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In Vitro Interaction of the Truffle *Terfezia terfezioides* with *Robinia pseudoacacia* and *Helianthemum ovatum*

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ABSTRACT. The type of the *in vitro* root interactions of *Terfezia terfezioides* with the plants *Robinia pseudoacacia* and *Helianthemum ovatum* was investigated including detailed anatomical and ultrastructural characterization. No difference in growth was detected at different phosphate concentrations on agar synthetic medium between the inoculated and control plants during a short-time cultivation. The fungal colonization of the roots increased with higher phosphate level in both plant species, but was always lower in *R. pseudoacacia* roots. Septate hyphae formed frequently intracellular branched coils in dead cortical cells. In *H. ovatum* intercellular hyphae were observed forming finger-like structures reminiscent of Hartig-net structures in ectomycorrhizae. A loose hyphal envelope covered the root surface of both colonized and noncolonized roots. The features resembled similar structures described earlier during the mycorrhizae of different *Terfezia* species. Our detailed anatomical and ultrastructural study shows that the *in vitro* root interactions of the *T. terfezioides* cannot be considered unambiguously as mycorrhiza.

Several species of the genus *Terfezia* (Ascomycetes, Pezizales) have been recorded to occur frequently in the Mediterranean and desert areas in connection with *Helianthemum* species (Cistaceae). Because of the market value of the sporocarps of some of these hypogeous species – commonly named desert truffles – their mycorrhizal state has been widely studied in the field and by *in vitro* experiments. In spite of the interest, some doubtful data were published, and detailed, reliable documentation of the anatomy of the mycorrhiza is rare.

Terfezia leptoderma TUL. formed ectomycorrhizae with *Helianthemum salicifolium* (L.) MILL. showing a well developed Hartig-net but no sheath (Dexheimer *et al.* 1985; Leduc *et al.* 1986). The association formed by *Terfezia claveryi* CHATIN and *Heliathemum salicifolium* was considered as endomycorrhiza, but it showed both ecto- and endomycorrhizal characteristics (Dexheimer *et al.* 1985). Intracellular hyphae adhering to the inner surface of the colonized root cells were more abundant than intercellular hyphae (Dexheimer *et al.* 1985). The mycorrhizae obtained from *T. claveryi* and *Helianthemum almeriense* PAU were reported as "both the extramatrical and intercellular hyphae were moniliform or beadlike" and "the intracellular hyphae formed coils which filled the whole lumen" (Morte *et al.* 1994).

The report of Malloch and Thorn (1984) on ectomycorrhizae formed by *Helianthemum* and *Terfezia* (and *Tirmania*) species is misleading. In the case of *H. salicifolium* the authors cited the work of Awamah *et al.* (1979), in which there is nothing what could be considered as ectomycorrhiza. In the case of *Helianthemum ledifolium*, Malloch and Thorn (1984) referred to the work of Alsheikh and Trappe (1983). This taxonomic study about *Phaeangium lefebvrei* PAT. contains a reference related to the potential mycorrhizal partner of *Helianthemum* spp. as *Terfezia* and *Tirmania* spp. described by Awameh and Alsheikh (1979) and Awameh *et al.* (1979). However, those interactions are not ectomycorrhizal. The an anatomical results of the mycorrhization of *H. ledifolium* (L.) MILL. and *H. salicifolium* by *Terfezia boudieri* CHATIN and *T. claveryi* CHATIN (and with two other *Tirmania* species) are poorly detailed, reporting "intracellular arbuscules" (Awamah *et al.* 1979). Later Alsheikh (1984) presented anatomical characterization of the same two *Helianthemum* and the same four fungi species collected in natural habitats. The hyphae were always observed to colonize the cortical cells forming "many short curved and densely interwoven extensions". Epidermal cells were also colonized, but they often collapsed. The author unequivocally distinguished the mycorrhizae from ecto-, ectendo- and arbuscular ones, found some similarity with ericoid mycorrhizae, but suggested to use the term "helianthemoid" mycorrhizae.

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Effects of substrate composition were investigated using the mycorrhizae of *Terfezia leonis* (TUL. et TUL.) TUL. et TUL. (nom.leg. *Terfezia arenaria* (MORIS) TRAPPE) and *Helianthemum sessiliflorum* (DEFS.) PERS. (Roth-Bejerano 1990; Kagan-Zur *et al.* 1994). The presented anatomical features of the mycorrhizae were ambiguous, particularly the demonstration of the Hartig-net (Roth-Bejerano 1990). Fortas and Chevalier (1992) studied the effect of phosphate on the synthesized mycorrhizae of *Heliathemum guttatum* MILL., *Terfezia arenaria* and *T. claveryi*. At high phosphate level, the truffles formed ectomycorrhizae with Hartig-net but no sheath. At lower phosphate levels ectendomycorrhizae were formed showing Hartig-net and coiling intracellular hyphae but again no sheath was observed.

The most reliable records of *Terfezia terfezioides* (MATT.) TRAPPE are from Europe, where the fungus has been mostly found in the Carpathian basin, in the catchment area of the Danube River, in mixed *Robinia pseudoacacia* L. forests on sandy soils (Babos 1981; Király *et al.* 1992; Ławrynowicz *et al*. 1997; Kovács and Bagi 2001; Kovács *et al.* 2001). The generic designation of *Terfezia terfezioides* (MATT.) TRAPPE (Trappe 1971) has been queried on the basis of molecular results (Percudani *et al.* 1999; Kagan-Zur *et al.* 2001; Díez *et al.* 2002). However, the close relationship between the species and the genus *Terfezia* is obvious (Percudani *et al.* 1999). Although the mycorrhizal relationship between *T. terfezioides* and *Robinia pseudoacacia* has been reported earlier (Király *et al.* 1992; Bratek *et al.* 1996), the knowledge on the interaction is insufficient. The main characteristic of the interaction is the coiling hyphae in the cortical cells, mantle and Hartig-net is lacking, and intercellular hyphae were not reported (Bratek *et al.* 1996). The insufficient documentation of the anatomical features and the mistakes in the comparison with the literature make this work unreliable as to the anatomy of the interaction between *T. terfezioides* and *R. pseudoacacia.* Even the mycorrhizal characteristic of the interaction is ambiguous; *e.g.*, in spite of the lack of the intercellular structures, Bratek *et al.* (1996) did not exclude that this was an ectendomycorrhiza. This latter mistake could have misled Percudani *et al.* (1999) who referred to the interaction as ectomycorrhizal based on the above article of Bratek *et al.* (1996). Díez *et al.* (2002) consider *T. terfezioides* as a mycorrhizal fungus using only the data of Bratek *et al.* (1996) for speculations regarding the plant interactions of this species.

The mycorrhiza of *Terfezia* with the black locust is peculiar as the tree was introduced to Europe. In the Carpathian basin, extensive afforestation with *R. pseudoacacia* L. started only in the 19th century (Magyar 1960). It was also reported earlier as an arbuscular-mycorrhizal plant (Harley and Harley 1987) and was used in mycorrhizal experiments with arbuscular-mycorrhial fungi (Moiroud *et al.* 1981; Olesniewicz and Thomas 1999), although ectomycorrhizae of the black locust were mentioned previously only once (Trappe 1962).

The purpose of this study was to obtain detailed anatomical data about the interaction of *T. terfezioides* with two plant species. In the case of *R. pseudoacacia* we aimed at characterizing in detail its relationship with the fungus. *Helianthemum ovatum* (VIV.) DUN. was chosen to study whether *T. terfezioides* has the potential to form mycorrhizae with this plant (*T. terfezioides* has never been found in the habitats of *H. ovatum*). The area from which the plant originated is a sandy grassland with semi-desert characteristics resembling the habitats of other *Terfezia* species. We compared the probable interaction with the previously described *Terfezia* mycorrhizae. Substrates with different phosphate contents were used to study whether the influence of this component on the interaction was as remarkable as in the case of other *Terfezia* species.

MATERIALS AND METHODS

Fungus and plant material. The *Terfezia terfezioides* (MATT.) TRAPPE strain was isolated from sporocarps collected near Kunfehértó (Hungary) in 1999. The strain and the sporocarps were kept in the collection of G.M. Kovács (*Department of Botany, University of Szeged*), under no. 10125. The strain was cultivated on modified MMN medium (in mg/L: K_2HPO_4 500, $(NH_4)_2HPO_4$ 250, $MgSO_4 \cdot 7H_2O$ 150, $CaCl_2 \cdot 2H_2O$ 50, NaCl 25, Fe-EDTA 20; in g/L: agar 10, glucose 10, maltose 3; pH adjusted before autoclaving to 8) (Marx 1969) in the dark at 25 °C.

The seeds of *Helianthemum ovatum* were collected in a semi-arid grassland area near Fülöpháza (Hungary) during the autumn 1999. The seeds of *Robinia pseudoacacia* were collected in the *Botanical Garden of the Department of Botany* (*University of Szeged*) during the autumn 2000. The seeds were dried at 23 °C and kept at room temperature in the dark until use.

In vitro *experiments.* Five different phosphate concentrations were used (12.5, 25, 50, 100 and 200 % of the original amount of $(NH_4)_2HPO_4$, *i.e.* 31.25, 62.5, 125, 250, 500 mg/L, and K_2HPO_4, *i.e*. 62.5, 125, 250, 500, 1000 mg/L) corresponding to 0.6, 1.2, 2.4, 4.8 and 9.6 mmol/L PO_4^{3-}, respectively); the other substrate parameters were not changed. The fungal inoculum was transferred to Petri dishes a few days before adding the seedlings.

The seeds were surface sterilized (33 % H_2O_2, 15 min), washed three times in sterilized tap water for 40 min and, after bruising the seed coats with a sterile needle, put on sterile paper towel sodden with sterile tap water in Petri dishes. After 12 h in darkness, they were kept in the light chamber (*see below*).

After germination of the seeds, the well-developed seedlings were placed on the substrates. The root of the plants was fitted into a shallow groove made across the outer parts of the mycelium. In case of *R. pseudoacacia* one-third of the substrate was cut out to make enough space for leaves. Controls were prepared from both the plants and the fungus with all the different phosphate concentrations. The Petri dishes with plants were kept in a conditioned chamber, with 12/12 h day–night periods (at 32 and 22 °C, respectively) illuminated with wide-spectral light (130 μmol photon per m^2 per s). The plants were left to grow under these conditions for 28–32 d.

Microscopic study. The root samples were fixed in 2 % glutaraldehyde in Sörensen buffer (pH 7.2) under vacuum and stored at 4 °C. The roots were washed 6 times for 10 min in the same buffer, and post-fixed in osmium tetroxide (1 % OsO_4 in Sörensen buffer, pH 7.2; 1 h). The samples were then again washed 6 times for 10 min in distilled water. After dehydration in acetone, they were embedded in Spurr's (1969) ERL (*ProSciTech*) embedding medium. The infiltration was carried out in 6 steps, in ERL–acetone proportions 1 : 2 (overnight at 4 °C), 1 : 1 and 2 : 1 (both for 30 min), twice for 30 min, and once overnight in pure ERL. For light microscopy semi-thin (0.5–0.8 μm) longitudinal sections were cut and stained with neofuchsin–Crystal Violet (Morgenstern 1969), and covered by microscopic mounting medium Enthelan (*Merck*). After the light microscopy, the ultra-thin (60–75 nm) longitudinal sections were cut from the suitable parts of the embedded roots for transmission electron microscopy (TEM). They were transferred to Formvar (*Rea*, USA) resin-covered copper grids and stained with uranyl acetate (1 %, 1 h) and lead citrate (2 %, 12 min), and were observed using the LEO 906 TEM (*Hi-Tech Instruments*). Serial sections were investigated microscopically using both methods to avoid artifacts and misinterpretations that may result from the cut angle or the thickness of the samples.

RESULTS

During 28–32 d of the experiment the plants grew well. The *H. ovatum* plantlets were 20–30 mm tall with ramified 20–40 mm long roots while *R. pseudoacacia* plants were 30–50 mm tall with 30–60 mm long ramified roots when sampled. No differences between the inoculated and control plants were found.

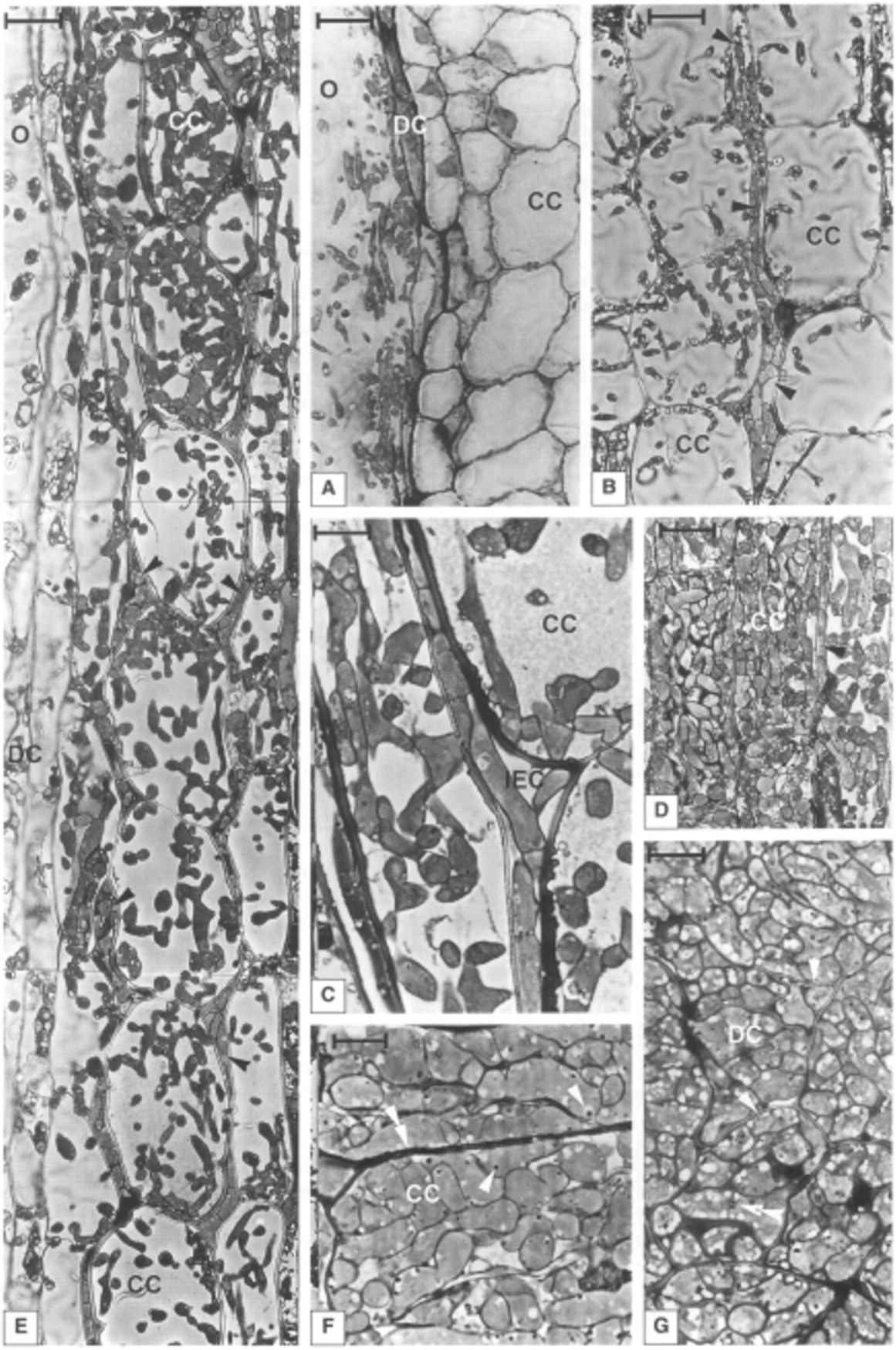
The cortical cells of the control plant roots were alive without any differences from the living root cells of the plants with the fungus. The hyphae of the control mycelial cultures did not show detectable differences from the outer hyphae of the systems with plants. The septate hyphae had vacuoles, lipid-droplets and Woronin-bodies, the latter often close to the septa (*see* Figs 3D, 4C, 4D).

The roots of *H. ovatum* and *R. pseudoacacia* were covered by mantle-like hyphal envelope (Figs 1A, 1E, 2A, 2B). The density of this structure increased slightly with increasing phosphate concentration. The hyphal envelope was not tightly connected to the roots and it was often lost during the embedding procedure.

The colonization of the *H. ovatum* roots at the lowest phosphate level was very poor. The hyphae colonized the rhizodermis and rarely some outer cortical cells (Figs 1A, 3B). The tangential walls of the hypodermis seemed to stop the hyphae growing into the cortex (Figs 1A, 3A, 3B). The noncolonized cortical cells were alive, containing large vacuoles and starch granules (Figs 3B, 3C). In spite of this, all the colonized plant cells were dead, as it was unambiguously determined based on the completely or mostly degraded cell particles (Fig. 3B). Neither membrane systems nor the cell contents or cytoplasm was detectable in the colonized plant cells – this phenomenon (the dead colonized plant cells) was the same irrespective of the phosphate concentration or the host plant (Figs 3B, 3E, 4B, 4D). Deposition of callose-like material at the penetrating sites was observed when the hyphae penetrated the walls of the living cortical cell layer; at the point of penetration the hyphae contained vesicles depleting their contents (Fig. 3A).

The colonization of *H. ovatum* roots at 1.2 mmol/L phosphate in the substrate was stronger. All the cortical cells were colonized and intercellular hyphae were also present (Fig. 1B). All the colonized cells were moribund at different levels of degradation but the cells of the central cylinder were alive. In one rootlet hyphae colonized the trachea (*data not shown*). At the hyphal penetration sites deposition of electron-dense material occurred in the cell wall. The intercellular hyphae abutted tightly on the cell walls.

All the cortical cells were colonized at 2.4 mmol/L phosphate; the colonization of the cells was intensive (Figs 1C, 1E). All the colonized cells were dead, sometimes with degraded cell compartments and with deposition of cell-wall material at the penetration of the fungus. The intercellular hyphae abutted on the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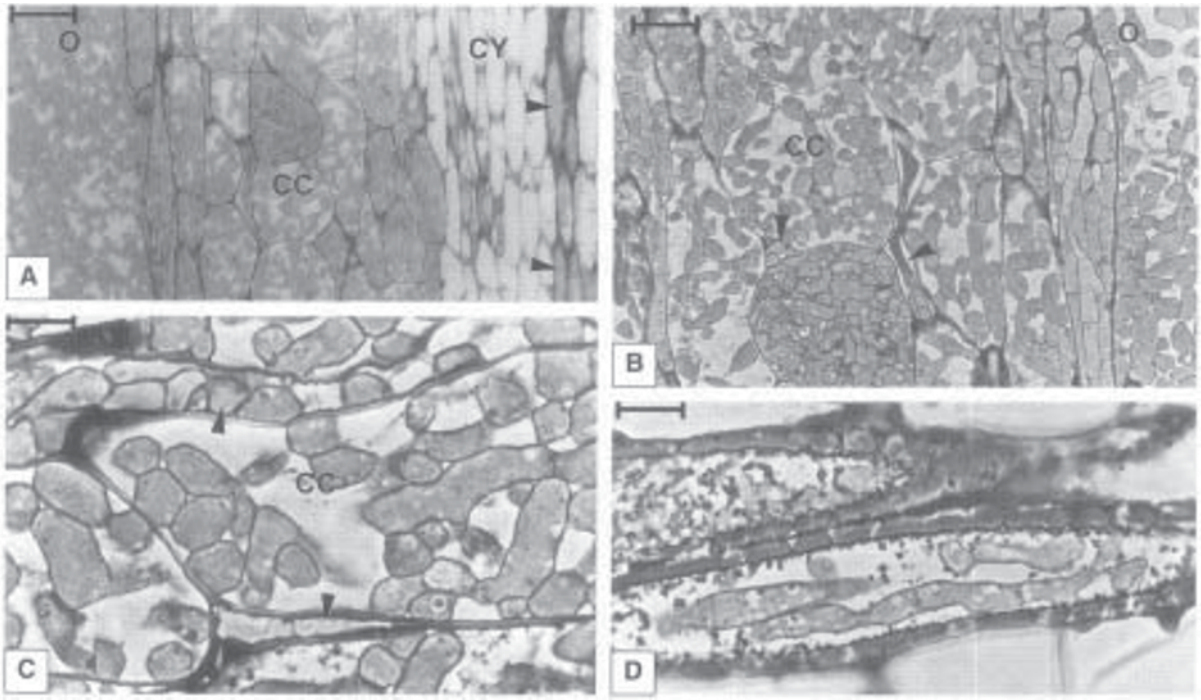
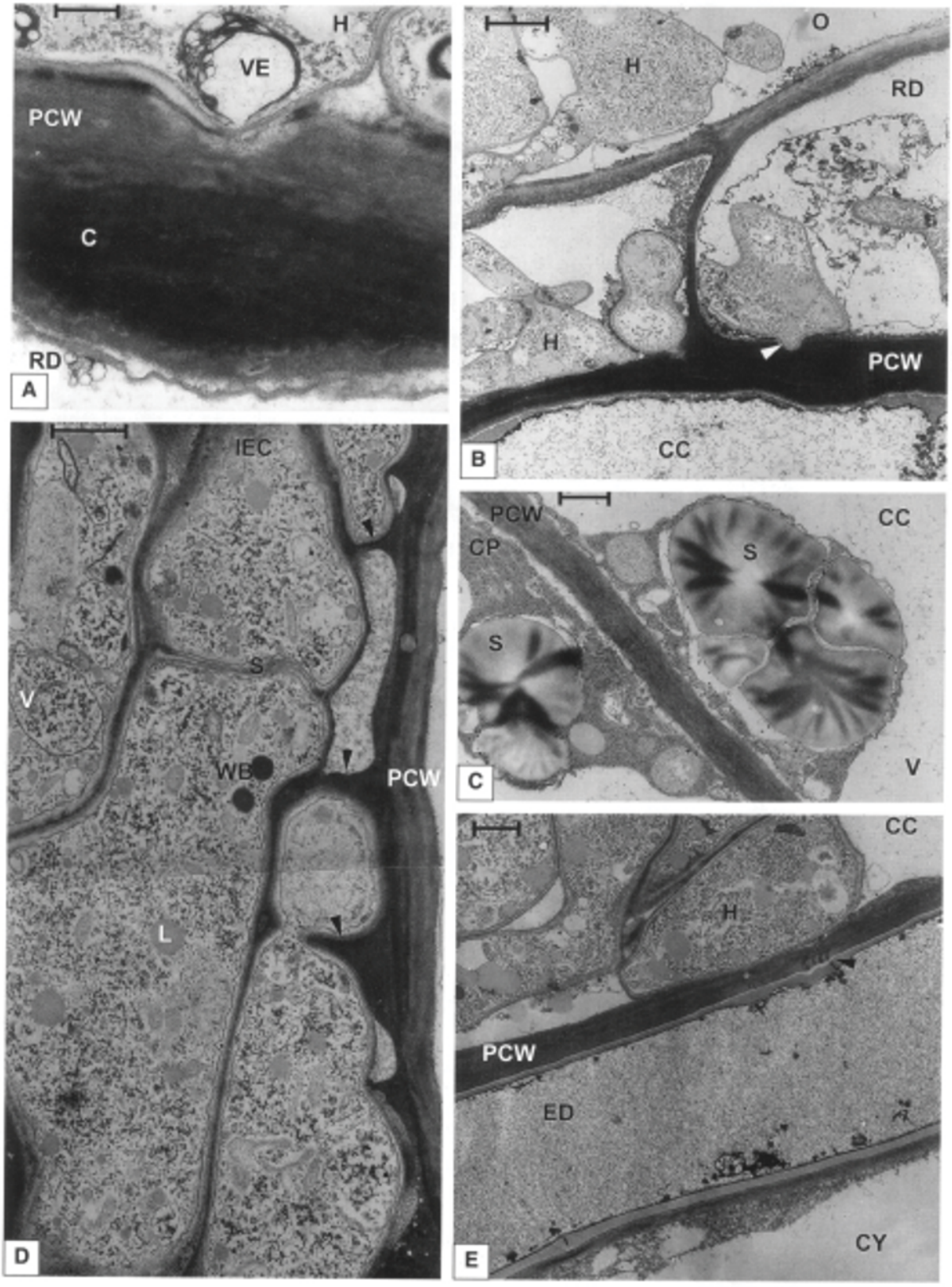


▲ **Fig. 2.** Light microphotographs* of the *in vitro* interaction of *Terfezia terfezioides* with *Robinia pseudoacacia* at 9.6 mmol/L phosphate. **A**: Radial section of the colonized rootlet; *arrowheads* – colonized vessel elements; bar corresponds to 40 μm. **B**: Longitudinal section of the root; *arrowheads* – intercellular hyphae; bar 20 μm. **C**: Colonized cortical cells; *arrowheads* – intercellular hyphae; bar 8 μm. **D**: Colonized trachea; bar 8 μm.

◀ **Fig. 1.** Light microphotographs* of the *in vitro* interaction of *Terfezia terfezioides* with *Helianthemum ovatum*. **A**: Radial section of the root of *H. ovatum* at 0.6 mmol/L phosphate; bar corresponds to 20 μm. **B**: Longitudinal section of the root cortex at 1.2 mmol/L phosphate; *arrowheads* – intercellular hyphae; bar 20 μm. **C**: Colonized cortical cells and intercellular spaces at 2.4 mmol/L phosphate; bar 8 μm. **D**: Colonized cortical cells at 4.8 mmol/L phosphate; *arrowhead* – intercellular hyphae; bar: 20 μm. **E**: Longitudinal section of a colonized rootlet at 2.4 mmol/L phosphate; *arrowheads* – intercellular hyphae; bar: 20 μm. **F**: Colonized cortical cells at 4.8 mmol/L phosphate; *arrowheads* – polyphosphate granules; *double arrowhead* – plant cell wall; bar 8 μm. **G**: Longitudinal section of colonized rhizodermal cells at 9.6 mmol/L phosphate; *arrowhead* – plant cell wall; *double arrowhead* – WB around the septa; bar 8 μm.

**Generally used abbreviations in figure legends*

C	callose	L	lipid
CC	cortical cells	O	outside of the root
CY	central cylinder	PCW	plant cell wall
ED	endodermis	RD	rhizodermis
H	hyphae	VE	vesicule
IEC	intercellular space	WB	Woronin-body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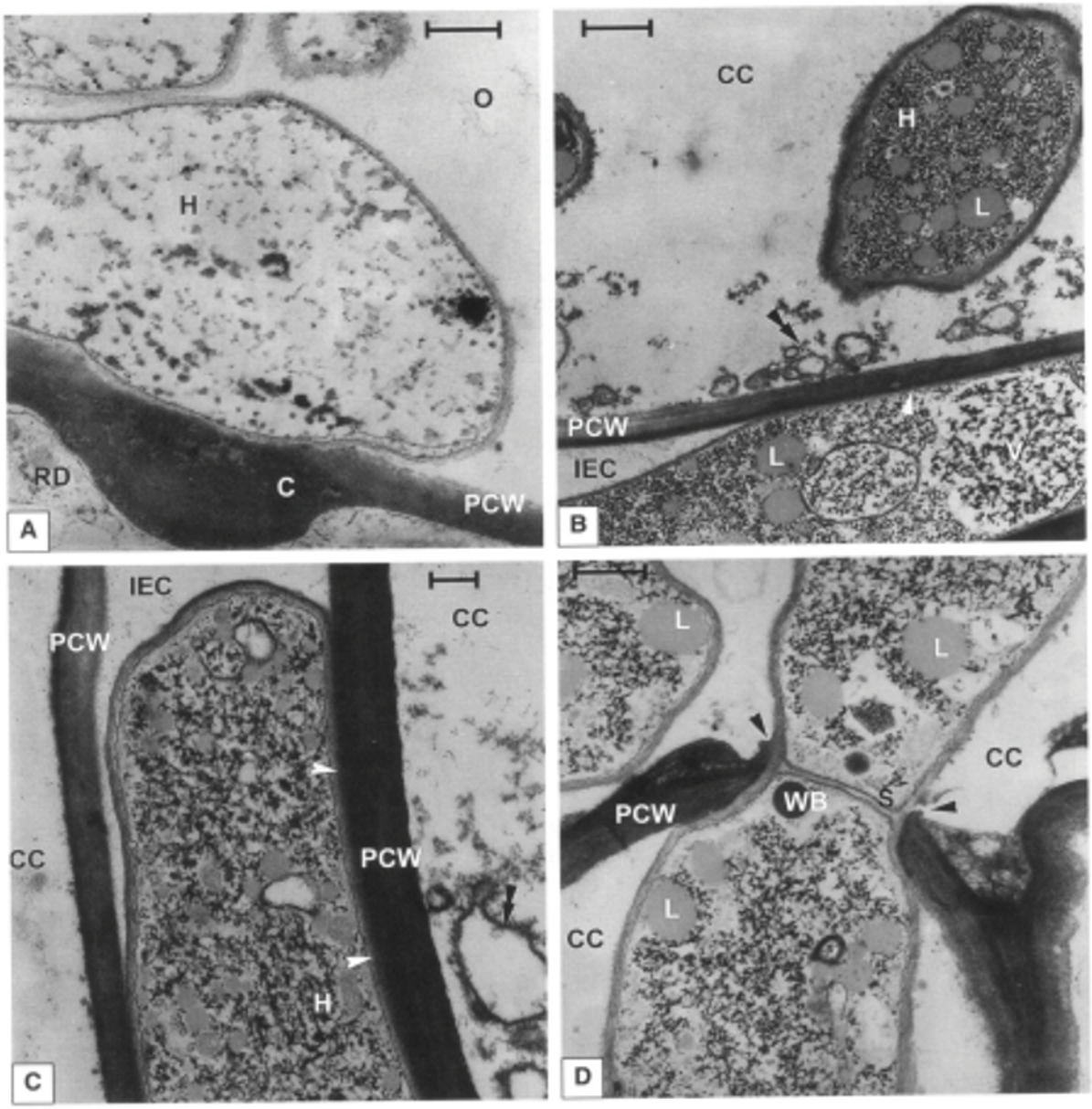

▲**Fig. 4.** Electron microphotographs of the *in vitro* interaction of *Terfezia terfezioides* with *Robinia pseudoacacia*. **A**: Dead hypha previously penetrating to a living rhizodermal cell at 2.4 mmol/L phosphate; bar corresponds to 500 nm. **B**: Colonized cortical cell and intercellular hyphae at 4.8 mmol/L phosphate; *arrowhead* – tight connection of the hyphae with the cell wall; *double arrowhead* – collapsed cell contents; bar 1 μm. **C**: Intercellular hyphae at 4.8 mmol/L phosphate; *arrowhead* – probable interface; *double arrowhead* – collapsed cell contents; bar 500 nm. **D**: Penetrating hyphae at 9.6 mmol/L phosphate; *arrowhead* – discontinuous PCW; WB in the center; bar 500 nm.

◀**Fig. 3.** Electron microphotographs of *in vitro* interaction of *Terfezia terfezioides* with *Helianthemum ovatum*. **A**: Outer hyphae penetrating to a rhizodermal cell at 0.6 mmol/L phosphate; VE in the top, C in the center; bar corresponds to 500 nm. **B**: Colonized dead rhizodermal cells and living cortical cell at 0.6 mmol/L phosphate; *arrowhead* – penetrating hyphae; bar 2 μm. **C**: Deposited starch in living cortical cells at 0.6 mmol/L phosphate; S – starch; bar 500 nm. **D**: Hartig-net at 2.4 mmol/L phosphate; *arrowheads* – finger-like structures, S – septa; bar 1 μm. **E**: Colonized cortical cells and the outer layer of the central cylinder at 2.4 mmol/L phosphate; *arrowhead* – plasmodesmata, ED diagonally; bar 1 μm.

cell walls, and finger-like lobed hyphae were detected (Fig. 3D). The cells of the central cylinder and the endodermal cells were alive; the hyphae started to penetrate the latter (Fig. 3E).

The colonization of the cortex was complete in case of 4.8 and 9.6 mmol/L phosphate; the rhizodermal and cortical cells were full with branching, septate, sometimes swelling hyphae (Figs 1D, 1F, 1G). All the colonized cortical cells were moribund, while the stele and the endodermis were alive.

The roots of *R. pseudoacacia* roots were not colonized at 0.6 mmol/L phosphate. The cortical cells were living containing large vacuoles but no starch. At 1.2 mmol/L phosphate some of the epidermal cells were colonized and at some places the hyphae also colonized the cortical cells. The colonized cells were dead, and no special anatomical features were detected in the intercellular hyphae. At 2.4 mmol/L phosphate most of the epidermal cells were colonized. In some cases thickened cell walls were detected at the penetration sites (Fig. 4A). The cortical cells were usually free of colonization. All colonized cells were dead. At 4.8 mmol/L phosphate concentration all the epidermal and cortical cells were colonized, the former strongly, the latter just slightly. All colonized cells were dead (Figs 4B, 4C) while the cells of the central cylinder were living. At 9.6 mmol/L phosphate all the epidermal and cortical root cells of *Robinia* were colonized and were filled nearly completely with the hyphae (Figs 2A–2D). Although the parenchymatic cells of the central cylinder were alive and free of colonization, the tracheas were often colonized with the hyphae (Figs 2A and 2D). No finger-like structures in the intercellular hyphae were observed at any phosphate concentration (Figs 4B, 4C). No continuity of host cell-wall materials on the penetrating hyphae was detected (Fig. 4D).

DISCUSSION

The influence of substrate phosphate concentration on the anatomy of the mycorrhizae of two *Terfezia* species with *Helianthemum guttatum* was described by Fortas and Chevalier (1992). The effect of the varying phosphate content in the case of the *T. terfezioides* presented here was different, but also conspicuous. The colonization of the cells and the filling out of the host cells by the hyphae of *T. terfezioides* increased with increasing phosphate content. However, the level of the colonization in case of *H. ovatum* at 4.8 and 9.6 mmol/L phosphate and in case of *R. pseudoacacia* at 9.6 mmol/L could be considered as unnatural. Reduced nutrient availability is commonly thought to improve mycorrhization (Smith and Read 1997). In our study a high concentration of phosphate together with exogenous sugar improved the rate of colonization. Roth-Bejerano *et al.* (1990) and Kagan-Zur *et al.* (1994) showed that at low phosphate concentration, mycorrhization between *H. sessiliflorum* and *T. leonis* was inhibited; without exogenous sugar (under very low nutrient supply), initial mycorrhization was detected after 5 weeks (Roth-Bejerano *et al.* 1990). Modified MMN media, but with composition different from that used in our study, were used for mycorrhization of *H. almeriense* with *T. claveryi* by Morte *et al.* (1994) and Morte and Honrubia (1995). With lower phosphate and sugar concentrations than in our study, Morte *et al.* (1994) obtained mycorrhizae after 4 weeks. Morte and Honrubia (1995, 1997) developed the MH medium for mycorrhization of these species. It has a relatively high exogenous sugar content (sucrose 15 g/L) but its phosphate concentration is less than one-fourth of the phosphate concentration used in our experiments. The increasing colonization found in our study might be explained by a higher ionic strength–osmotic effect. The enhancing effect of the phosphate on the colonization could be explained as being due to a faster interaction of *T. terfezioides* with the plants. This could be ascribed to a positive effect of the exogenous glucose on the mycorrhization at high phosphate content, as was also reported in case of ectomycorrhizal fungi (Guerin-Laguette *et al.* 2000).

Although the structures formed by the fungi could be compared, the interaction described here cannot be considered unambiguously as mycorrhiza. The main anatomical features of the intracellular structures formed by *T. terfezioides* show some characteristics of the mycorrhizae of *Terfezia* species. While the ultrastructural studies of the endomycorrhizae between *T. claveryi* and *H. salicifolium* revealed living colonized host cells and functioning interface (Dexheimer *et al*. 1985), we found that all colonized cells of both plants appeared to be moribund. As the intracellular structures in *R. pseudoacacia* are similar to those in the *H. ovatum* roots and in the other *Terfezia* associations described previously, the term "terfezioid" would probably be more reasonable than the "helianthemoid" suggested by Alsheikh (1984). The similarity of the intracellular structures found in the roots of *Citrullus vulgaris* SCHRAD. formed by *Terfezia pfeilii* HENN. (Kagan-Zur *et al*. 1999) also could support this distinction.

Although the ectomycorrhizae of *R. pseudoacacia* have been reported (Trappe 1962), the black locust is mostly known as an arbuscular-mycorrhizal tree (Harley and Harley 1987). The *Helianthemum* species commonly forms ectomycorrhizae (Read *et al*. 1979; Harley and Harley 1987). *H. ovatum*, in the area from where the seeds originated, also forms ectomycorrhizae (Kovács and Jakucs 2001). This could be the reason why the finger-like structures were observed only in the roots of *H. ovatum*. On the other hand,

a functional contact between the intercellular hyphae and host cell walls cannot be excluded in *R. pseudoacacia*, because this could function as the only interface between the host and the fungus. An unambiguous effect of the plant species on mycorrhization was also reported in *T. leptoderma* which formed neither Hartig-net nor unambiguous mycorrhizae with two *Cistus* species but did so with *H. salicifolium* (Leduc *et al.* 1986).

No detectable effect of the inoculation of *T. terfezioides* was found during short experiments. The colonization of the endodermis and the hyphae in the tracheas make questionable the mycorrhizal characteristic of the interaction. However, it is not possible to exclude that these traits are artifacts. The defense reaction at the penetrating sites and the collapse of the plant cells make also doubtful the clear mycorrhizal connection, although these features also occur in mycorrhizae (Scannerini and Bonfante-Fasolo 1983; Smith and Read 1997). On the other hand, the finger-like structures and the colonization, in spite of the sugar content in the substrate, could be interpreted as an indication of mycorrhizal interaction. Similar uncommon mycorrhizal features were also reported in the case of other ascomycetous fungi (Buscot and Kottke 1990).

The *H. ovatum–T. terfezioides* system seems to be a useful model for further *in vitro* experiments because of its small size and a relatively easy handling of the plant in aseptic conditions.

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