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The ascomycete *Meliniomyces variabilis* isolated from a sporocarp of *Hydnotrya tulasnei* (*Pezizales*) intracellularly colonises roots of ecto- and ericoid mycorrhizal host plants

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Attempts to isolate the ascomycete *Hydnotrya tulasnei* from fresh hypogeous sporocarps into a pure culture yielded beside *H. tulasnei* also a strain of *Meliniomyces variabilis* (MVA-2). Both phenotype and genotype analyses showed that MVA-2 was highly similar to another *M. variabilis* strain, isolated previously from a root tip of *Picea abies*. The mycorrhizal potential of both *H. tulasnei* and *M. variabilis* is still dubious. Therefore, we tried an in vitro synthesis of root-fungus association between *H. tulasnei*, both *M. variabilis* strains and typically ectomycorrhizal (*P. abies* and *Pinus sylvestris*) and typically ericoid mycorrhizal (*Vaccinium corymbosum*) host plants. For comparison, a strain of *Rhizoscyphus ericae* was included. Both *M. variabilis* strains formed intracellular structures characteristic of ericoid mycorrhiza in *V. corymbosum* roots, and also colonised the roots of *P. abies* and *P. sylvestris* seedlings, modifying their morphology. Superficially, *Picea* and *Pinus* root tips resembled early stages of EcM development, but transversal sections revealed absence of the Hartig net and frequent intracellular colonisation of the cortex. The reference strain of *R. ericae* showed similar behaviour in *Picea*, *Pinus* and *Vaccinium* roots, only the intracellular colonisation was more intensive and morphology of roots of both conifers was less changed when compared to *M. variabilis*. *H. tulasnei* failed to colonise the roots of *P. abies* and *V. corymbosum*, possibly due to sub-optimal conditions for its growth.

Key words: ericoid mycorrhiza, ectomycorrhiza, Variable White Taxon, *Rhizoscyphus ericae* (= *Hymenoscyphus ericae*) aggregate

Vohník M., Fendrych M., Kolařík M., Gryndler M., Hršelová H., Albrechtová J. a Vosátka M. (2007): Askomycet *Meliniomyces variabilis* izolovaný z plodnice druhu *Hydnotrya tulasnei* (*Pezizales*) vnitrobuněčně kolonizuje kořeny typicky ektomykorhizních a erikoidně mykorhizních rostlin. – Czech Mycol. 59(2): 215–226.

Při pokusech o aseptickou izolaci mycelia z čerstvých podzemních plodnic *Hydnotrya tulasnei* byl také získán kmen houby *Meliniomyces variabilis* (MVA-2). Fenotypová a genotypová analýza ukázala, že tento kmen je blízce příbuzný jinému kmeni *M. variabilis*, izolovanému z kořenové špičky smrku ztepilého (*Picea abies*). Protože je mykorhizní potenciál *H. tulasnei* a *M. variabilis* stále nejasný, použili jsme získané kmeny pro aseptickou inokulaci kořenů typicky ektomykorhizních (smrk ztepilý a borovice lesní) a erikoidně mykorhizních [brusnice chocholičnatá (*Vaccinium corymbosum*)] hostitelských rostlin. Pro srovnání jsme také zkoumali typicky erikoidně mykorhizní houbu *Rhizoscyphus ericae*. Oba kmeny *M. variabilis* v kořenech brusnice tvořily vnitrobuněčné struktury charakteristické pro erikoidní mykorhizu. Oba kmeny také kolonizovaly kořeny smrku a borovice, jejichž kořenové špičky vzhledem připomínaly časná stádia vývoje ektomykorhizy. Z příčných řezů však bylo patrné, že u nich není vyvinuta mezibuněčná Hartigova síť. Naopak, primární kůra kořenových špiček byla houbovými hyfami kolonizována vnitrobuněčně. Referenční kmen *R. ericae* se choval podobně, jím způsobená vnitrobuněčná kolonizace však byla intenzivnější a morfologie kolonizovaných kořenů smrku a borovice byla modifikována méně než v případě *M. variabilis*. *H. tulasnei* nebyla schopna kolonizovat kořeny experimentálních rostlin, pravděpodobně díky nevhodným kultivačním podmínkám.

INTRODUCTION

The genus *Hydnotrya* (*Pezizales*, *Discinaceae*) comprises ascomycetes with hypogeous sporocarps (Montecchi and Sarasini 2000), which are suspected to be ectomycorrhizal (Newton and Haigh 1998, Tedersoo et al. 2006). However, the mycorrhizal status and host plant preferences of *Hydnotrya tulasnei* Berk. & Br. are still unclear. Tedersoo et al. (2006) molecularly detected *H. tulasnei* in root samples from a mixed forest (*Picea abies*, *Tilia cordata*, *Betula pendula*, *Populus tremula*) in Estonia and described a putative *H. tulasnei* morphotype. However, the authors detected neither the exact host species (except beech at two localities in Denmark) nor isolated *H. tulasnei* into a pure culture. To our knowledge, there are no reports about an in vitro re-synthesis of ectomycorrhiza (EcM) using *H. tulasnei*.

In August 2004 and 2005, two *H. tulasnei* sporocarps were found in a mixed forest in northern Bohemia and in a coniferous forest in southern Bohemia, respectively. An attempt was made to isolate *H. tulasnei* from both sporocarps into a pure culture for re-synthesis. However, each of the sporocarps yielded a different mycelium. One isolate morphologically resembled *Meliniomyces variabilis* Hambleton & Sigler, formerly known as Variable White Taxon (Hambleton and Currah 1997), the other was the putative *H. tulasnei*.

M. variabilis belongs to the *Hymenoscyphus ericae* (Read) Korf & Kernan (= *Rhizoscyphus ericae* (Read) Zhuang & Korf) aggregate (Vrålstad et al. 2000),

which comprises fungi forming ericoid mycorrhiza (ErM) and/or EcM (Vrålstad et al. 2002a). Although *M. variabilis* is commonly isolated from roots of ericaceous, broad-leaved and coniferous hosts, its mycorrhizal status is still dubious (Hambleton and Sigler 2005 and references therein). Based on re-synthesis tests, where it formed intracellular loops in rhizodermal cells of ericaceous host plants, it was suspected to be an ericoid mycorrhizal symbiont (Piercey et al. 2002, Berch et al. 2002).

Because the mycorrhizal status and host preferences of both *H. tulasnei* and *M. variabilis* are obscure, we conducted a study aiming 1) to identify both fungal isolates obtained from *H. tulasnei* sporocarps, and 2) to elucidate their mycorrhizal potential by testing their ability to colonise roots of typically ectomycorrhizal (*P. abies* and *Pinus sylvestris*) and ericoid mycorrhizal (*Vaccinium corymbosum*) plant hosts.

MATERIALS AND METHODS

Isolation of fungal strains

Two *H. tulasnei* sporocarps were subjected to isolation of the fungal mycelium. The first was collected in August 2004 in the Táborsko region, southern Bohemia (CZ), in a forest dominated by *Picea abies*. The second was collected in August 2005 in a mixed forest between the villages Držkov and Zásada near Jablonec nad Nisou, northern Bohemia (CZ). Both sporocarps were identified based on typical macro- and micro-morphological characteristics according to Montecchi and Sarasini (2000). The specimens were deposited in the mycological herbarium of the National Museum, Prague (PRM). For additional information, see Tab. 1.

Small pieces of fungal tissue were aseptically extracted from the internal part of each sporocarp and transferred into Petri dishes with standard MS medium (Murashige and Skoog 1962) containing 0.16 mg IAA, 0.04 mg kinetin and 0.8 mg IBA per litre as growth regulators. The dishes were Parafilm™-sealed and cultivated at room temperature in the dark. Each sporocarp produced only one distinct type of fungal mycelium, differing in growth rates and colony morphology. In the following text, the isolate from the first sporocarp is referred to as MVA-2, the second sporocarp as HTU-1.

The *M. variabilis* strain MVA-1, in morphology similar to MVA-2, was also included in this study. It was isolated in September 2003 from a root tip of *P. abies* seedling from a *P. abies* stand at Modrava, Šumava National Park, southern Bohemia (M. Vohník, unpublished data). The isolation procedure included three washings of root tips (which superficially resembled EcM tips) in sterile water followed by a 10 min. washing in 10 % SAVO (household bleach containing active chlorine) and two washings in sterile water. The root tips were then placed on PDA (39 $g \cdot l^{-1}$, Fluka) and cultivated at room temperature in the dark. MVA-1 was deposited in the Culture Collection of Fungi (CCF; Faculty of Science, Charles University, Prague) and its ITS sequence was deposited in GenBank (Tab. 1). The ITS sequence (549 bp) of MVA-1 showed 99 % similarity with 522 bp of *M. variabilis* UAMH 8864 (= GenBank AY838789).

As a reference ErM fungus from the *R. ericae* aggregate, we included a strain (= RER-1) of *R. ericae* from Pearson and Read (1973).

Tab. 1. Additional data about the fungal strains used in this study and their interaction with the roots of three host species. **HTU-1** = *Hydnotrya tulasnei*; **MVA-1** and **MVA-2** = *Meliniomyces variabilis*; **RER-1** = *Rhizoscyphus ericae*; **PRM** = Herbarium Mycologicum Musei Nationalis Pragae, Prague, CZ; **CCF** = Culture Collection of Fungi, Prague, CZ; "–" = no interaction; **NT** = not tested; **INT** = intracellular colonisation; **ErM** = formation of ericoid mycorrhiza; **NA** = not available.

Isolate	**Source of isolate**	**Date and location**	**Sporocarp specimen accession number**	**Culture accession**	**GenBank accession number**	***Picea abies***	***Pinus sylvestris***	***Vaccinium corymbosum***
HTU-1	sporocarp of *Hydnotrya tulasnei*	August 2004; *Picea abies* – dominated forest, Táborsko region, S Bohemia (CZ)	PRM 902032	Available from M. Gryndler	AM261522	–	NT	–
MVA-1	root tip of a *Picea abies* seedling	September 2003; *Picea abies* stand, Modrava, Šumava NP, S Bohemia (CZ)	NA	CCF 3583	AM261523	INT	INT	ErM
MVA-2	sporocarp of *Hydnotrya tulasnei*	August 2005; mixed forest between Držkov and Zásada near Jablonec n. N., N Bohemia (CZ)	PRM 905514	Available from M. Gryndler	AM261524	INT	INT	ErM
RER-1	*Calluna vulgaris* hair root	Pearson and Read, 1973	NA	UAMH 6735	AJ319078	INT	INT	ErM

Identification of fungal strains

The mycelium of HTU-1 and MVA-2 was scraped with a lancet from margins of colonies actively growing on PDA (MVA-2) or half-strength PDA (HTU-1) and processed with a Mo-Bio UltraClean™ Microbial DNA Isolation Kit following the manufacturer's instructions. The isolated DNA was amplified using ITS1 and ITS4 primers according to Kolařík et al. (2004) and sequenced in the DNA sequencing laboratory, Faculty of Science, Charles University in Prague, using a BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI 3100 Genetic Analyser, following the manufacturer's instructions. The obtained ITS1-5.8SrDNA-ITS2 sequences were compared with published sequences using the BLAST 2.1 similarity search (Altschul et al. 1997). BioEdit 7.0.4.1 (Hall 1999) was used for comparison and alignment of sequences.

Aseptic synthesis: *Meliniomyces variabilis* and *Rhizoscyphus ericae*

One compartment of each split Petri dish was filled with MMN and the other was left empty. Despite a relatively high content of glucose, MMN proved to be a suitable medium for ErM and EcM synthesis with members of the *R. ericae* aggregate in our previous experiments (M. Vohník, unpublished data). Also, there was no qualitative or quantitative difference with respect to ErM and EcM formation between MMN and 10× diluted MMN (1 g of glucose per litre). After solidification, the medium was overlaid with a sterile cellophane membrane to prevent growth of roots into the medium. The central septum of each dish was perforated to allow insertion of experimental plants. Agar plugs obtained from margins of actively growing fungal colonies were transferred into each dish and cultivated for one month at room temperature in the dark. After this period, one aseptic experimental plant was transferred into each dish in such a way that its roots were laid on the surface of the fungal colonies and the shoots were placed in the empty compartments. There were three sets of dishes (with *P. abies*, *P. sylvestris* and *V. corymbosum*), each containing three dishes with MVA-1, three dishes with MVA-2, three dishes with RER-1 and three non-inoculated dishes.

The roots of the experimental plants were covered with a piece of sterile moistened filter paper. The dishes were Parafilm™-sealed and placed in a vertical position into a growth chamber (16/8h day/night, 23 °C, 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$). After two (*V. corymbosum*) or three (*P. abies* and *P. sylvestris*) months, the experimental plants were extracted from the dishes and their roots were separated and processed as follows: i) *V. corymbosum* roots were cleared with 10 % KOH (20 min. at 121 °C), washed with tap water, acidified (1 min. in 3 % HCl), washed with tap water, stained with trypan blue (1 hour at 121 °C) and de-stained overnight in lacto-glycerol; ii) *P. abies* and *P. sylvestris* roots were hand sectioned and thin sections were stained with aniline blue. The stained roots and thin sections were observed using a microscope equipped with differential interference contrast optics at high magnifications (400× and 1000×). For thin sections of *P. abies* and *P. sylvestris*, epifluorescent microscopy was employed according to Cudlín (1991).

Aseptic synthesis: *Hydnotrya tulasnei*

During sub-cultivation of the isolated fungi, HTU-1 failed to grow on MMN. From the media screened (MMN, MS, PDA, half-strength PDA), HTU-1 grew best on half-strength PDA. Thus, we used this medium overlaid with a cellophane membrane or filter paper in a synthesis test. After six weeks, HTU-1 grew on half-strength PDA in parallel dishes without cellophane membranes or filter paper, but did not produce any significant growth in the dishes where membranes or filter paper were inserted. In the former case, HTU-1 produced submersed mycelium identical to the original colonies derived from the sporocarp. Apparently, cellophane membrane and filter paper prevented submersed growth of the HTU-1 mycelium, hence completely inhibiting its development. In this situation, it seemed best to insert three *P. abies* + three *Vaccinium myrtillus* seedlings directly onto the surface of the original HTU-1 colony formed on MS medium. The original HTU-1 colony was still viable and served as a source of viable mycelium for dishes without membranes or filter paper.

RESULTS

Isolation and identification of fungal strains

Two fungal strains differing in morphology and ITS sequences were isolated in this study, each from one of the *H. tulasnei* sporocarps. The isolation from the first sporocarp yielded white to yellow smooth fungal colonies with moist appearance and sharp and narrow margins, growing superficially on the medium (= MVA-2, Fig. 1a). The second sporocarp yielded slowly growing, brown to orange submersed diffuse mycelium (= HTU-1, Figs. 1c and 1d). The ITS sequence (569 bp) of MVA-2 showed 99 % similarity with 465 bp of *M. variabilis* UAMH 8864 (= GenBank AY838789) and 99 % similarity with 492 bp of MVA-1. The ITS sequence (735 bp) of HTU-1 showed 99 % similarity with 731 bp of "*Pezizales* sp. B48" (= GenBank AJ534700 = *H. tulasnei*, Tedersoo et al. 2006). Thus, genotype analysis confirmed that HTU-1, isolated from the morphologically identified *H. tulasnei* sporocarp belonged to this species and both genotype and phenotype analyses confirmed that MVA-1 and MVA-2 represented different strains of *M. variabilis*. The ITS sequences of HTU-1 and MVA-2 were deposited in GenBank (Tab. 1). HTU-1 and MVA-2 cultures are available from M. Gryndler.

Aseptic synthesis: *Meliniomyces variabilis* and *Rhizoscyphus ericae*

MVA-1, MVA-2 and RER-1 intracellularly colonized rhizodermal cells of *V. corymbosum* micro-cuttings and formed characteristic ErM structures (Fig. 2). Turgescent rhizodermal cells were filled with dense hyphal coils, and colonised parts of the root system were embedded in hyphal wefts (Fig. 3). RER-1 formed more intensive root colonisation than both *M. variabilis* strains and its intracellular hyphae were thinner, resulting in more compact loops. Colonised micro-cuttings grew and performed well, but there was no conspicuous macroscopic difference between the inoculated and non-inoculated plants.

MVA-1, MVA-2 and RER-1 also colonised *P. abies* and *P. sylvestris* roots. Root morphology of the colonised seedlings was different from the non-inoculated seedlings (Figs. 4a and 4b). When contact between the *P. abies* primary root and the fungal colony was established, numerous short lateral roots without root hairs, morphologically resembling EcM root tips, were produced (Figs. 5 and 6). In contrast, non-inoculated plants produced only few short lateral root tips covered with root hairs. Lateral roots of *P. sylvestris* inoculated with MVA-1, MVA-2 and RER-1 were often dichotomously branched, which never occurred in the non-inoculated roots. Even though a loose mantle of fungal hyphae often surrounded the lateral root tips of both *P. abies* and *P. sylvestris* (Fig. 7), cross-sections revealed intracellular colonisation of their cortex cells and absence of the Hartig net (Fig. 8). Intracellular colonisation was most intensive in the case of RER-1. How-

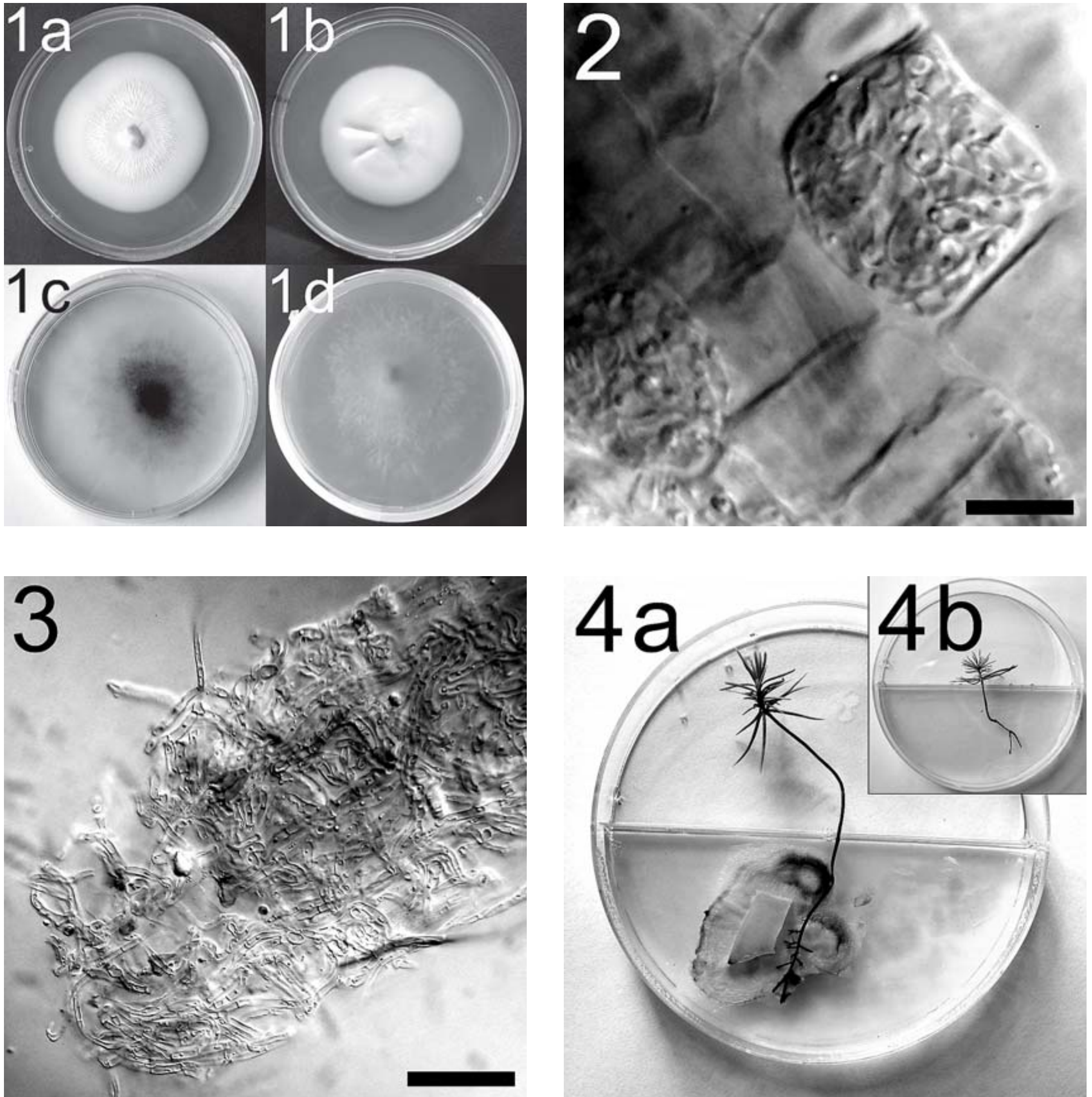
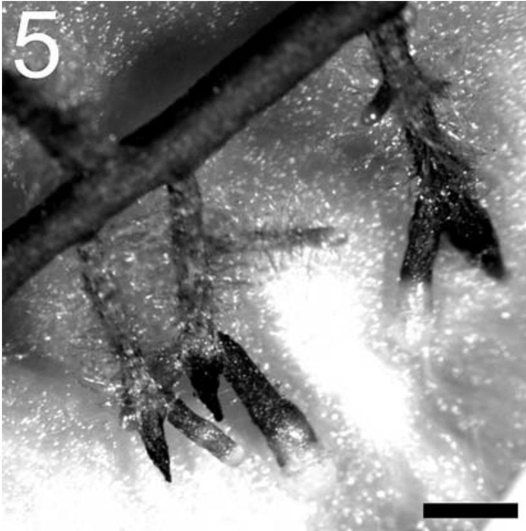
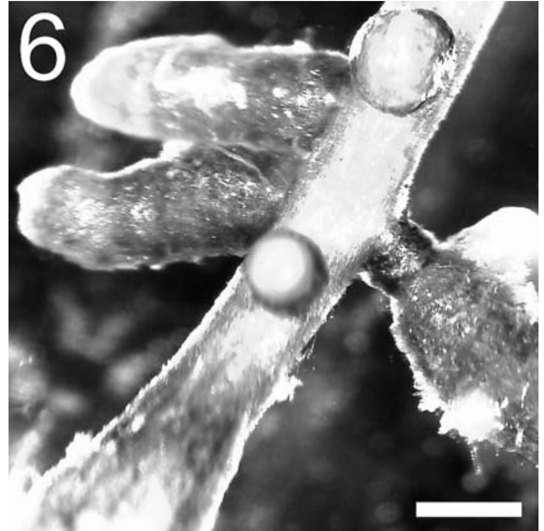
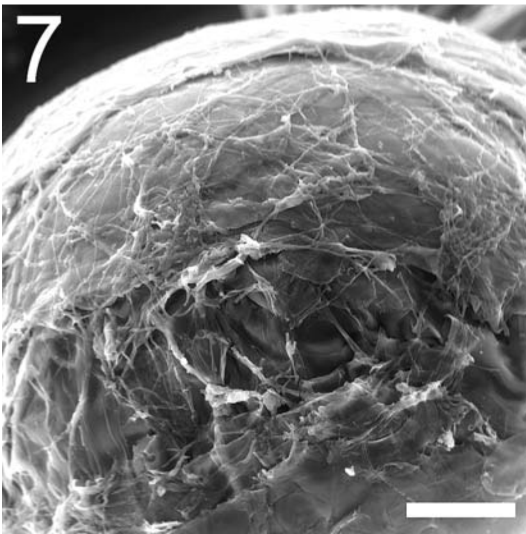


Fig. 1a. *Meliniomyces variabilis* MVA-2 after five weeks on PDA (all cultures in 9 cm Petri dishes). **Fig. 1b.** *M. variabilis* MVA-1 after five weeks on PDA. **Fig. 1c.** *Hydnotrya tulasnei* HTU-1 after three months on MS medium, view from above. **Fig. 1d.** *Hydnotrya tulasnei* HTU-1 after three months on MS medium, view from below. **Fig. 2.** Intracellular hyphal coils formed by MVA-1 in the roots of *Vaccinium corymbosum* (bar = 10 µm). **Fig. 3.** *Vaccinium corymbosum* root tip covered by the hyphal mantle of MVA-1 (bar = 50 µm). **Fig. 4a.** Experimental system – a seedling of *Picea abies* inoculated with MVA-2. **Fig. 4b.** Non-inoculated *P. abies* seedling.







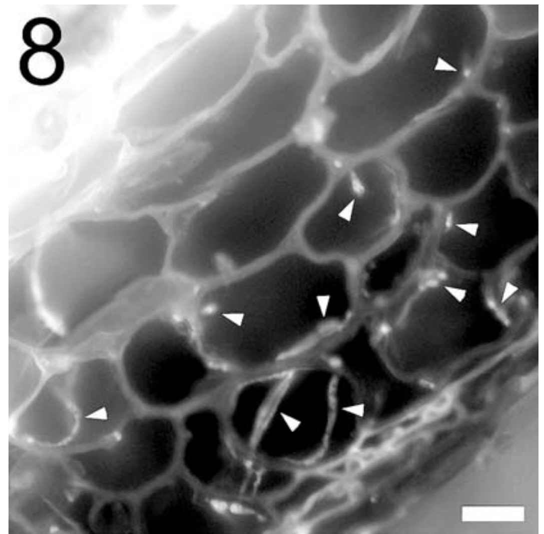


Fig. 5. The morphology of lateral *Picea abies* roots changed after contact with MVA-1 and MVA-2 had been established (bar = 2 mm). **Fig. 6.** Typical morphology of lateral *Picea abies* roots, inoculated with MVA-1 and MVA-2 (bar = 1 mm). **Fig. 7.** Surface of a tip of a lateral *Picea abies* root, covered by a loose weft of MVA-1 hyphae (bar = 100 µm). **Fig. 8.** Thin section of a lateral *Picea abies* root, showing numerous hyphae of MVA-1 (arrowheads), colonising cortical cells (bar = 10 µm).

ever, the effect of RER-1 on root morphology (the "EcM appearance") was weaker than in the case of MVA-1 and MVA-2. The seedlings of *P. abies* colonised by MVA-1 and MVA-2 grew visibly better than the non-inoculated control seedlings, indicating a possible beneficial effect of *M. variabilis* on host plants. However, due to a low number of replicates (n = 3) we did not perform a statistical analysis.

Aseptic synthesis: *Hydnotrya tulasnei*

HTU-1 failed to form EcM with *P. abies* roots. After three months, its hyphae grew around the roots of *P. abies* seedlings, but never penetrated their inter– of intracellular space. No EcM structures like a Hartig net or a developed mantle were observed. A similar situation was noticed in *V. myrtillus* seedlings, where no interaction between roots and HTU-1 was observed.

DISCUSSION

Our study is the first report on the isolation of the putative ErM/EcM fungus *M. variabilis* from the inside of a fresh young sporocarp of another putative EcM fungus, *H. tulasnei*. Significance of this finding together with possible co-existence of *H. tulasnei* and *M. variabilis* could be resolved by screening more *H. tulasnei* sporocarps. Future investigators should consider the presence of endophytic fungi (e. g. *M. variabilis*) even in fresh, young *H. tulasnei* sporocarps.

To our knowledge, this is the first report of isolation of the *H. tulasnei* mycelium into a pure culture. Bearing in mind limitations in interpreting the results of the *H. tulasnei – P. abies* re-synthesis, we can only speculate about the mycorrhizal status of *H. tulasnei*. Its resolution appears to depend especially on finding an experimental scheme respecting the demands of both *H. tulasnei* and the host plant used.

M. variabilis is frequently isolated from EcM roots, namely from the "*Piceirhiza bicolorata*" morphotype (e.g. Vrålstad et al. 2000, 2002b). Also MVA-1 was isolated from a root tip of *P. abies*, which superficially resembled EcM (see Materials and Methods). On the other hand, *M. variabilis* has to date never formed true EcM in re-synthesis tests (e. g. Vrålstad et al. 2002a, Piercey et al. 2002, this study).

The presence of MVA-1 and MVA-2 (and to a limited extent also RER-1) changed the superficial morphology of *P. abies* and *P. sylvestris* roots into the EcM appearance: lateral roots of *P. abies* could resemble early stages of *Piceirhiza bicolorata*, and *P. sylvestris* roots were dichotomously branched. However, changes in root morphology resulting in the EcM appearance are not necessarily connected with EcM formation, and do not need to be caused by EcM fungi, as shown for example by Gay (1990) in IAA-affected lateral roots of *Pinus halepensis*. This seems to be the case also in our study, because a Hartig net was

missing in the lateral roots with EcM appearance and instead, intracellular colonisation occurring across the whole cortex was present. It can be questioned whether the three-month cultivation period was sufficient for EcM establishment in our experiment. However, Vrålstad et al. (2002a) succeeded in EcM establishment with members of *R. ericae* aggregate within three months and the same period was sufficient for a Hartig net development between *P. abies* and *Cadophora finlandica*, another member of the *R. ericae* aggregate, and *Cenococcum geophilum*, a common ascomycetous EcM fungus, under an identical scheme as used in this study (M. Vohník et al., unpublished data). It is also uncommon that EcM development would start with intracellular colonisation of the cortex. Moreover, the intracellular colonisation pattern of both *M. variabilis* strains in *P. abies* and *P. sylvestris* roots resembled the pattern formed by the typical ErM fungus *R. ericae*, which has never formed EcM.

Intracellular colonisation of roots of typically EcM coniferous plants by *M. variabilis* was previously reported by Schild et al. (1988) and Piercey at al. (2002). *M. variabilis* also has the potential to intracellularly colonise roots of typically ErM plants (Berch et al. 2002, Piercey et al. 2002, this study). While the significance of intracellular colonisation in ericaceous roots can be deduced from the well-established ericoid mycorrhiza, the importance of intracellular colonisation in coniferous roots is yet unknown.

However, the apparent ability of *M. variabilis* to colonise intracellularly both typically ErM and EcM host plants, and the fact that this colonisation mode has at least no negative effect on colonised hosts (Piercey et al. 2002, this study) may have important eco-physiological consequences. Similarly to many boreal and temperate ecosystems, where ericaceous shrubs form the understorey in coniferous forests, *Vaccinium* species frequently dominate the understorey in *P. abies* stands in southern Bohemia. The hypothesized connection between ErM and EcM plants via the mycelium of a common fungus is expected to play an important role in their lives (Vrålstad 2004). *Cadophora finlandica* (Wang & Wilcox) Harr. & McNew is proposed to be a candidate for such a fungus (Vrålstad 2004), because it forms both ErM (intracellular loops) with ericaceous and EcM (the Hartig net) with coniferous plants (Villarreal-Ruiz et al. 2004). On the other hand, Piercey et al. (2002) established intracellular association between *M. variabilis* (UAMH 8863) and the typically ErM plant *Rhododendron groenlandicum* (Oeder) Kron & Judd and the typically EcM plant *Picea mariana*. A similar re-synthesis was reached with MVA-1 and MVA-2 in the roots of *P. abies*, *P. sylvestris* and *V. corymbosum* in this study. Thus, *M. variabilis* could play the same role as proposed for *C. finlandica*. To confirm this deduction, it is needed i) to screen whether the intracellular association between *M. variabilis* and coniferous plants regularly occurs at natural sites and ii) to resolve whether this association has a mutualistic character.

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