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STUDIES ON THE ROOT ASSOCIATIONS OF THE TRUFFLE *TERFEZIA TERFEZIOIDES*

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The paper contains an overview of the results of the studies made on the truffle *Terfezia terfezioides*, particularly the investigations related to the associations of this fungus with plants. Twelve plant species originated from a natural habitat of the fungus were supposed to be connected with *T. terfezioides* based on the anatomy of the endogenous fungal structures in their roots. Aseptic experiments were carried out on modified MMN substrates with different phosphate concentrations to study the interaction of *T. terfezioides* with *Robinia pseudoacacia* and *Helianthemum ovatum*. The colonization of the roots of black locust was always weaker than that of *Helianthemum*. The main characteristics were the intracellular coiled, branched, frequently septated hyphae in dead root cells. The intercellular hyphae formed Hartig-net with finger like structures only in *Helianthemum*. the interactions could not be considered unambiguously as mycorrhizae. There was no difference between the RFLP profiles of the nr DNA ITS of nineteen fruit bodies collected at the same time from the habitat and the ITS of three randomly chosen specimens were identical on sequence level, too. These invariability makes to design species specific PCR primers possible to check unambiguously the host plants.

Keywords: *Terfezia terfezioides*, host plants, *in vitro* associations, ITS

Introduction

Terfezia terfezioides (Ascomycota, Pezizales) is a hypogeous fungus described first time from Italy [1] and deposed into the genus *Terfezia* only 64 years later [2]. Since the first report the truffle was found many times in Europe mostly in the

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catchment area of the Danube river [3, 4, 5, 6]. The characteristic natural habitat of the fungus is the mixed black locust forests on sandy soils [3, 4, 6].

The mycorrhizal characteristics of other *Terfezia* species have been widely studied particularly because of their market value [7]. The natural mycorrhizal plant partners of the genus usually belong to the family Cistaceae [7, 8]. Some *in vitro* mycorrhizal systems of *Terfezia* species were used to study the effect of the substrate on the mycorrhizae [9] or the effect of drought stress on mycorrhized plants [10]. Although many data – however sometimes misleading results – have been published about the plant connections of other *Terfezia* species, the knowledge about the root interactions of *T. terfezioides* is insufficient. After some suggested host plants [4] only the black locust (*Robinia pseudoacacia*) were published as mycorrhizal partner of the truffle [11]. The main aim of the field works of our studies was to obtain data about the probably host plants of *T. terfezioides* based on the endogenous fungal structures of the roots of the plants collected from one new habitat of the truffle. On the other hand sterile strains of the fungus were isolated from fruit bodies and this culture was used to study *in vitro* the features of the root associations with *R. pseudoacacia* and *Helianthemum ovatum*. The former was chosen to obtain more reliable data about the interaction the latter was chosen to check whether the truffle is compatible with this plant which is from the family Cistaceae and originated from a semi-desert habitat resembling the habitats of other *Terfezia* species.

The internal transcribed spacer (ITS) region of the nuclear ribosomal genes has been commonly used to identify fungal partners in mycorrhizal connections [12, 13, 14] since specific PCR primers of the region and their PCR reaction conditions were designed [15, 16, 17, 18]. The mycorrhizal partner of *T. pfeilii* was also identified with the help of that molecular marker [19]. This and also other studies of the ITS region of different *Terfezia* species documented a strange intraspecific – moreover intrahifal – variability of the DNA region revealing in RFLP profiles and in sequences, too [19, 20, 21, 22]. This variability made reasonable the study of the variability of the ITS of *T. terfezioides* within one habitat to check whether the region was reliable enough to design species specific primers for further studies of the host plants of the fungus.

Material and methods

The area from where the root samples for natural host study and the fruit bodies of *T. terfezioides* for strain isolation and molecular studies originated lies on the Great Hungarian Plain next to Kunfehértó.

The root samples were collected between October 1999 and October 2000. For microscopical investigations the samples were prepared following the methods of

Grace and Stribley [23] with small modifications. The rootlets were cleared with KOH, stained with lactic acid-aniline blue, and covered in lactic acid. The detailed procedure can be found in Kovács and Bagi [24]. One plant was considered as associated with *T. terfezioides* if endogenous fungal structures with the main features of the *Terfezia mycorrhizae* were found in its roots.

The sterile strain of the truffle was isolated from ascocarps in October 1999 and has been kept on modified MMN medium (250 mg/L $(NH_4)_2HPO_4$, 500 mg/L K_2HPO_4, 150 mg/L $MgSO_4 \times 7\ H_2O$, 50 mg/L $CaCl_2 \times 2\ H_2O$, 25 mg/L NaCl, 20 mg/L FeEDTA, 10 g/L glucose, 3 g/L maltose, 10 g/L agar, 2 ml/L Wickerham's vitamin, streptomycin, the pH adjusted to 8 before autoclaving) in the dark at 25°C. The sterile plantlets of *R. pseudoacacia* and *H. ovatum* originated from seeds. The plantlets were inoculated with mycelium in Petri dishes on MMN with different phosphate content, 12.5, 25, 50, 100 and 200% of the original phosphate concentration of the substrate. The dishes with plants were left to grow in conditioned chamber for 28–32 days. Semi-thin (0.5–0.8 μm) longitudinal sections were cut for light microscopy and stained with neofuchsin-crystal violet and ultra-thin (60–75 nm) longitudinal sections were cut for transelectron microscopy, stained with uranyl acetate and contrasted with lead citrate. For detailed description of the culture conditions and microscopical preparation see Kovács et al. [25].

For DNA investigation 19 ascocarp collected in the same time were used. The DNA was extracted from desiccated fruit bodies according to Gardes et al. [16] with slight modifications. The amplification of the ITS region was carried out with ITS1F and ITS4 primer pair according to Gardes and Bruns [18]. The RFLP analysis of the ITS was made using C*fo*I, *Eco*RI, *Hinf*I and *Xho*I enzymes and the digestion products were analysed by electrophoresis on agarose gel. The direct sequencing of the PCR products was carried out with the primers used for amplification. The detailed procedure can be found in Kovács et al. [26].

Results and discussion

Roots of forty-nine plant species were studied collected from the habitat of *T. terfezioides*. Based on the anatomical structures twelve of them were supposed to be connected with the truffle, such as *Anthriscus sylvestris, Brachypodium sylvaticum, Celtis occidentalis, Clinopodium vugare, Crataegus monogyna, Euonymus europaeus, Glechoma hirsuta, Muscari racemosum, Robinia pseudoacacia, Rubus caesius, Ulmus minor* and *Viola odorata*. The previously reported habitats of the fungus were always *Robinia* forests [3, 4]. However, *Robinia* is not mentioned in the description of the environment of the holotype [see in 3]. The hypothesized partner species of *Terfezia*

are frequent plants in mixed black locust forests. The herbaceous species, e.g. *B. sylvaticum, V. odorata* or *G. hirsuta* are common underwood formers in Robinietum plant communities, just like some of the woody plants, e.g. *C. occidentalis*, *C. monogyna, P. spinosa* or *Ulmus* species. *C. occidentalis* is mentioned almost in all descriptions of the habitats of *T. terfezioides* [3] and *C. monogyna* was the most frequent shrub found in plant coenological studies together with this fungus [4].

With the knowledge of the results of the in vitro synthesized associations of the truffle the previous consideration of the "mycorrhizal partners" of *T. terfezioides* [24] should be questioned. Both the inoculated *H. ovatum* and *R. pseudoacacia* plantlets showed no difference compared with the control ones during the period of the experiments. The anatomical features of the colonisations showed the characteristics of mycorrhizae of other *Terfezia* species [8, 19, 27] like the intracellular coiled, branched, frequently septated hyphae. There were significant differences between the colonization level of the roots of the two plant species at all the used phosphate concentrations. The colonization of *H. ovatum* was always stronger, e.g. at the lowest phosphate level the roots of *Robinia* were not colonized while the roots of *Helianthemum* were. The colonization of both plants proved to be extremely strong in case of *Helianthemum* at the 100% phosphate level, in case of *Robinia* only at the highest concentration. The phosphate concentration had strong effect on the synthesized mycorrhizae (the studied truffles formed ectomycorrhizae with Hartig-net without sheath at high phosphate content and ectendomycorrhizae without sheath with Hartig-net and with coiling intracellular hyphae at lower phosphate concentration) of *H. guttatum* with *T. arenaria* and *T. claveryi* [9], such odd influence was not detected. All the colonized cells were dead and defence reaction of the host cells were frequently observed on the plant cell walls. Finger-like structures, the most reliable features of the Hartig-net [28] were found only in the roots of *Helianthemum*. The comparison with previously published *Terfezia* root associations is rendered difficult, not just by the taxonomical differences, but the insufficient, unreliable documentations of the works [11, 29] or the different aims of the studies [30]. Moreover, the interactions of *T. terfezioides* studied in the experiments presented here could not be considered unambiguously as mycorrhizae. Further *in vitro* experiments should be carried out to clear the character of the interactions of *T. terfezioides* with plants. This interaction could be not clear mycorrhizal which is not unique among ascomyceteous fungi.

Although some *Terfezia* species show intraspeciefic variability in their ITS sequences [19, 20, 21, 22], the RFLP profiles of the ITS region of the nineteen studied fruitbodies were the same. *Eco*RI has not digested the amplified region (740 bp approx.), while C*fo*I, *Hinf*I and *Xho*I digested the region into 2 (400 and 300 bp approx.), 3 (340, 190 and 145 bp approx) and 2 (555 and 185 bp approx.) detectable

bands, respectively. The ITS sequences of the randomly chosen three samples were also identical (Gene bank AJ 305169, AJ 306555, AJ 306556). Based on the results not just the certain RFLP profiles could help to find the natural hosts of *Terfezia terfezioides*, but such a conservative genetic marker makes possible the design of species-specific PCR primers like in the case of ascomycetous *Tuber melanosporum T. brumale* and *T. indicum* [31] and *T. borchii* [32].

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