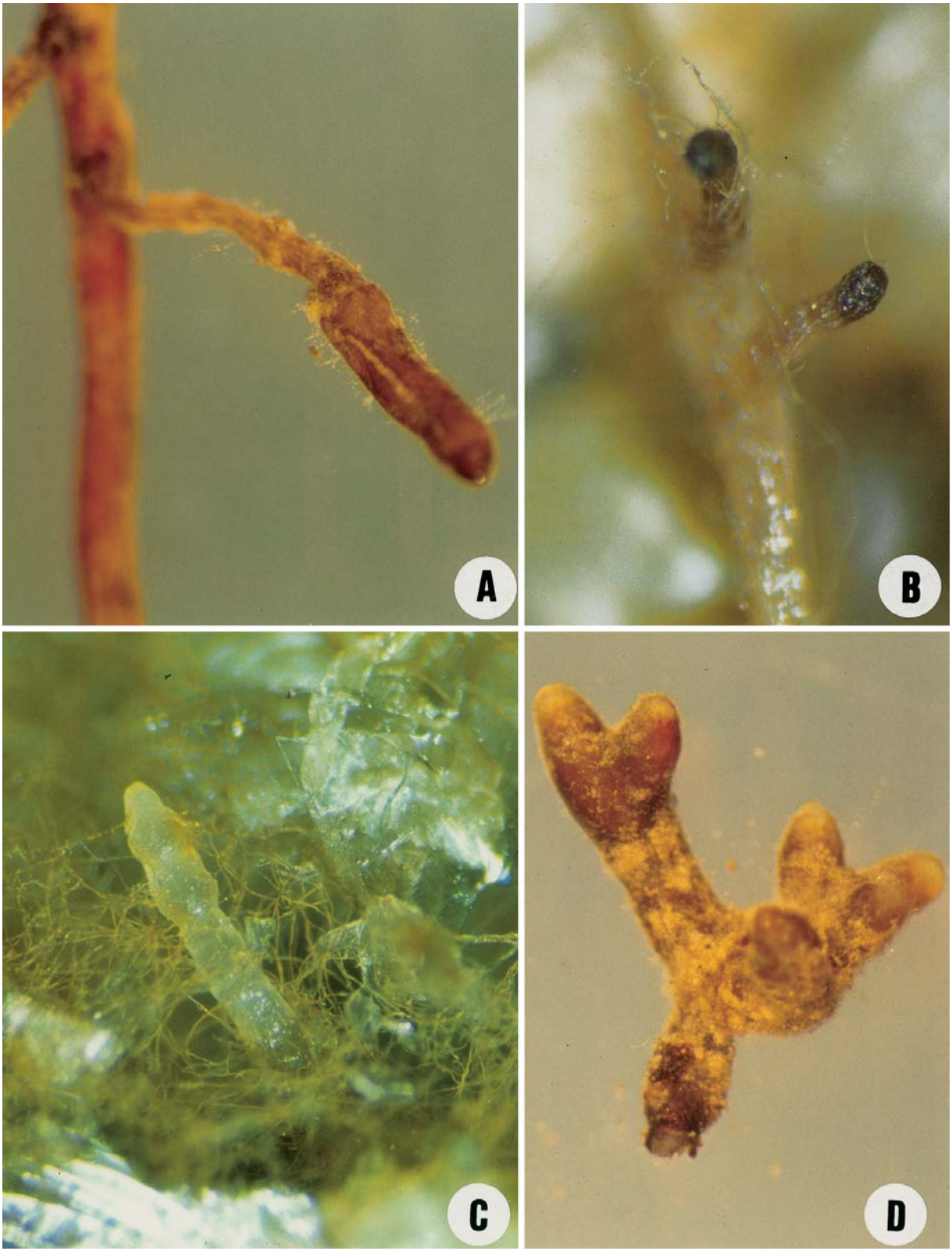
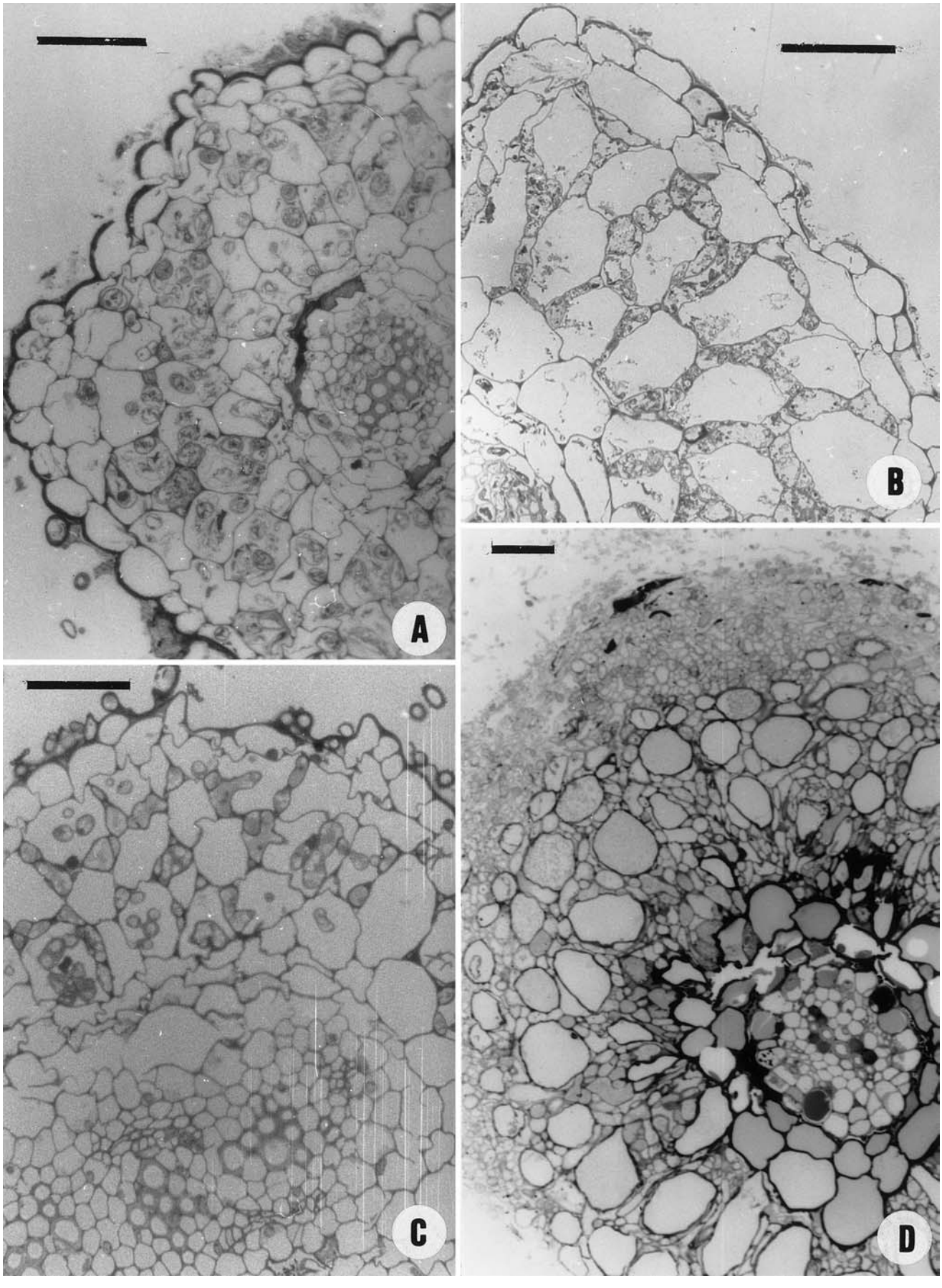


Fig. 1A–D Different mycorrhizal systems in *Helianthemum almeriense* roots. **A** Club-shaped mycorrhiza. **B** Capitate mycorrhiza. **C** Moniliform mycorrhiza. **D** Branched mycorrhiza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root. Finally, in in vitro conditions, colonization was also intercellular, and only occasional hyphae were observed inside cells (Fig. 2D). In this case, fungal hyphae developed in the cortex within the intercellular spaces. Groups of hyphae were frequently found between cells, causing an important dilation of the intercellular spaces so that cortical cells showed an elongated form. This cortex organization was like a typical Hartig net. The cuticle was absent or broken off. The main characteristic of this mycorrhiza formed in vitro was the presence of a well-developed sheath, 50 μm thick (Fig. 2D). In some cases, intracellular hyphae were observed in epidermal root cells. These cells were larger than non-colonized cells (data not shown).

As regards hyphal penetration of roots, some observations suggested that hyphae passed throughout the empty spaces between sloughing cells in the outer layer (epidermis) and then entered the first layer of cortical cells. Epidermal cells were colonized only in some cases. Fungal hyphae did not invade the endodermis or the central cylinder in any case.

No differences were observed in the ultrastructural features of natural or control inoculated mycorrhizal roots (in vitro or in vivo), or according to the fungal species or type of inoculum used. Infected host cells retained all their organelles. The nucleus of host cells was frequently in a central position, surrounded by fungal hyphae (Fig. 3A). The cytoplasm of these colonized cells was very dense and reduced to a thin layer endowed with mitochondria, rough endoplasmic reticulum, ribosomes and dictyosomes (Fig. 3B).

Two different types of interface were observed: a host cell wall-fungal wall interface, where the fungal wall was in direct contact with the host cell wall, and a host plasmalemma-fungal wall interface (Fig. 3C). The presence of the host cell wall-fungal wall interface was very frequent in *T. claveryi* mycorrhiza and rare in *P. lefebvrei* mycorrhiza. A reduced interfacial region between both symbionts was observed with a fibrillar material in contact with the hyphal wall and, in some cases, in contact with the host plasmalemma (Fig. 3D).

Intercellular hyphae were inside an electron-dense interhyphal matrix (Fig. 4A). In the cytoplasm of the intracellular hyphae, the fungal nucleus was usually observed in a peripheral position (Fig. 4A). These intracellular hyphae showed some mitochondria with transverse cristae, ribosomes (Fig. 3D) and Woronin bodies close to the septum (Fig. 3A). In older mycorrhiza, active intracellular hyphae were often observed growing within wall layers of empty hyphae which varied in number (Fig. 4B). The fungal cytoplasm was occupied by large vacuoles at the beginning of hyphal degeneration (Fig. 4C). In the last stage of fungal senescence, all the emptied hyphae were clumped together (Fig. 4D) and were surrounded by the host cell plasmalemma.

A warty ornamentation was observed on the external hyphal wall only in the mycorrhiza synthesized in in vitro and pot conditions by *H. almeriense* with *P. lefebvrei* (Fig. 5A). External hyphae were endowed with warts of different size with an electron-dense content. This warty ornamentation was observed only in the external root hyphae and was absent in the area of contact between the hyphae and the root surface (Fig. 5B). Moreover, only *P. lefebvrei* intracellular hyphae showed large quantities of electron-dense globules, probably of a lipidic nature (Fig. 5C). These globules were observed in mycorrhizae formed by both types of *P. lefebvrei* inoculum used, and were not observed in *T. claveryi* hyphae.

Fig. 2 **A** Mycorrhizal roots of *H. almeriense* collected from field areas; colonization was mainly intracellular. **B** Mycorrhizal roots of *H. almeriense* and *Terfezia claveryi* in greenhouse conditions, where intercellular colonization forms a Hartig net. **C** Mycorrhizal root of *H. almeriense* and *Picoa lefebvrei* in greenhouse conditions; colonization was both inter- and intracellular. **D** Mycorrhizal roots of *H. almeriense* synthesized under in vitro conditions with *T. claveryi* mycelium; a well-developed sheath was observed and colonization was also intercellular. *Bars*=40 μm

Discussion

It is known that plants of the genus *Helianthemum* are able to form ectomycorrhiza (Chevalier et al. 1984; Fortas and Chevalier 1992; Kovacs and Jakucs 2001), arbuscular endomycorrhiza (Read et al. 1977), ericoid mycorrhiza (Dexheimer et al. 1985) and ectendomycorrhiza (Cano et al. 1991; Fortas and Chevalier 1992). The results of the present work show that *H. almeriense* was able to form a sheathing ectomycorrhiza with both *T. claveryi* and *P. lefebvrei* in in vitro conditions. This is the first report of the presence of a sheath in *Helianthemum*-desert truffle mycorrhizal associations. This sheath formation could be due to the vigorous mycelium growth on the MH mineral-rich medium, which contains sucrose as a carbon source, and also result from the small physical space inside the test tube which would force hyphae to surround the roots formed in vitro. However, an ectomycorrhiza and an ectendomycorrhiza without a sheath were observed in mycorrhiza synthesized with both fungi in pots under greenhouse conditions, similar to those described in *H. salicifolium* and *T. claveryi* mycorrhiza (Dexheimer et al. 1985). Finally, they also formed an endomycorrhiza without a sheath in field conditions. Since the phosphorus content of the soil used for the mycorrhiza synthesized in pots was almost half that used for in vitro synthesis, this may have influenced the type of mycorrhiza formed, although other factors varying between the experimental conditions could have contributed to this phenomenon. These results support those obtained by Fortas and Chevalier (1992) for *Helianthemum guttatum* and *Terfezia* and *Tirmania* species that substrate fertility affects mycorrhiza morphology (Fortas and Chevalier 1992).

The moniliform structure of the intracellular hyphae has been also observed in mycorrhizal roots of different *Helianthemum* species with different *Terfezia* species (Awameh et al. 1979) and for the *R. pseudoacacia*/*T. terfezioides* association (Bratek et al. 1996). The intra-

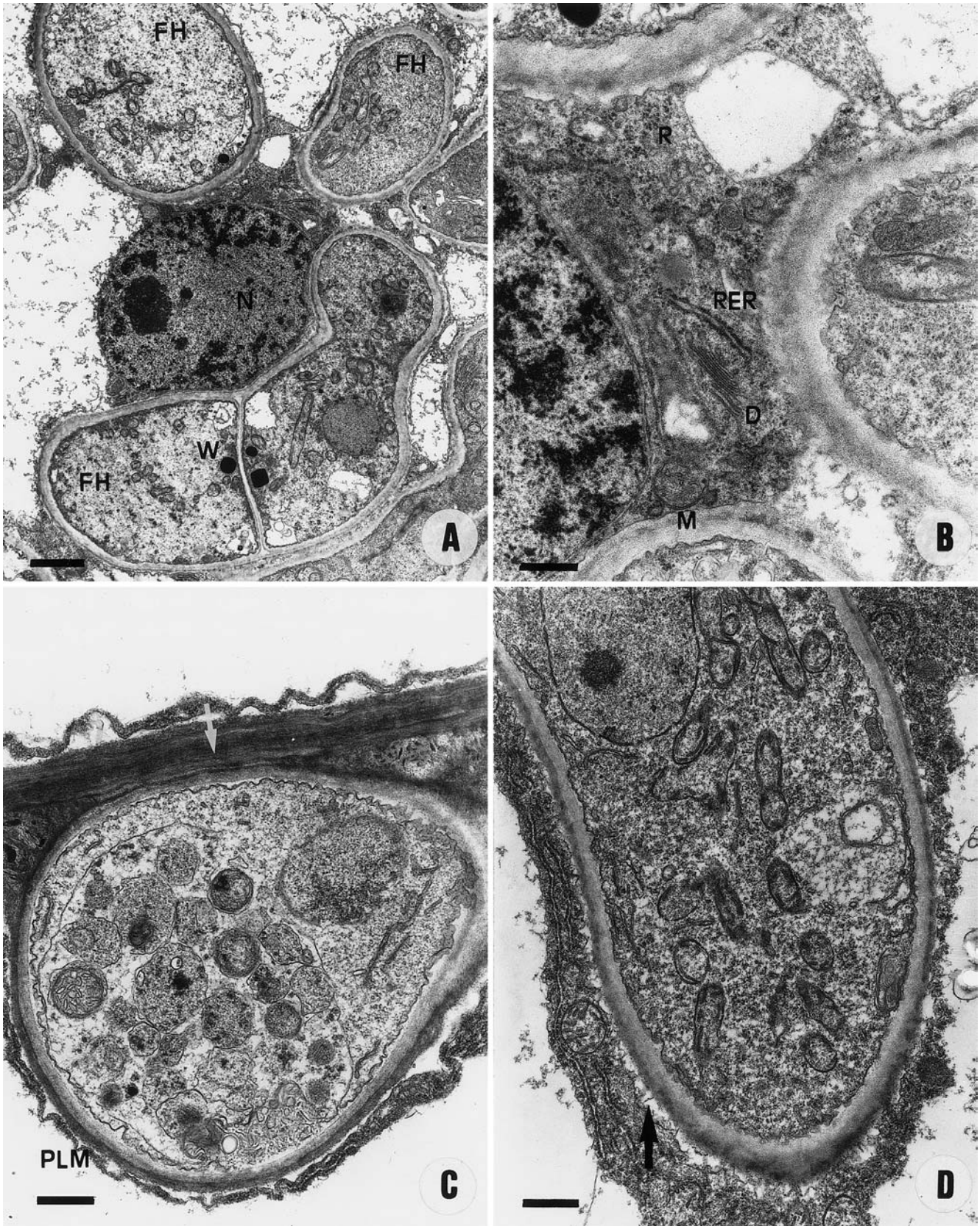


Fig. 3A–D Intracellular hyphae of *T. claveryi*. **A** The nucleus (*N*) of the host cell in a central position, surrounded by fungal hyphae (*FH*) with Woronin bodies (*WB*) close to the septum. **B** Detail of the host cytoplasm with mitochondria (*M*), rough endoplasmic reticulum (*RER*), ribosomes (*R*) and dictyosomes (*D*). **C** Types of interface: host cell wall-fungal wall interface (*arrow*), and host plasmalemma (*PLM*)-fungal wall interface. **D** A fibrillar material (*arrow*) in contact with the hyphal wall in the interfacial region between both symbionts. **A** *Bar*=2.2 μm; **B** *bar*=0.5 μm; **C** *bar*=0.7 μm; **D** *bar*=1.1 μm

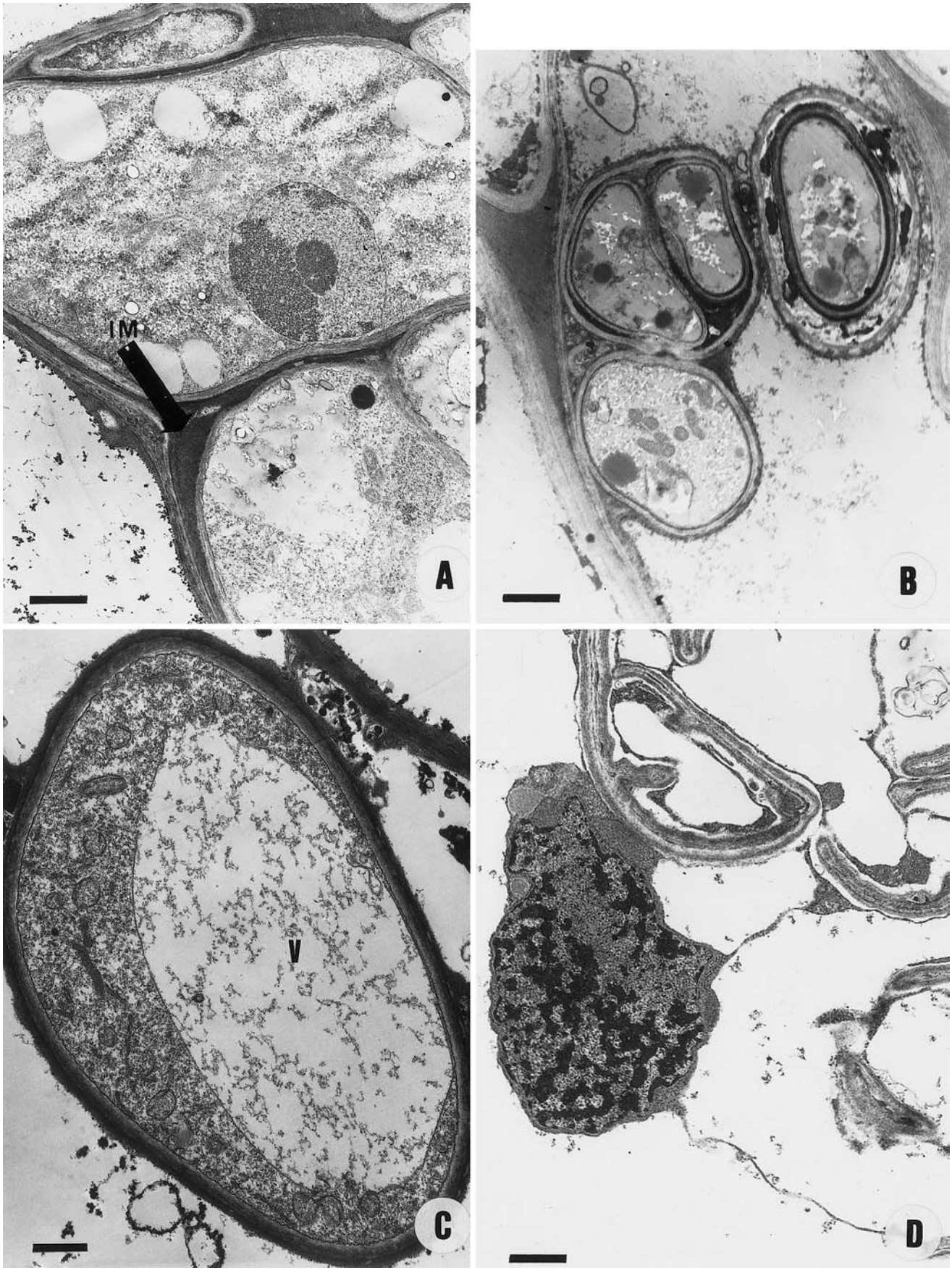
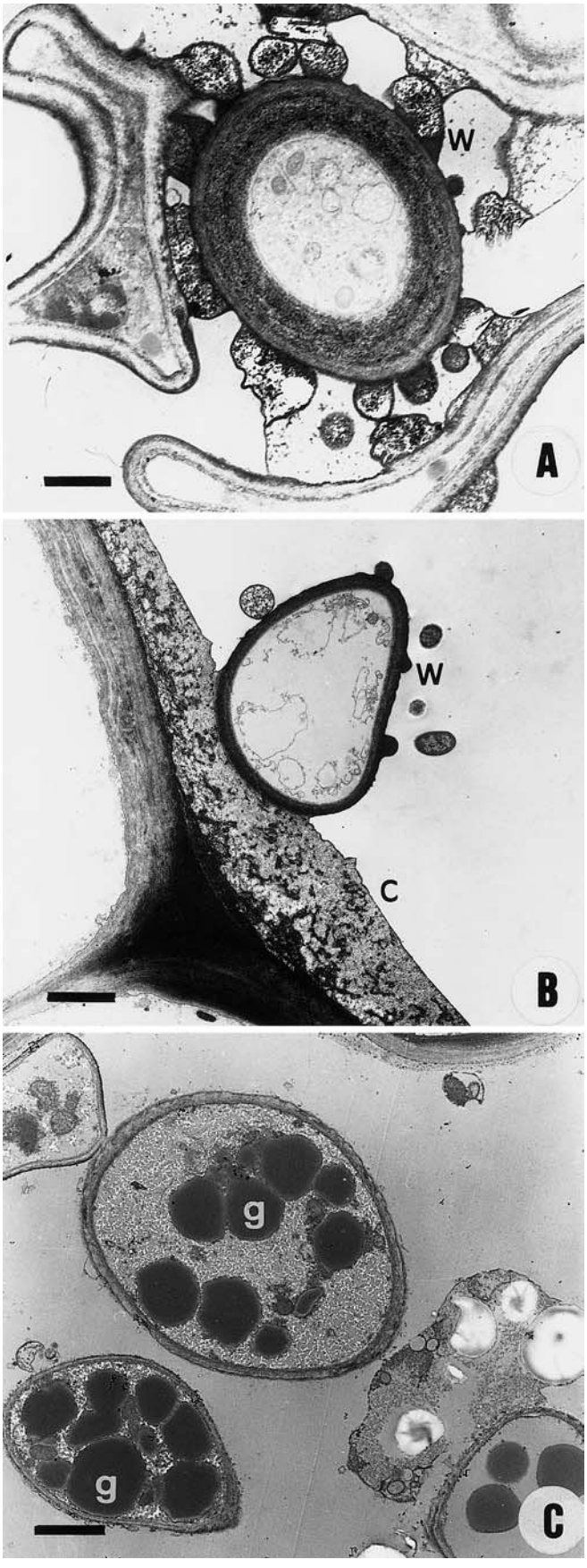


Fig. 4A–D Inter- and intracellular hyphae of *T. claveryi.* **A** A highly electron-dense interhyphal matrix (*IM*) in intercellular hyphae. **B** Active intracellular hyphae growing inside old hyphae. **C** The fungal cytoplasm is occupied by a big vacuole (*V*). **D** The empty hyphae are clumped together. **A** *bar*=1.5 μm; **B** *bar*=1.4 μm; **C** *bar*=1 μm; **D** *bar*=1.9 μm



cellular organization of these hyphae was very similar to the "coils" observed in arbuscular mycorrhiza although they were not ramified like arbuscules, as was observed by Awameh et al. (1979). A few colonized epidermal cells were observed. These could show an initial phase of mycorrhiza development, where the fungus might use epidermal cells to reach the cortical cells. This epidermal cell colonization was also observed by Fortas and Chevalier (1992).

The two different types of interface observed, a host cell wall-fungal wall interface and a host plasmalemma-fungal wall interface, correspond to interface types IT 7 and IT 24, respectively, according to Bracker and Littlefield's (1973) classification. The presence of numerous intracellular hyphae of *T. claveryi* in direct contact with the host cell wall (host cell wall-fungal wall interface) was very frequent and regular. This has been previously observed in the *H. salicifolium*/*T. claveryi* association (Dexheimer et al. 1985) and *H. guttatum*/*T. claveryi* association (Fortas 1990) and may be considered a characteristic of *T. claveryi* mycorrhiza (Dexheimer et al. 1985).

In older mycorrhiza, hyphae were observed within additional wall layers, which may be interpreted as hyphae developing within other older hyphae, as observed by Gianinazzi-Pearson et al. (1981) in *Glomus tenuis* (Greenhall) Hall mycorrhiza.

The *P. lefebvrei* mycorrhiza organization was characterized by intracellular hyphae with large amounts of electron-dense globules, probably of a lipidic nature. Hyphal lipid accumulation is common in long-term in vitro cultured mycelium and has been described in other types of mycorrhiza (Bonfante-Fasolo and Gianinazzi-Pearson 1982). However, these globules were also observed in the mycorrhiza formed from *P. lefebvrei* spores. The chemical composition of *P. lefebvrei* ascocarps showed a very high lipid content (199.4 g kg^{-1}), 3 times more than *T. claveryi* ascocarps (69.5 g kg^{-1}) (Murcia et al., in press), which seems to be a characteristic of *P. lefebvrei.* The same ornamentation on the hyphal wall as described in the mycorrhizal symbiosis with *P. lefebvrei* was observed on the hyphal peridial tomentum of ascoma of this fungus (Moreno et al. 2000). This warty ornamentation is very similar to that described in other fungi, including some E-strain ascomycetes, such as *Humaria hemisphaerica* (Wigg.: Fr) Fuckel and *Tricharina gilva* (Boud.) Eckblad (Ingleby et al. 1990).

Fig. 5A–C Mycorrhizae synthesized in in vitro conditions by *H. almeriense* with *P. lefebvrei*. **A** Warty ornamentation (*W*) on the external hyphal wall. **B** Warty ornamentation on the external root hyphae; these Ws were not present in the contact area between the hyphae and the plant cuticle (*C*). **C** Globules (*g*) with an electron-dense content: very abundant in intracellular hyphae. **A** *bar*=1.2 μm; **B** *bar*=1.5 μm; **C** *bar*=2.8 μm

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