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SHORT NOTE

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Mycorrhizae between black locust (*Robinia pseudoacacia)* and *Terfezia terfezioides*

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Abstract In the present paper we report a mycorrhizal association between the hypogeous white truffle *Terfezia terfezioides* and the black locust (*Robinia pseudoacacia*) growing at various sites in Hungary. The mycorrhiza can be considered as being of the endo- or ectendo-type, since both mantle and Hartig net are absent. Morphological features of the septate hyphae colonizing cortical root cells were investigated by light microscopy on cryosections and on ultra-thin sections studied by transmission electron microscopy. Artificial infection of micropropagated black locust plantlets with the mycelium of the fungus was successful and had the same characteristics as naturally occurring associations.

Key words Mycorrhiza · Truffles · *Terfezia terfezioides* · *Robinia pseudoacacia*

Introduction

Black locust (*Robinia pseudoacacia,* Fabaceae) was introduced from North America into Europe several centuries ago. This plant can grow on both poor sandy soils and on humus-rich soils. In the 19th century, it was widely used in Hungary for binding wind-blown sands. The current extent of black locust forests is greater in Hungary than in all other European countries.

Terfezia terfezioides (Matt.) Trappe 1971 (Ascomycetes, Terfeziaceae) (Fischer 1938; Trappe 1971), a white truffle, has been reported about 30 times in Hungary, in almost all cases under black locust trees growing on calcareous sandy soils (Hollós 1933; Novák and Zeller 1959; Szemere 1965; Babos 1981; Király and Bratek 1992). The fungus has also been detected in Italy (Montecchi and Lazzari 1985, 1993; Brotzu 1994) but is regarded as rare. Other species of this genus and its close relatives (*Tirmania* spp.) are widespread in semiarid areas and deserts of the Middle East and North Africa (Awameh 1981). Leduc et al. (1986) and Fortas and Chevalier (1992) demonstrated the existence of mycorrhizae formed by white truffles (e.g. *Terfezia arenaria, Terfezia claveryi, Tirmania pinoyi*) on *Helianthemum* and *Cistus*. These fungi can form an ectendo mycorrhiza lacking a mantle and, depending on the phosphorus content of the soil, with or without a Hartig net.

As we have found *T. terfezioides* 10 times in Hungary, always among black locust roots and presumed a mycorrhizal interaction (Király et al. 1992; Bratek et al. 1994). Up to the present, black locust had only been reported to form arbuscular mycorrhizae (Harley and Harley 1987). In this paper, we report on our investigation of the association between *T. terfezioides* and black locust.

Materials and methods

Sampling

Samples of black locust roots were taken on several occasions from three different localities in Hungary (Horány, Örbottyán, Gyál). Roots were collected from an area of about 1 m^2 surrounding *Terfezia* fruitbodies found in truffle beds. The roots were identified using macromorphological features (yellow colour, thread-like rhizodermis and the distinctive smell of the black locust root) and by anatomical characteristics of root sections. Samples were fixed in FEA (formaldehyde, 70% ethanol, acetic acid 5:90:5) for light microscopy and 2% glutaraldehyde for transmission electron microscopy (TEM).

Light microscopy

Approximately 10 fixed, slightly swollen root tips were cut from each root sample and placed into distilled water containing 2% glycerol for 1 h. Cross and longitudinal cryosections (30 μm) were prepared and examined by bright-field light microscopy (Opton) and Nomarski interference contrast microscopy (Olympus). Per-

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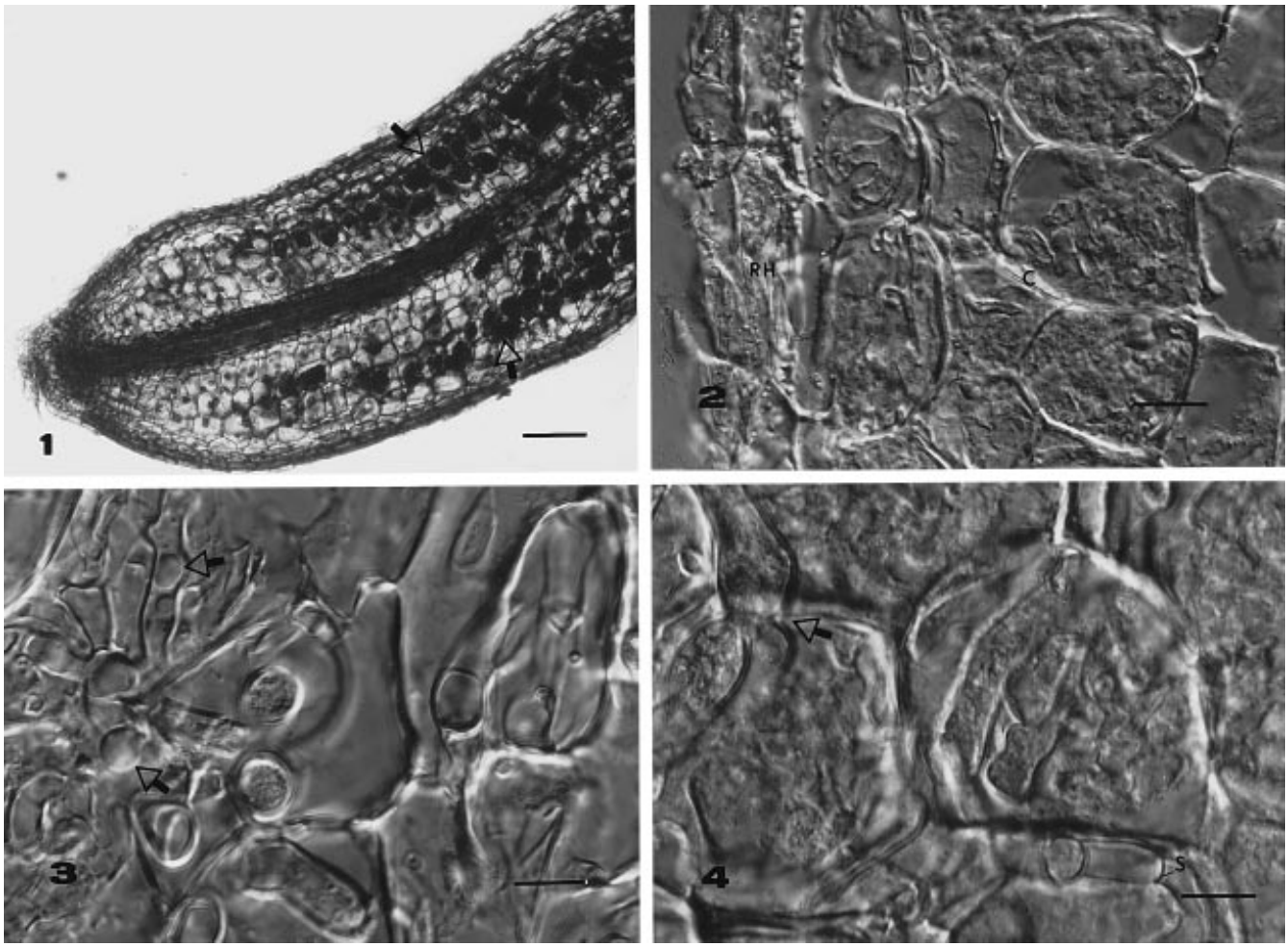

Fig. 1 Longitudinal section of black locust root stained with aniline-blue. Root hairs are missing. The root apex is free from infection. Hyphae colonize the cortex cells (*arrows*) of the proximal zone. Bright field microscopy; *bar* 100 μm

Fig. 2 Root cortex cells colonized by hyphae. Some branching cellular hyphae entirely fill the cells. The rhizodermis is not colonized. Nomarski optics (*RH* rhizodermis, *C* cortical cell with cellular hyphae); *bar* 20 μm

Fig. 3 Finger-like branching of intercellular hyphae in colonized cortical cells. Lipid droplets of hyphae can be seen (*arrows*). Nomarski optics; *bar* 10 μm

Fig. 4 Invading hyphae penetrating the cell wall of cortical cells (*arrow*). Nomarski optics (*S* septum); *bar* 10 μm

manent sections were stained with 1% aniline-blue dissolved in Amman's solution (lactic acid, glycerol, phenol, distilled water 2:4:2:2).

Transmission electron microscopy

Fixed roots were post-fixed in 1% osmium tetroxide, washed in 0.07 M phosphate buffer (pH 7.2), dehydrated stepwise in ethanol (25%, 50%, 75%, 90%, 96%, abs.) and propylene oxide. Samples were embedded in Durcupan and polymerized at 60 °C. Ultra-thin sections were stained for 4 min with 5% uranyl acetate dissolved in methanol and for 6 min with 2.6% lead citrate. Sections were examined using a TESLA BS 500 electron microscope at 60 kV.

Infection of sterile black locust plantlets

Micropropagated, sterile plantlets of black locust obtained by shoot multiplication were transferred to pots containing sterilized soil of the original habitat. Sterile mycelium cultures of *T. terfezioides* were isolated from fruitbodies by cutting out small pieces from their middle section under sterile conditions and transferring to Hagem's-Modess agar plates. Inocultaion of 5-cm-high plantlets with two different fungal isolates (P_{60} and P_{83}, from Örbottyán and Horány, respectively) was repeated five times using sterile mycelial discs 5 mm in diameter. The plants were grown for 40–80 days at 22–26 °C illuminated by daylight lamps (75 W/m^2) and regularly watered with sterile Hoagland solution. Samples of the roots were taken weekly, fixed and sectioned as described above for studying colonization. Control plants without inoculation were kept under the same conditions.

Results

Mycorrhizae between black locust and *T. terfezioides* were seen in about 80% of the root samples collected from natural truffle beds and in about 20% of artificially infected roots 2 months after infection. Colonized root tips were slightly swollen compared with uncolonized ones and root hairs were absent from colonized roots. Colonization by the fungus was seen in cryosections, unstained or stained with aniline-blue, by light

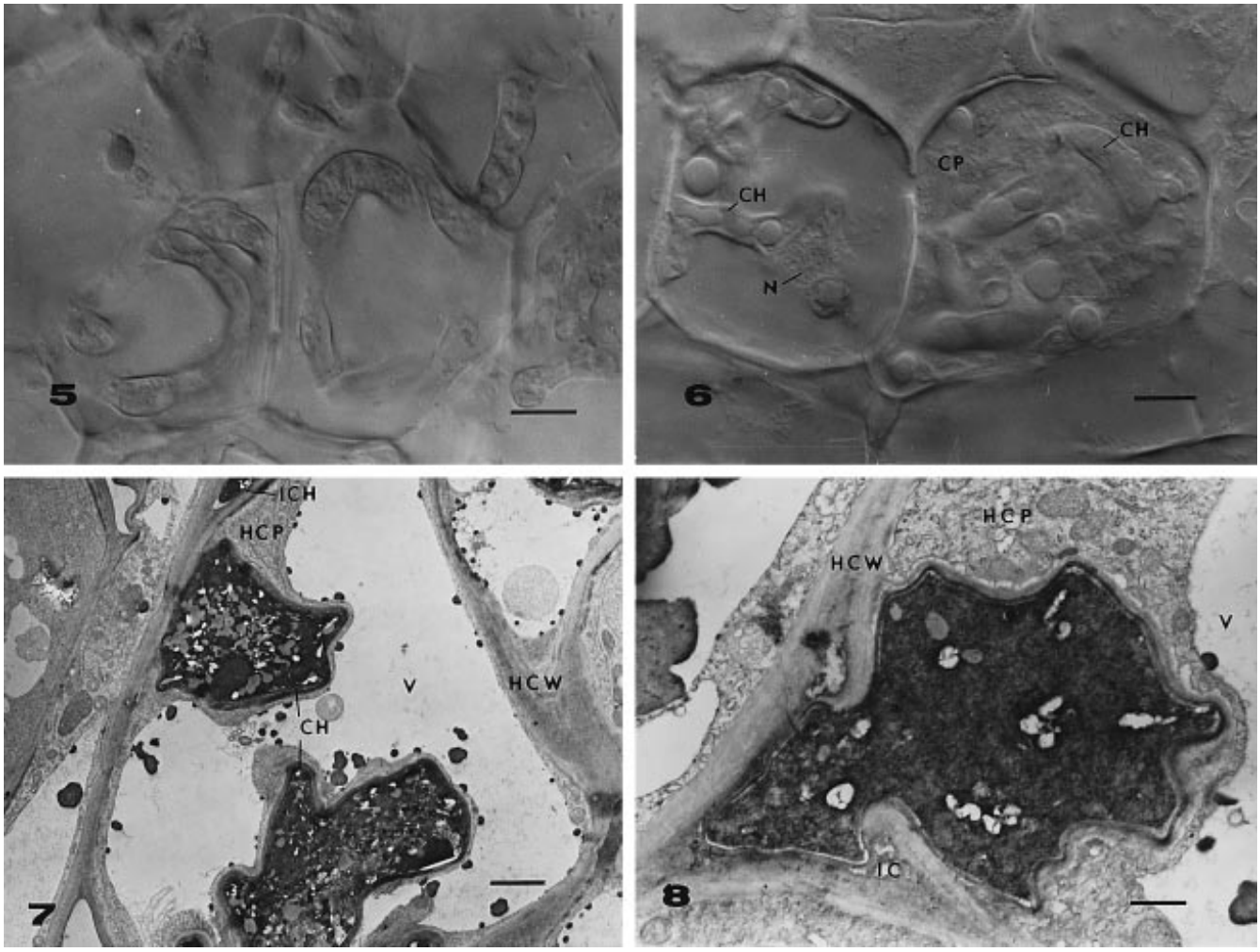

Fig. 5 Early forms of colonization. Occasional cellular hyphae hug the inside of the cortex cell walls. Nomarski optics; *bar* 10 μm

Fig. 6 Cytoplasm and nucleus of colonized plant cells (*CH* cellular hyphae, *CP* cytoplasm of host cell, *N* nucleus of the host cell); *bar* 10 μm

Fig. 7 Electronmicrograph of colonized cortical cells with intracellular and intercellular hyphae. Electron-dense intracellular hyphae are surrounded by the cytoplasm of the host cell (*CH* intracellular hypha, *ICH* intercellular hypha, *HCP* host cytoplasm, *HCW* host cell wall, *V* vacuole); *bar* 2 μm

Fig. 8 Intercellular hypha penetrates the plant cell wall and enters the cell. The cytoplasm of the host cell containing organelles seems to be normal (*HCP* host cytoplasm, *HCW* host cell wall, *IC* intercellular space, *V* vacuole); *bar* 1 μm

microscopy. Neither a mantle nor a Hartig net was detectable. The hyphae of the fungus invaded the cortical cells of the locust root and formed branches; about 2.5–3 mm of the distal part of the root was infected and root apices were free of the fungus (Fig. 1). The first hyphae appeared in the cortical cells 450–500 μm from the root tip in the zone where primary xylem elements differentiate. In these cells, the average width of the hyphae was 6–8 μm. Early stages of infection could be seen near the root tip, where some inner cortical cells were colonized by slightly moniliform hyphae hugging the inside surface of the cell wall (Fig. 5). The hyphae were septate (Fig. 4) without clamps and formed finger-like branches (Fig. 3). The rhizodermis and the hypodermal cortex cell layers in this zone were free of hyphae, and only occasional intercellular hyphae entering from the surface of the root were observed. Fungal hyphae did not invade the central cylinder.

Further from the root tip, the number of infected cells was higher, the branching of invading hyphae denser, and hyphae almost entirely filled the cortical cells (Figs. 2, 4). Under Nomarski optics, dense cytoplasm and lipid droplets in the hyphae as well as the cytoplasm of the plant cell could clearly be seen (Fig. 6). In the proximal zone, some degeneration of the old, branched hyphae was observed. In sections examined by TEM, no sign of destruction of the infected cortex cells could be detected. The cytoplasm, endoplasmatic reticulum and ribosomes of the plant cells surrounding the invading hyphae appeared to be normal. Hyphae inside the cells were highly electron dense and few cell organelles were apparent (Figs. 7, 8).

Artificial infection of micropropagated black locust plants by *T. terfezioides* was successful. Invasion of *Terfezia* hyphae was detected after 6–8 weeks in the cortical cells of the roots. The hyphae seen in sections of artificially infected roots were morphologically similar to those of naturally colonized roots.

Discussion

We observed atypical mycorrhizae formed by *T. terfezioides* with black locust, similar to those described by Dexheimer et al. (1985) between *Helianthemum salicifolium* and *T. claveryi* and defined as a type of endomycorrhiza. The morphological characteristics of the invading hyphae in the cortical cells of the black locust roots also resembled those observed by Leduc et al. (1986) in roots of *Helianthemum* and *Cistus* infected by the desert truffles *Terfezia* and *Tirmania*. These authors found the intracellular type of mycorrhizae in plants growing under phosphorus-poor conditions. In phosphorus-rich soils, the same hypogeous fungi formed a Hartig net in *Helianthemum* roots but without any mantle (Fortas and Chevalier 1992). They were defined as "ectendo-mycorrhiza". Thus, depending on the nutritional conditions, white truffles form different kinds of mycorrhizae. According to Roth-Bejerano et al. (1990), the establishment of an *Helianthemum-Terfezia* association on agar media can be stimulated by addition of activated charcoal, which, because of its absorption properties creates nutrient-poor conditions.

Our investigations indicate the existence of a natural endomycorrhizal relation between black locust and *T. terfezioides* different from the VA type, as neither vesicles nor arbuscules were seen and intracellular hyphae were septate. Artificial infection of micropropagated black locust plantlets with mycelium of the isolated fungus were morphologically identical to those obtained from truffle-beds, confirming the existence of this type pf mycorrhiza.

Although nothing is known about the physiological background of the endomycorrhizal assocation between black locust and *T. terfezioides*, a mutualistic relation can be supposed since no signs of parasitism were observed in TEM investigations. Degeneration of hyphae in the proximal region of the infected root zone may reflect a kind of digestion of the hyphae by the cortical cells, similar to that characteristic of degenerating pelotons in orchidaceous mycorrhizae (Harley and Smith 1984) and fungi in the Gentianaceae (Stahl 1900; Neumann 1934).

The mycorrhizal relation between *R. pseudoacacia* and *T. terfezioides* raises the question of whether these two species arrived in Europe from America together or whether this truffle is native to Middle Europe. *T. terfezioides* is unknown in North America, where only its close relative, *T. spinosa* Hk., is found; however, it is not entirely certain that these two species differ (J.M. Trappe, personal communication).

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