Katedra fyziologie rostlin Přírodovědecké fakulty
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Interakce v rhizosféře erikoidně mykorhizních rostlin

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Disertační práce

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OBSAH

ÚVOD DO DISERTAČNÍ PRÁCE II

1. **ČÁST I**: EKOFYZIOLOGIE ERIKOIDNÍ MYKORHIZY A DSE-ASOCIACE NA KOŘENOVÉM SYSTÉMU SPOLEČNÉHO HOSTITELE IV

1. 1. **ČÁST I**: Úvod V

1. 2. **ČLÁNEK 1**: *Morphological diversity and proportions of ericoid mycorrhiza and DSE-association in roots of European rhododendrons* X

1. 3. **ČLÁNEK 2**: *In vitro interaction between dark septate endophytic Phialocephala fortinii and ericoid mycorrhizal Rhizoscyphus ericae and its effect on the growth and colonization rates of Vaccinium myrtillus* XI

1. 4. **ČLÁNEK 3**: *The inoculation with Oidiodendron maius and Phialocephala fortinii alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in Rhododendron cv. Azurro* XII

1. 5. **ČÁST I**: Diskuse XIII

2. **ČÁST II**: KOLONIZAČNÍ POTENCIÁL *MELINIOMYCES VARIABILIS* A VYBRANÝCH EKTOMYKORHIZNÍCH A SAPROTOROFNÍCH HUB V KOŘENECH TYPICKY ERM A ECM ROSTLIN XVIII

2. 1. **ČÁST II**: Úvod XIX

2. 2. **ČLÁNEK 4**: *An ascomycete Meliniomyces variabilis isolated from a sporocarp of Hydnotrya tulasnei (Pezizales) intracellularly colonizes roots of ecto- and ericoid mycorrhizal host plants* XXVIII

2. 3. **ČLÁNEK 5**: *Interactions between Cenococcum geophilum, Geomyces pannorum, Meliniomyces variabilis and roots of Rhododendron and Vaccinium* XXIX

2. 4. **ČÁST II**: Diskuse XXX

3. **ČÁST III**: INTERAKCE MEZI ERIKOIDNĚ MYKORHIZNÍMI A DSE HOUBAMI A KRYTENKAMI XXXIII

3. 1. **ČÁST III**: Úvod XXXIV

3. 2. **ČLÁNEK 6**: *Testate Amoebae vs. Mycorrhizal Fungi: A Possible Novel Interaction in Mycorhizosphere of Ericaceous Plants?* XXXVII

3. 3. **ČÁST III**: Diskuse XXXVIII

SHRNUTÍ XLI

SUMMARY XLII

CITOVANÁ LITERATURA XLIII

Úvod do disertační práce

Tuto disertační práci (DP) jsem vypracoval v rámci doktorského studia Fyziologie rostlin na **Katedře fyziologie rostlin PřF UK**. Vlastní práce probíhala v období 2004-2006 na **Oddělení mykorhizních symbióz BÚ AVČR v Průhonicích**. Mým školitelem byl **Mgr. Miroslav Vosátka, CSc**. a konzultantkou **Doc. RNDr. Jana Albrechtová, PhD**.

Tato DP je rozdělena do tří částí:

Část I: Ekofyziologie erikoidní mykorhizy a DSE-asociace na kořenovém systému společného hostitele

Část II: Kolonizační potenciál *Meliniomyces variabilis* a vybraných ektomykorhizních a saprotrofních hub v kořenech typicky erikoidně mykorhizních a ektomykorhizních rostlin

Část III: Interakce mezi erikoidně mykorhizními a DSE houbami a krytenkami

Tyto tři části, ač se zabývají samonosnými tématy, jsou vzájemně spjaty jednak svým **vznikem**, jednak **fenoménem širokého spektra půdních (mykorhizních nebo pravděpodobně mykorhizních) hub, kolonizujících mykorhizosféru vřesovcovitých rostlin**. Každá část je opatřena úvodem, relevantními manuskripty/publikacemi a diskusí výsledků. Tato DP tedy má alternativní formu (oproti klasické ve smyslu dokumentu "Žádost o povolení obhajoby disertační práce"). Popis experimentálních metod a postupů, konkrétní výsledky, poděkování a finanční podpora jsou uvedeny samostatně v rámci jednotlivých manuskriptů/publikací. Je mou milou povinností poděkovat také nadaci "Nadání Josefa, Marie a Zdeňky Hlávkových" za částečné financování nákladů, spojených se zahraničními prezentacemi dosažených výsledků.

Část I této DP se při argumentaci opírá o tři původní práce:

Článek 1: Vohník, M., Albrechtová, J., Vosátka, M. Morphological diversity and proportions of ericoid mycorrhiza and DSE-association in roots of European rhododendrons. (manuskript)

Článek 2: Vohník, M., Albrechtová, J., Vosátka, M. In vitro interaction between dark septate endophytic *Phialocephala fortinii* and ericoid mycorrhizal *Rhizoscyphus ericae* and its effect on the growth and colonization rates of *Vaccinium myrtillus*. (manuskript)

Článek 3: Vohník, M., Albrechtová, J., Vosátka, M. 2005. The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro. *Symbiosis* 40: 87-96

Část II této DP se při argumentaci opírá o dvě původní práce:

Článek 4: Vohník, M., Fendrych, M., Kolařík, M., Gryndler, M., Hršelová, H., Albrechtová, J., Vosátka, M. An ascomycete *Meliniomyces variabilis* isolated from a sporocarp of *Hydnotrya tulasnei* (Pezizales) intracellularly colonizes roots of ecto- and ericoid mycorrhizal host plants. (manuskript)

Článek 5: Vohník, M., Fendrych, M., Albrechtová, J., Vosátka, M. Interactions between *Cenococcum geophilum*, *Geomyces pannorum*, *Meliniomyces variabilis* and roots of *Rhododendron* and *Vaccinium*. (manuskript přijat k publikaci v časopisu *Folia Microbiologica*)

Část III této DP se při argumentaci opírá o jednu původní práci:

ČLÁNEK 6: Vohník, M., Burdíková, Z., Albrechtová, J., Vosátka, M. Testate Amoebae vs. Mycorrhizal Fungi: A Possible Novel Interaction in Mycorhizosphere of Ericaceous Plants? (manuskript)

Předkládaná DP je sepsána v českém jazyce se shrnutím v angličtině. S radostí využívám pravděpodobně poslední příležitosti užít svého mateřského jazyka při sepisování práce tohoto formátu a rozsahu. Věřím, že takto bude má práce srozumitelnější českému čtenáři, který v současné době nemá k dispozici českých textů, zabývajících se zkoumanou problematikou. Doufám, že dostatečnou znalost angličtiny, která je jazykem současné vědy, jsem prokázal např. při sepisování manuskriptů výše uvedených vědeckých článků.

Cítím již v úvodu potřebu určitým způsobem vysvětlit a snad i ospravedlnit poněkud širší záběr mé DP. Proč vysvětlit? Protože její původní, vědeckou radou oboru Fyziologie rostlin schválený koncept počítal pouze se současnou Částí I. Při její realizaci však přede mnou často vyvstávaly zajímavé skutečnosti a nutkavé otázky, které přesahovaly rámec původního konceptu. Tyto otázky jsem mohl ignorovat, nebo se pokusit je zodpovědět. Proč ospravedlnit? Protože jsem zvolil druhou možnost. A to s vědomím, že tak budu muset část času a energie investovat mimo původní koncept. Je na jiných, aby objektivně zhodnotili, do jaké míry byla tato volba správná. Subjektivně mohu říci, že mne naplňovala pocity štěstí i zklamání, radosti i smutku, zdaru i zmaru - jak by vědecká práce nejspíš měla činit. A jak ostatně činí.

Abych učinil všem mi známým požadavkům zadost, prohlašuji, že jsem tuto DP ani její podstatnou část nepředložil k získání jiného nebo stejného akademického titulu. Prohlášení spoluautorů jsou připojena ve zvláštní příloze, která není součástí DP.

RNDr. Martin Vohník

V Průhonicích, 20. 12. 2006

1. ČÁST I:

Ekofyziologie erikoidní mykorhizy a DSE-asociace na kořenovém systému společného hostitele

Rhododendron ferrugineum kvetoucí v létě 2005 pod konečnou stanicí *Tramway du Mont Blanc*

1. 1. Část I: Úvod

Mykorhiza je jednou z nejvýznamnějších symbióz v rostlinné říši. Vznikla současně s přechodem rostlin na souš a odhaduje se, že je rozšířena až u 80 procent vyšších rostlin (např. Smith a Read 1997). Obecné schéma její funkce, tedy transport asimilátů z hostitelské rostliny do symbiotické houby a minerálních živin z houby do rostliny, bylo postupně rozšířeno o vliv mykorhiz na vodní provoz rostlin, zvýšenou toleranci hostitelských rostlin k vysokým koncentracím těžkých kovů a jiným (a-)biotickým stresovým faktorům, ochranu hostitelských rostlin před půdními patogeny apod. Některé studie dokazují, že druhové složení mykorhizních hub může přímo ovlivňovat druhové složení rostlin na stanovištích (např. van der Heijden a kol. 1998).

Diverzita mykorhiz je vysoká: z morfologicko-funkčního hlediska rozlišujeme několik typů endomykorhiz [z nichž arbuskulární mykorhiza (AM) představuje vůbec nejrozšířenější typ mykorhizy], dále ektomykorhizu (EcM) a několik typů ektendomykorhiz. Mezi endomykorhizní typy symbióz patří i erikoidní mykorhiza (ErM), vyskytující se u zástupců čeledi *Ericaceae*. Zvláštním typem asociace kořenů vyšších rostlin a vláknitých půdních hub je tzv. DSE-asociace, pojmenovaná podle hub (*dark septate endophytes*), které ji tvoří (Jumpponen a Trappe 1998). Na rozdíl od původních představ (např. Melin 1922) je v současné době akceptováno, že i tento typ asociace může mít, alespoň za určitých okolností, charakter symbiotický, tedy prospěšný pro oba zúčastněné partnery (Jumpponen 2001, Mandyam a Jumpponen 2005).

Během ontogeneze hostitelské rostliny nezřídka dochází k výměně nebo alespoň posunu od jednoho mykorhizního typu k jinému. Kupříkladu semenáčky některých listnatých stromů (olší, eukalyptů apod.) jsou převážně AM, kdežto dospělé rostliny jsou převážně EcM. Často je také jedna mykorhiza (ve smyslu kořene kolonizovaného symbiotickými houbami) tvořena větším počtem různých houbových symbiontů, což je zřejmé zejména v případě EcM, ale i AM. U EcM existuje časová plasticita složení houbových symbiontů na jednotlivém kořenovém systému. Tzv. *early stages* mykobionti jsou v souvislosti s vývojem habitatu, ve kterém hostitelská rostlina roste, nahrazováni tzv. *late stages* mykobionty.

Na přirozených stanovištích se vyskytují rostliny i houby stejných či různých mykorhizních preferencí souběžně a vzájemně spolu interagují. Na kořenech jednotlivých mykorhizních rostlin pak můžeme nalézt **(i)** pouze jeden mykorhizní typ nebo **(ii)** souběžně několik mykorhizních typů (AM + EcM, EcM + ektendomykorhiza, AM + DSE-asociace apod.). Uvědomíme-li si však skutečnost, že DSE houby jsou rozšířeny kosmopolitně a mají schopnost kolonizovat kořeny většiny vyšších rostlin, nezbývá než konstatovat, že varianta (i) je méně pravděpodobná. Příkladem rostlin, u kterých bylo pozorováno více různých typů mykorhiz, jsou zástupci čeledi *Ericaceae*, na/v jejichž kořenech byly kromě typické ErM pozorovány i DSE-asociace, AM a EcM.

Na kořenech vřesovcovitých rostlin odebraných z přirozených stanovišť se vyskytuje především ErM. Přítomnost DSE-asociace je dokumentována spořeji, což dle mého názoru může mít několik důvodů (viz diskuse Část I). Přesto se zdá, že DSE jsou v přírodě s kořeny

vřesovcovitých běžně asociováni (viz http://www.k-state.edu/biology/bio/faculty/jumpponen/species_table.htm)

Přítomnost AM u vřesovcovitých je dokumentována zejména prací Chaurasia a kol. (2005), zabývající se mykobionty kořenů Himalájských rododendronů. Ačkoliv autoři v kořenech nalezli jen vezikuly (byť v hojné míře: řádově desítky vezikul na cm^{-1}, uváděná kolonizace 28 – 42% kořenové délky), označili pozorovanou kolonizaci za AM. Ta je však charakterizována zejména přítomností arbuskulí (např. Smith a Smith 1990). Autoři se pouze okrajově zmiňují o očekávatelné přítomnosti ErM, naproti tomu uvádějí přítomnost DS hyf u dvou druhů rododendronů. Netroufám si odhadnout ekofyziologický význam takto vysokého výskytu vezikul v kořenech vřesovcovitých. Může jít např. o projev efektu chůvy (*nurse effect*), způsobeného okolními AM rostlinami, možná o záměnu kořenů rododendronů s okolními rostlinami. Z vlastní zkušenosti vím, že kořeny rododendronů jsou často propleteny s kořeny sousedících AM rostlin, což velmi ztěžuje jejich identifikaci. Ta se pak většinou opírá o morfologii kořenů – zejména o přítomnost/nepřítomnost kořenových vlásků, velikost kořenů apod. Poměrně malý vzorek kořenů himalájských rododendronů, který jsem měl možnost zkoumat, byl téměř 100% kolonizován DSE, v kořenech evropských rododendronů se vyskytuje ErM a DSE, byť jsou tyto kořeny často obklopeny AM hyfami (viz Článek 1). AM symbiózu u *Ericaceae* nicméně dokládá i práce Urcelay (2002), který popisuje výskyt AM (vezikuly i arbuskuly), DSE (mikrosklerocia) a ErM (hyfová klubíčka) u *Gaultheria poeppiggi* DC v centrální Argentině.

EcM u vřesovcovitých je dokumentována např. prací Dighton a Coleman (1992), kteří zkoumali typy mykorhizních asociací u *Rhododendron maximum* L., nebo studií Smith a kol. (1995), kteří zkoumali, zdali mohou vřesovcovité rostliny *Gaultheria shallon* Pursh a *Rhododendron macrophyllum* G. Don sdílet společné mykobionty s koniferami *Pseudotsuga menziesii* (Mirb.) Franco a *Tsuga heterophylla* (Raf.) Sarg. (viz Část II).

Část I této disertační práce je zaměřena na interakce ErM a DSE-asociace (popř. hub, které tyto asociace tvoří) na/v kořenech společné hostitelské rostliny, proto se o obou typech na tomto místě zmíním podrobněji.

Souhrnný popis anatomie, morfologie a ekofyziologie ErM podávají např. Read (1983), Read (1996), Smith a Read (1997), Perroto a kol. (2002) nebo Cairney a Meharg (2003). Fundamentální náhled do problematiky ErM lze získat z monumentální série "*The biology of mycorrhiza in the Ericaceae*", která vycházela v časopisu *New Phytologist* v letech 1973 – 1998 (Abuarghub a Read 1988, 1988b, Bajwa a Read 1985, Bajwa a kol. 1985, Bradley a kol. 1982, Kerley a Read 1995, 1997, 1998, Leake a Read 1989, 1989b, Leake a kol. 1990, Pearson a Read 1973, 1973b, Shaw a Read 1989, Shaw a kol. 1990, Stribley a Read 1974, 1974b, 1976, 1980 a Stribley a kol. 1975). Zdá se, že roky 1973 a 1998 ohraničovaly jakousi zlatou éru výzkumu ErM, ve třetím tisíciletí zejména experimentální zkoumání ErM skomírá. Z chudého výčtu publikací např. Sokolovski a kol. (2002) zjistili, že rhizodermální buňky *Calluna vulgaris* (L.) Hull. jeví

zvýšenou kapacitu pro příjem aminokyselin, jsou-li kolonizovány typickou ErM houbou *Rhizoscyphus* (dříve *Hymenoscyphus*) *ericae* (Read) Zhuang & Korf.

Srovnání ErM a DSE-asociace zejména z hlediska vykonané experimentální práce vyznívá jednoznačně v neprospěch DSE-asociace. Současné publikované práce se sice často studiem nejhojněji zkoumané DSE houby *Phialocephala fortinii* Wang & Wilcox zabývají, většinou však z mykologicko-populačně genetického pohledu (např. Grünig a kol. 2001, 2004), bez zkoumání její asociace s kořeny vyšších rostlin. Souhrn základních (a často i jediných) informací o DSE-asociaci lze získat z přehledových článků Jumpponen a Trappe (1998), Jumpponen (2001) a Mandyam a Jumpponen (2005). Jumpponen (2001) navrhuje, aby byla DSE-asociace považována za mutualistickou, tedy i mykorhizní. Činí tak na základě výčtu několika prací, které dokázaly určitou prospěšnost *P. fortinii* vůči inokulované hostitelské rostlině, a také na základě konceptu tzv. *mutualisticko - parazitického kontinua* (např. Johnson a kol. 1997).

Z hlediska náhledu na mykorhiznost DSE-asociace je existence mutualisticko – parazitického kontinua důležitá, proto se u něj krátce pozastavím. Dle tohoto konceptu mohou i mykorhizní houby za určitých okolností působit na svého hostitele neutrálně či negativně, tj. paraziticky. Např. ve vztahu *sink - source* je mykorhizní houba, resp. její mycelium za ideálních podmínek pro rostlinu jak sink (putují k ní uhlíkaté produkty fotosyntézy), tak source (putují od ní roztoky především minerálních živin), přičemž oba projevy jsou vyvážené. Analogicky je i rostlina za normálních podmínek pro houbové mycelium sink i source. Vlivem vnitřních nebo vnějších faktorů může u mykorhizní houby převážit, byť i dočasně, projev sinku, houba pak začíná na rostlině parazitovat. Tato možnost platí i z pohledu rostliny, např. minimálně ranná vývojová stádia orchidejí jsou příkladem parazitizmu hostitelské rostliny na její "mykorhizní" houbě. Předpokládá se však (snad s výjimkou orchideoidní mykorhizy, ale viz Cameron a kol. 2006), že se jedná pouze o oscilace z normálního stavu. Je zřejmé, že mykorhiznost houby, stojící např. na pomezí mykorhiza – parazitismus, prakticky nelze vyvrátit, pouze potvrdit.

Je třeba uvést, že pozitivní vliv DSE na hostitelské rostliny může být zprostředkovaný, tedy nemykorhizní, jak ostatně uvádí i Mandyam a Jumpponen (2005). DSE houby mohou např. mineralizovat organický substrát v rhizosféře a zpřístupňovat ho tak rostlinám, což může vést k jejich zlepšenému růstu. Takové houby je příhodné nazývat jako rostlině prospěšné (*a beneficial associate*), spíše než mykorhizní. Tento výraz lze použít i v případě, kdy houba přímo kolonizuje kořeny rostlin. Např. Newsham (1999) prokázal, že DSE *Phialophora graminicola* (Deacon) Walker kolonizuje kořeny trávy *Vulpia ciliata* ssp. *ambigua* (Le Gall) Stace & Auquier a podporuje její růst a příjem živin. Autor ji označil právě jako "*a beneficial associate of P. graminicola*", nikoliv jako houbu mykorhizní. V ideálním případě by rozhodnutí o mykorhiznosti měl předcházet důkaz o obousměrném vyváženém toku látek mezi houbou a její hostitelskou rostlinou, toho však, pokud je mi známo, nebylo v případě DSE-asociace dosaženo. Alternativně lze uvažovat o mykorhiznosti v případě, že zkoumaná houba tvoří v kořeni morfologické struktury, které se toku látek obvykle účastní a které jsou pro jednotlivé typy mykorhiz charakteristické (viz

Článek 4 a 5). Takové struktury ale doposud nebyly u DSE-asociace s definitivní platností nalezeny (viz diskuse k Článku 1).

DSE-asociace byla v minulosti označována různými názvy, např. i termínem pseudomykorhiza. Protože jsem tento název v minulosti sám (nejspíš nesprávně) používal, cítím povinnost na závěr úvodu o DSE-asociaci vyjasnit jeho původ a správné použití. Termín *pseudomycorrhiza* (PSM) použil na začátku minulého století Melin pro houbami kolonizované kořeny konifer, jejichž morfologie, nikoliv však anatomie, připomínala EcM. V PSM kořenech chyběla zejména Hartigova síť, tedy fyziologicky aktivní rozhraní (*interface*) mezi rostlinou a EcM houbou. Takové kořeny byly kolonizovány především agregátem hub zvaným *Mycelium radicis atrovirens* (MRA). Již Melin (1922) uvádí, že houby MRA tvořící PSM měly na kolonizované konifery negativní vliv. Přívlastek pseudomykorhizní tak nabyl i fyziologického, byť negativního, rozměru. S postupujícím časem a s rostoucím zájmem o houby asociované s kořeny rostlin byli zástupci komplexu MRA izolováni i z kořenů ne-EcM rostlin, kde jejich negativní působení nebylo (alespoň relativně vůči EcM-PSM rostlinám) zřejmé. Řada autorů začala používat termín PSM pro všechny kořeny kolonizované MRA. Tento termín tak byl používán v souvislosti se specifickou skupinou hub, které kolonizují kořeny, spíše než s morfologickou strukturou popsanou Melinem.

S rozvojem molekulárních technik se ukázalo, že hlavním zástupcem MRA je *P. fortinii*, tedy typická DSE houba. Pravděpodobně díky anatomii a morfologii ektomykorhiz se přitom označení DSE používá spíše v souvislosti s tmavými přepážkovanými hyfami, vnitrobuněčně kolonizujícími kořeny především ne-EcM rostlin. Je také třeba si uvědomit, že taxonomická identita řady zástupců MRA byla pomocí molekulárních metod určena až zpětně. *P. fortinii* tak může být řazena jak do MRA (zejména pokud je asociována s kořeny EcM rostlin), tak mezi DSE (zejména pokud je asociována s kořeny jiných rostlin). Pokud navíc specifickým způsobem modifikuje kořeny typicky EcM rostlin, může být považována za houbu pseudomykorhizní.

Trend (snad nesprávného) používání termínu PSM se změnil s prací Jumpponen a Trappe (1998) a Jumpponen (2001). Zejména Jumpponen (2001) poukazuje na možný mykorhizní charakter asociací, vzniklých mezi kořeny a DSE. Používání termínu PSM však evokuje spíše negativní účinek asociovaných hub, navíc je díky svému původnímu (ve smyslu Melin 1922) užití matoucí. Proto se v současné době pro asociaci kořenů a DSE používá termín DSE-asociace, a to zejména je-li od DSE houby "očekáván" pozitivní (nebo alespoň neutrální) efekt na hostitelskou rostlinu. Termín PSM by pak měl být používán ve smyslu Melina, tedy ve smyslu původním. PSM je tak možno považovat za specifický podtyp DSE-asociace. Příklad používání termínu PSM výstižně ilustruje (nejen terminologickou) náročnost zkoumání (ne-)mykorhizních hub, asociovaných s rhizosférou/s kořeny mykorhizních rostlin. Excelentní historický přehled tohoto zkoumání podává Summerbell (2005b), který také publikoval několik původních experimentálních prací na dané téma (např. Summerbell 1987, 1989 a 2005).

Na kořenech vřesovcovitých rostlin se ErM a DSE mohou vyskytovat samostatně, nebo společně. Přestože jsou vřesovcovité rostliny považovány za primárně ErM, současná přítomnost

obou asociací u nich byla zjištěna mikroskopicky (např. Urcelay 2002, Rains a kol. 2003, Peterson a kol. 2004, Cázares a kol. 2005), i za použití molekulárních technik (Hambleton a Currah 1997, Midgley a kol. 2004, Bougoure a Cairney 2005). Není však zřejmé, je-li společný výskyt pravidlem, nebo výjimkou. Také ekofyziologický význam tohoto soužití zůstává nejasný. Teoreticky se projev interakce ErM a DSE-asociace může pohybovat kdekoliv v rámci již zmíněného mutualisticko-parazitického kontinua.

Výsledky mé předchozí práce (Vohník a kol. 2003) dokladují neutrální vliv kolonizace kořenů houbou *P. fortinii* na růst *Rhododendron* cv. Belle-Heller. Mé jiné nepublikované experimenty naznačovaly, že některé kmeny *P. fortinii* v kombinaci s některými ErM houbami ovlivňují fyziologické parametry (biomasa, příjem živin) hostitelských rostlin pozitivně, naproti tomu jiné kmeny *P. fortinii* v kombinaci se stejnými ErM houbami působí negativně. Má nepublikovaná pozorování kořenů vřesovcovitých rostlin z přirozených stanovišť přitom naznačovala, že současný výskyt ErM a DSE-asociace je spíše pravidlem, než výjimkou. Část I této disertační práce si proto kladla následující cíle:

1. **Dokumentovat případný souběžný výskyt ErM a DSE-asociace v kořenech evropských rododendronů s důrazem na sledování a porovnání morfologických struktur, charakteristických pro obě asociace.**
2. **Pokusit se nalézt morfologické struktury DSE-asociace, které by mohly představovat fyziologicky aktivní rozhraní mezi DSE a jejich hostitelskými rostlinami.**
3. **Kvantifikovat výskyt ErM a DSE-asociace v kořenech evropských rododendronů v závislosti na zeměpisné šířce.**
   Tyto cíle jsou zpracovány v Článku 1 této DP.

4. **Sledovat interakce mezi ErM a DSE-asociací, popř. houbami, které tyto asociace tvoří, s důrazem na jejich vliv na růstové parametry hostitelských rostlin.**
   Tento cíl je zpracován v Článcích 2 a 3 této DP.

1. 2. ČLÁNEK 1

Morphological diversity and proportions of ericoid mycorrhiza and DSE-association in roots of European rhododendrons

(manuskript)

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Morphological diversity and proportions of ericoid mycorrhiza and DSE-association in roots of European rhododendrons

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Abstract

Ericaceous species form ericoid mycorrhiza (ErM), but also host dark septate endophytes (DSE). The co-existence of ErM and DSE-association is a poorly known phenomenon that may significantly influence both plant and fungal partners. To investigate this co-existence and to screen colonization patterns of ErM and DSE-association, we sampled roots of six European *Rhododendron* species from different sites across a latitudinal profile of Europe. Structures of ErM and DSE-association were simultaneously present in all field samples, however, differed in their frequency depending on the *Rhododendron* species. Additionally, the structures intermediate between ErM and DSE-association were found. These included loose intracellular coils formed by hyaline to dark septate hyphae. The highest ErM colonization (> 40%) was found in roots of *Rhododendron hirsutum*, *R. ponticum* and *R. ferrugineum*, whereas the lowest was in roots of *R. lapponicum* (< 10%) and *R. luteum* (<20%). The highest DSE colonization was in roots of *R. lapponicum* (>50%) and *R. kotschyi* (>30%), whereas the lowest (<10%) was in roots of *R. hirsutum*, *R. ferrugineum* and *R. ponticum*. The highest colonization by the intermediate structures (>20%) was in roots of *R. lapponicum*, whereas the lowest (<5%) was in roots of *R. ponticum*. The highest total colonization was in roots of *R. lapponicum* (>80%) and *R. kotschyi* (>70%), whereas the lowest (<50%) was in roots of *R. luteum*. There was a high variability of colonization types among different individuals of the same species. DSE colonization was negatively correlated with ErM colonization and occurrence of non-colonized cells, and positively correlated with colonization by the intermediate structures. ErM was negatively correlated with colonization by the intermediate structures. We discuss possible ecological meaning of our results in context of ErM – DSE interactions.

Key words: ericoid mycorrhiza, DSE-association, *Rhododendron*, colonization pattern

Introduction

Ericaceous species form a unique root-fungus association called ericoid mycorrhiza (ErM), which has a determinative effect on plant fitness (Smith & Read, 1997; Cairney & Meharg, 2003).

Even though members of *Ericaceae* are considered primarily ericoid mycorrhizal (Hambleton & Currah, 1997), their roots are often colonized by ubiquitous dark septate endophytes (DSE; Jumpponen & Trappe, 1998), which have obscure effects on their hosts (Jumpponen, 2001; Mandyam & Jumpponen, 2005).

Although the simultaneous presence of ErM and DSE in roots of ericaceous plants can be expected (Peterson *et al*., 2004) and both ErM and DSE were detected within a root system of ericaceous plants either microscopically (Urcelay, 2002; Rains *et al*., 2003; Peterson *et al*., 2004; Cázares *et al*., 2005) or using molecular techniques (Hambleton & Currah, 1997; Midgley *et al*., 2004; Bougoure & Cairney, 2005a), only little is known about the ErM-DSE co-existence. This contrasts with the fact that the interaction between ErM and DSE may have important eco-physiological consequences for their host plants. For example, Vohník *et al.* (2005) showed that inoculation with *Oidiodendron maius* Barron increased phosphorus uptake by *Rhododendron* cv. Azurro in comparison with non-inoculated plants. This effect of *O. maius* was not altered in the presence of *Phialocephala fortinii* Wang & Wilcox strain PFO-H, whereas in the presence of *P. fortinii* strain PFO-F phosphorus uptake was reduced to the level of non-inoculated plants.

At different field sites, it can be expected that ErM fungi and DSE colonize different proportions of ericaceous root systems. To date, it is unknown what influences the equilibrium between ErM fungi and DSE in the rhizosphere of ericaceous plants, and how is this equilibrium established. Answering this question would improve our understanding of principles of the ErM-DSE co-existence, which may have parallels in other types of root-fungus associations.

One factor determining the co-existence of ErM and DSE-association may be a latitudinal profile. From a "mycorrhizal" point of view, increasing latitude is reflected by a shift in major mycorrhizal types in the respective plant community, i.e., arbuscular mycorrhizae (AM) are replaced with ectomycorrhizae (EcM), which are further substituted by ErM. This pattern also applies to AM, EcM and ErM host plants (Read & Perez-Moreno, 2003). Similarly, the frequency of DSE in roots is supposed to increase along an altitudinal gradient. In Austrian Alps, Haselwandter and Read (1980) found DSE colonization rates increasing with altitude in non-ericaceous plants. At the same time, the authors found ErM colonization rates decreasing with altitude, but did not note the presence of DSE in roots of ericaceous plants.

DSE are supposed to be the most frequent and important root associates in "increasingly stressed environments" (Mandyam & Jumpponen, 2005), e.g. in alpine and sub-arctic conditions (Haselwandter & Read, 1980; Read & Haselwandter, 1981; Stoyke & Currah, 1991; Schadt *et al.*, 2001). On the other hand, ericaceous plants living in a symbiosis with ErM fungi often dominate such "harsh environments" (Cairney & Meharg, 2003). This indicates that at least under certain conditions both ErM fungi and DSE co-exist and therefore can simultaneously colonize ericaceous hosts.

Species of *Rhododendron* L. seem to be a good model for studying such co-occurrence. Their roots constitute a habitat for fungi forming ErM (Pearson & Read, 1973; Moore-Pankhurst &

Englander, 1981; Duddridge & Read, 1982; Douglas *et al*., 1989; Currah *et al*., 1993a), DSE-association (Currah *et al.*, 1993b; Vohník *et al.*, 2003, Vohník *et al*. 2005), and possibly also EcM (Dighton & Coleman, 1992) and AM (but only hyphae and vesicules were found to date: Dighton & Coleman, 1992; Chaurasia *et al.*, 2005). The distribution of rhododendrons enables studying their fungal associations on a broad environmental scale: rhododendrons are autochthonous in Asia, Australia, Europe and North America, and were introduced in Africa and South America. They occupy habitats differing in altitude (0 - 5000 m a. s. l.), latitude (northern Greenland to northern Australia) and substrate composition. *Rhododendron* species vary in habitus and life-style, ranging from 15 cm tall arctic dwarf shrubs to tropical epiphytes to 25 m tall arboreous *Rhododendron arboreum* Adams. from the Himalayas (Dostálková, 1981).

Rhododendrons´ habitat and lifestyle heterogeneity can also be exemplified in European rhododendrons. According to Dostálková (1981), there are nine autochthonous species in Europe (for their list, distribution and habitat preferences see Dostálková, 1981) which, together with other ericaceous species (*Calluna vulgaris* Hull, *Loiseleuria procumbens* (L.) Desv., *Vaccinium myrtillus* L., *Vaccinium vitis-idaea* L.) represent an important part of local flora, forming distinctive plant communities that often dominate areas of their occurrence (Dostálková, 1981).

To contribute to understanding of the co-existence of ErM and DSE-association, we screened roots of 6 autochthonous European *Rhododendron* species from various habitats ranging from sub-arctic to alpine to Mediterranean. We paid special attention to the presence of typical ErM/DSE-association structures as well as to structures that were difficult to attribute to either of the two types, because such structures might hamper the evaluation of proportions of ErM and DSE colonization.

Materials and Methods

Collecting of root samples

Roots of the following *Rhododendron* species were collected, stained and screened for colonization: *Rhododendron. ferrugineum* L. (according to the origin labeled as FER-Aut, FER-Esp, FER-Fra throughout the following text), *Rhododendron hirsutum* L. (HIR), *Rhododendron kotschyi* Simk. (KOT), *Rhododendron lapponicum* Wahlemb. (LAP), *Rhododendron luteum* Sweet (LUT) and *Rhododendron ponticum* L. (PON).

Most root samples were taken from localities in alpine or sub-alpine habitats: FER at different sites in Alps and Central Pyrenees, HIR at Slovenian Velika Planina plateau, KOT in Romanian Carpathian Mts. Roots of LUT were from deciduous forest (tree dominants *Fagus sylvatica* L., *Quercus petraea* (Mattusch.) Liebl., *Carpinus betulus* L. and *Castanea sativa* Mill.) near Boštanj, Slovenia. LAP samples were from subarctic Kilpisjärvi, Finland. PON occurs as a Tertiary relict in southern Iberian Peninsula (Mejías *et al*., 2002) and was collected in a stream valley in sclerophyllous evergreen forest near Puerto de Galis, Spain. Collecting sites varied across ~33 latitudinal degrees, ~2300 m of altitude and ~14°C difference in mean annual temperature,

sub-arctic Kilpisjärvi (69°03'N, ~480 m a. s. l., Finland) and Mediterranean Puerto de Galis (36°08'N, ~420 m a. s. l., Spain) representing the northern- and southernmost locality, respectively. The collection site in the Romanian Carpathian Mts., 2505 m a. s. l., was the highest locality, whereas Boštanj (Slovenia) with 220 m a. s. l. was the lowest locality. For details about the localities see Table 1.

From each locality, roots of five different individuals were sampled. Where possible (FER, KOT), collection sites differed in altitude. Samples of hair roots with adhering substrate (approx. volume 30 ml) were carefully taken from a 5-10 cm depth and stored in lacto-glycerol (lactic acid : water : glycerol = 1 : 1 : 1) in a fridge (8°C) until screened.

Clearing and staining of roots, evaluation of colonization

Root samples were divided into halves; one half was washed under running tap water from attached substrate and directly observed under microscope, the other was additionally cleared with 10% KOH and stained with trypan blue according to Brundrett *et al.* (1996). The first half was used to screen the mycelium occurring in the rhizosphere of *Rhododendron* roots, including the extraradical mycelium of mycorrhizal fungi. The second half was used for evaluation of root colonization. Stained roots were observed at high magnification (400-1000x) with DIC, using an Olympus BX60 microscope. Pictures were taken with an Olympus DP70 camera.

ErM was recognized on the base of the characteristic fine intracellular coils and loops, either hyaline or blue-stained. DSE- association was recognized on the base of **i)** microscletoria, either dark-brown, hyaline or blue-stained; **ii)** a dense parenchymatous mantle (sensu Wurzburger & Bledsoe, 2001); **iii)** extra- or intracellular thick, light to dark brown septate hyphae; **iv)** extra- or intracellular lightly brown to hyaline septate hyphae, but only if connected with hyaline/brown/blue-stained intracellular microsclerotia or a parenchymatous mantle. Besides the typical ErM and DSE structures listed above, we found structures intermediate between the ErM and DSE colonization patterns (INT; See Results). Occurrence of other fungal hyphae (AM, basidiomycetous) attached to the screened roots is also noted in Results.

Morphology of ErM and DSE was described for each screened *Rhododendron* species. Additionally, percentual and proportional colonization of hair roots of five individuals per FER-Esp, HIR, KOT, LAP, LUT and PON by ErM, DSE and the intermediate structures was counted. For each *Rhododendron* individual, ten hair root segments (approx. length 5 mm) were randomly chosen from its root sample and ErM/DSE/INT colonization was counted on the cell-by-cell basis. In each segment, at least 250 cells were evaluated. 84,715 hair root cells were evaluated in total. Percentual by ErM, DSE-association, the intermediate structures and percentual

The ratios between ErM- and DSE-colonized cells, between DSE-colonized and non-colonized cells, between ErM-colonized and non-colonized cells, between INT-colonized and non-colonized cells, and between colonized (ErM + DSE + INT) and non-colonized cells were counted. The ratios did not have homogenous variances and normal distribution. Therefore, Kruskal-Wallis

ANOVA was used to evaluate the effect of the *Rhododendron* species on these ratios. Kolmogorov-Smirnov two-sample test was used to evaluate the difference among screened *Rhododendron* species. We further evaluated whether different individuals of the same *Rhododendron* species significantly differed in these ratios using Kruskal-Wallis ANOVA, and whether any correlation between ErM, DSE and INT colonization existed, using Spearman R correlation.

Results

Morphology of ericoid mycorrhiza and DSE-association

The ErM colonization pattern was represented by typical intracellular coils or loops. However, morphology of ErM colonization had several modifications: (i) the fine structure of the blue-stained coils was poorly visible (Fig. 1); (ii) the fine structure of the blue-stained coils was well visible (Fig. 2); (iii) fine coils did not stain with trypan blue and remained hyaline. Such hyaline coils were connected with extracellular hyaline hyphae (Fig. 3) or dark, septate hyphae (Fig. 7); (iv) fine coils did not stain with trypan blue and remained dark-colored. There were differences in the diameter of hyphae forming such coils, in density of such coils, and in proportion of the cell they occupied (Figs 4 & 5). Frequently, both blue-stained and dark-pigmented coils occurred in parallel in one root segment (Figs 3 & 6); (v) very fine blue-stained coils, which usually did not occupy the whole lumen of the colonized cell (Fig. 8); (vi) relatively thick, blue-stained hyphae formed very loose intracellular coils (Fig. 9). A dense weft of hyaline to blue-stained hyphae covering the whole surface of the root was rarely observed; if present, it was in roots colonized by ErM to a high (up to 100%) degree.

Common features of the DSE colonization pattern were intracellular microsclerotia, either hyaline or blue-stained or dark-pigmented (Fig. 10). The DSE colonization pattern was also characterized by presence of a loose or dense parenchymatous net, which appeared to develop either on the root surface or under the layer of the rhizodermal cells (Fig 11). The cellular structure of the parenchymatous net resembled the Hartig net (Fig 12). In hair roots with the parenchymatous net, ErM colonization was infrequent. If present, it occupied a cell layer directly below the net (Fig. 15c). Sometimes, thick DS hyphae surrounded screened roots and formed primordia of hyaline hyphae appearing to be formed to penetrate into rhizodermal cells, but intracellular colonization was absent or scarce (Fig. 13). The DSE colonization pattern was also characterized by thick, septate, hyaline to blue-stained intraradical hyphae (Fig. 14). In parts of the screened roots where ErM and DSE-association were simultaneously spatially present, only the weft of DS hyphae was observed around the roots. Sometimes, vesicles reminding modified microsclerotia were present in the hair roots (Fig. 17).

Even though morphology of ErM and DSE-association is generally considered to be distinct, in our study, some of their characteristics were difficult to distinguish when both root-fungus associations were present together in a common root system. Thick, dark brown hyphae

forming intracellular microsclerotia were often connected to fine, dark to hyaline hyphae, which formed loose loops in other rhizodermal cells (Fig. 10). Loose intracellular loops were also formed by thick hyaline hyphae (Fig. 16). Because of frequent occurrence of structures, which could be either ErM or DSE-association, in some root samples, we decided to introduce a new category, the intermediate structures (INT). Characteristically, they were formed by light to dark brown septate hyphae, which were thinner that typical DS hyphae, but thicker than typical ErM hyphae, and had a shape of relatively loose hyphae (Figs 15, 18, 19 & 20). Sometimes, INT resembled ErM (cf. Figs 9 & 19); sometimes they resembled DSE-association (cf. Figs 10, 14 & 15 with 18, 19 & 20). For example in FER-Fra samples, mostly loose dark brown to black intracellular coils of thick DSE hyphae were present in hair roots. These coils were then often connected with DS hyphae on the root surface (Fig. 18). In contrast, PON samples were dominated by dark dense coils, which were apparently ErM structures (Figs 4 & 5). Interestingly, it seemed that some hyphae formed both what appeared to be ErM and DSE-association (Figs. 15 & 21). Such atypical colonization pattern had two distinct types. The less frequent first type, found in KOT hair roots, was characterized by INT in the rhizodermal cells, accompanied by dense blue-stained ErM-like coils in the layer of cells below the rhizodermis (Figs 15a – c). The more frequent second type, found in FER-Aut, FER-Fra and KOT hair roots, was characterized by the parenchymatous net developed between the non-colonized rhizodermis and the ErM-like colonized layer of cells below rhizodermis (Figs 21a – e).

In all screened *Rhododendron* samples, definitive evidence of ErM and DSE co-occurrence was the simultaneous presence of dense hyaline/dark/blue-stained coils typical for ErM and microsclerotia or the parenchymatous net or thick, dark brown septate hyphae, typical for DSE-association. The spectrum of morphological features occurring in respective *Rhododendron* species is listed in the Table 1.

Proportions of ErM and DSE colonization in the field samples

Average percentual colonization of FER-Esp hair roots by DSE, ErM and INT was 6.8%, 41.6% and 6.2%, respectively. It was 5.6%, 58.8% and 10.5% in HIR hair roots; 32.4%, 30.2% and 7.6% in KOT hair roots; 55.0%, 8.5% and 22.4% in LAP hair roots; 22.7%, 16.8% and 7.5% in LUT hair roots; 8.6%, 52.0% and 3.5% in PON hair roots.Average total colonization (DSE + ErM + INT) of hair roots was 54.6% in FER-Esp, 74.9% in HIR roots, 70.2% in KOT, 85.9% in LAP, 47% in LUT and 64.1% in PON (Table 1).

There was a significant effect of the *Rhododendron* species on the ratio between ErM-colonized and DSE-colonized cells ($H = 199.5$, $p = 0.000$), DSE-colonized and non-colonized cells ($H = 175.1$, $p = 0.000$), ErM-colonized and non-colonized cells ($H = 129.0$, $p = 0.000$), INT-colonized and non-colonized cells ($H = 128.4$, $p = 0.000$), and colonized and non-colonized cells (147.5, $p = 0.000$). The highest ErM : DSE ratio was in HIR (mean 82.007 ± SD 82.61; significantly different group c) followed by FER-Esp (53.231 ± 73.87; c), PON (44.176 ± 58.52; c),

LUT (3.126 ± 9.54; b), KOT (1.708 ± 2.24; b) and LAP (0.168 ± 0.14; a). The highest DSE ratio was in LAP (5.761 ± 4.08; d) followed by KOT (1.828 ± 1.84; c), LUT (0.557 ± 0.57; b), PON (0.358 ± 0.59; a), HIR (0.280 ± 0.45; a) and FER-Esp (0.165 ± 0.21; a). The highest ErM ratio was in HIR (3.000 ± 2.67; e) followed by PON (1.902 ± 1.32; d), KOT (1.596 ± 1.67 cd), FER-Esp (1.126 ± 0.87; c), LAP (1.054 ± 1.56; b) and LUT (0.355 ± 0.30; a). The highest INT ratio was in LAP (2.502 ± 1.99; d) followed by HIR (0.491 ± 0.50; c), KOT (0.444 ± 0.60; c), LUT (0.221 ± 0.36; ab), FER-Esp (0.170 ± 0.19; b) and PON (0.095 ± 0.12; a). The highest ratio between colonized and non-colonized cells was in LAP (9.318 ± 7.11; d) followed by KOT (3.869 ± 3.67; c), HIR (3.771 ± 2.73; c), PON (2.356 ± 1.64; c), FER-Esp (1.461 ± 0.96; b) and LUT (1.134 ± 0.93; a) (Table 2).

During the screening of morphology of hair roots' colonization, there was high variability in proportions of ErM, DSE-association and INT between samples of the same species. While ErM dominated a sample from the root system of one individual, another sample from an individual a few meters away seemed to be colonized preferentially by DSE. Another frequent situation was the dominance of ErM in a part of the root sample, while the other part was densely colonized by DSE. These observations were supported by the statistical analysis. There was a significant difference in the ratio between ErM-colonized and DSE-colonized cells among different individuals of FER-Esp ($H = 19.5$, $p = 0.0006$), HIR ($H = 24.3$, $p = 0.0001$), LAP ($H = 23.3$, $p = 0.0001$), LUT ($H = 19.3$, $p = 0.0007$) and PON ($H = 26.2$, $p = 0.0000$). There was a significant difference in the ratio between DSE-colonized and non-colonized cells among different individuals of FER-Esp ($H = 18.6$, $p = 0.0009$), HIR ($H = 22.1$, $p = 0.0002$), KOT ($H = 30.9$, $p = 0.0000$), LAP ($H = 14.1$, $p = 0.0071$), LUT ($H = 19.3$, $p = 0.0007$) and PON ($H = 25.7$, $p = 0.0000$). There was a significant difference in the ratio between ErM-colonized and non-colonized cells among different individuals of FER-Esp ($H = 10.7$, $p = 0.0299$), KOT ($H = 27.1$, $p = 0.0000$) and LAP ($H = 28.0$, $p = 0.0000$). There was a significant difference in the ratio between INT-colonized and non-colonized cells among different individuals of FER-Esp ($H = 12.3$, $p = 0.0156$), HIR ($H = 29.1$, $p = 0.0000$), KOT ($H = 32.9$, $p = 0.0000$), LAP ($H = 16.5$, $p = 0.0025$), LUT ($H = 23.4$, $p = 0.0001$) and PON ($H = 24.5$, $p = 0.0001$). There was a significant difference in the ratio between colonized (ErM + DSE + INT) and non-colonized cells among different individuals of FER-Esp ($H = 11.5$, $p = 0.0215$), LAP ($H = 18.9$, $p = 0.0008$), LUT ($H = 12.1$, $p = 0.0169$) and PON ($H = 11.5$, $p = 0.0212$).

In all individuals of all species, there was a negative correlation between DSE- and ErM-colonization ($r = -0.704$, $p = 0.0000$), a positive correlation between DSE- and INT-colonization ($r = 0.442$, $p = 0.0000$), a negative correlation between DSE- and non-colonization ($r = -0.315$, $p = 0.0000$) and a negative correlation between ErM- and INT-colonization ($r = -0.424$, $p = 0.0000$). There was no correlation between ErM- and non-colonization ($r = 0.0266$, $p = 0.646$).

Other hyphae present in the rhododendrons´ rhizosphere

Non-ErM- or DSE-association-forming hyphae were primarily of two kinds: i) basidiomycetous thick hyphae with clamp connections and ii) trypan blue-stained thick non-septate hyphae, sometimes connected with AM spores.

Hyphae with clamp connections occurred in FER-AUT, HIR and LUT samples. In LUT samples, we noticed the frequent presence of hyaline to brown basidiomycetous hyphae connected with structures, which showed morphological similarity to papulasporas (*Papulaspora* Preuss; Domsch *et al*., 1980). These structures were very frequent on the surface of decayed leaves found in the rhizosphere of LUT samples. Hyphae connected with these structures were associated with the surface of LUT roots, and it appeared that they occasionally formed intracellular loose loops resembling the intermediate structures, in the rhizodermal cells.

AM hyphae occurred in FER-AUT, HIR and PON samles. They never penetrated root tissues and were only loosely associated with screened *Rhododendron* roots. We never observed any arbuscules or AM vesicles in *Rhododendron* root samples. Occasionally we found structures resembling vesicles sensu Barrow (2003; Fig. 17). These vesicles resembled atypical developmental stages of microsclerotia, which are characteristic structures DSE-association.

In healthy HIR roots we found intracellular coils formed by thick hyaline hyphae of unknown origin (Fig. 16). We did not observe similar structures in other root samples.

Discussion

Since early observations on mycorrhizal status of alpine plant communities (Haselwandter & Read, 1980; Read & Haselwandter, 1981) it became evident that plants simultaneously possess different fungal associations in their roots. Some authors pointed out the importance of other than traditional root-fungus associations (AM, EcM, ErM), DSE-association, for host plants (Haselwandter & Read, 1980; Ahlich & Sieber, 1996; Jumpponen & Trappe, 1998; Jumpponen, 2001). Haselwandter and Read (1980) and Read and Haselwandter (1981) however indicated only ErM in ericaceous species, including *R. ferrugineum* and *R. hirsutum*, in alpine habitats in Austrian Alps.

In contrast, we found both ErM and DSE-association in roots of all sampled *Rhododendron* species, even though proportions of both associations differed in different species. The highest ErM proportions were found in roots of HIR, PON and FER-Esp, which were collected in southern part of Europe, and the lowest ErM proportions were found in roots of LAP, which was the northernmost species. On the other hand, the highest DSE colonization was found in LAP roots and the lowest in HIR, FER-Esp and PON roots. Statistical analysis revealed that DSE colonization was negatively correlated with ErM colonization, i.e. ErM colonization decreases with increasing DSE colonization.

Surprisingly, ErM-DSE co-occurrence was not frequently reported in early studies focusing on the mycorrhizal status of natural communities using light microscopy. One reason for this lack might be that ericaceous species only rarely represented significant proportion of screened

host plants and if included, then they were apparently expected to be only ericoid mycorrhizal. This trend however has recently changed (Urcelay, 2002; Rains *et al*., 2003; Peterson *et al*., 2004; Cázares *et al*., 2005; this study). For example, Rains *et al*. (2003) showed that both ErM fungi and DSE inhabited 6 of 7 ericaceous species from a lower montane cloud forest. Cázares *et al*. (2005) found both ErM fungi and DSE in 5 of 6 ericaceous species from a sub-alpine glacier forefront.

Another reason might result from morphological similarities between ErM and DSE-association. According to common descriptions, ErM and DSE-association should be easily distinguishable. However, we found that existence of some common features hampered their distinguishing in the field samples. While it was always possible to distinguish typical ErM (fine intracellular coils or loops) and it is unlikely that DSE would produce such structures (but see Usuki & Narisawa, 2005), and the same applies for ErM fungi and microsclerotia, we observed transient intracellular structures that cannot be reliably distinguished as strictly ErM or DSE. These mainly comprised loose or dense intracellular coils formed by DS hyphae. The same loose to dense coils were connected to extraradical DS hyphae forming microsclerotia, which indicated they represented DSE-association. Moreover, colonization by the intermediate structures was highest in LAP and was positively correlated with DSE colonization in all screened species. This is another indication that most of the intermediate structures were actually formed by DSE fungi.

Loose to dense intracellular coils formed by DS hyphae, and similar to what we call the intermediate structures, are reported from ericaceous roots worldwide (e.g. Cairney & Ashford 2002, Fig. 5, p. 312; Peterson *et al*. 2004, Fig. 166, p. 90) and are interpreted as ErM (Cairney & Ashford, 2002) or DSE (Peterson *et al.*, 2004). This may cause bias in results of microscopic observations of field samples, because loose to dense DS intracellular coils may be interpreted as ErM or DSE-association, or be ignored, according to the subjective feeling of an observer. Obviously, INT might originate not only from ErM fungi or DSE, but also from saprotrophic/parasitic root endophytes, but when INT occurred in the root segment, they usually occupied majority of the screened cells, which otherwise looked healthy and were collected from healthy plants.

The similarities between ErM and DSE fungi, including colonization patterns, "harsh habitats" preference or the possibility to survive sufficiently without host plants as saprotrophs, open an intriguing question whether some of them can behave at once as ErM- and DSE-fungi. For example, *H. chaetospira* forms intracellular loops and microsclerotia in roots of *Brassica campestris* L. (Ohki *et al.*, 2002) and intracellular loops of ErM type, but no microsclerotia, in rhizodermal cells of *R. obtusum* var. *kaempferi* (Usuki & Narisawa, 2005). Thus, "*H. chaetospira* may occur in the roots of apparently healthy plants as either DSE or ErM fungi depending on the host species" (Usuki & Narisawa, 2005). This has to be revealed at both structural and physiological levels - the statement of Usuki and Narisawa (2005) is however based only on *in vitro* re-synthesis trials and morphological observations. By definition, also typical DSE fungus *P.*

fortinii forming intracellular coils in ericaceous roots and enhancing fitness of the host plant could be assigned as an ErM fungus.

The observational approach used in our study unfortunately limits reliable identification or distinguishing of fungal partners in the root at a species level, because different ErM/DSE fungi can develop very similar structures. Also seasonal variability in colonization degree (Hutton *et al*., 1994; Ruotsalainen *et al*., 2002) and morphological pattern (Smith & Read, 1997) may obscure the evaluation. The objective of our study was, however, not to determine root endophytic species, but to screen ErM-DSE co-occurrence. Irrespectively of seasonal and morphological variability in fungal colonization, we found ErM and DSE-association simultaneously occupying the roots of all screened rhododendrons. This in our opinion indicates the eco-physiological relevance of this phenomenon, even if the respective ErM and DSE fungi remained undetermined.

The question about the identity of fungi in a root can be answered when respective fungi are isolated from root fragments, cultivated and determined, either morphologically or using DNA analysis. Using this culture-based approach, numerous authors found that ErM fungi and DSE co-exist within root systems of ericaceous species (Stoyke *et al*., 1991, 1992; Hambleton & Currah, 1997; Addy *et al*., 2000; Midgley *et al*., 2004; Bougoure & Cairney, 2005a). The discrimination of DSE fungi in other molecular studies (e.g. Usuki *et al*., 2003; Allen *et al*., 2003) might be due to the sterilization of the root surface before isolation of fungal mycelium or extraction of fungal DNA from the root, because our observations indicate that a significant proportion of DS hyphae are present extracellularly around the root and thus are destroyed by the sterilization procedure. These facts notwithstanding, only a little attention was paid to DSE in molecular studies focused on the community of fungal endophytes of ericaceous roots, possibly because they did not form "typical ErM intracellular coils" in re-synthesis trials, thus were regarded as non-mycorrhizal (e.g. Midgley *et al*., 2004). On the other hand intracellular coils are not regarded as typical structures of DSE-association, and their absence should not lead to the conclusion that DSE are non-mycorrhizal. Instead, structures typical for DSE-association (see below) should be searched.

Employing culture-independent molecular techniques can dispatch some disadvantages of the culture-dependent approach. Both approaches however reveal different fungi from the same root sample when used separately (Allen *et al*., 2003; Midgley *et al*., 2004; Bougoure & Cairney, 2005a, b), and it is recommended to combine them to find the most probable composition of root endophytic fungi (Bougoure & Cairney, 2005a). For example, Allen *et al*. (2003) found assemblage of fungi isolated from ErM roots to be dominated by "intracellular coils forming" *Capronia*-like fungi, but assemblage of DNAs from the same root segments was dominated by uncultivable (thus with unconfirmed mycorrhizal status) *Sebacina*-like basidiomycetes.

It should be skeptically considered that DSE-association is both *in vitro* and in the field recognized mainly by the presence of intracellular microsclerotia, which likely serve as fungal propagules with a high content of storage compounds (Yu *et al*., 2001), and are not supposed to act as sites where possible nutrient exchange between DSE and colonized plant takes place. Moreover,

we found considerably higher occurrence of microsclerotia under aseptic conditions, where in some parts of the roots nearly all rhizodermal cells were filled (Fig. 18b), thus likely physiologically impaired, than in the field where such a situation never occurred. Additionally, microsclerotia can be formed by different fungi, not regarded as "true" DSE, e.g. by *Heteroconium chaetospira* (Grove) M. B. Ellis in cortical cells of *Brassica campestris* L. (Ohki *et al.*, 2002).

Another character of DSE association is a presence of DS hyphae either in the root or on the root surface. This character however appears to be a vague criterion, since many fungi can form DS hyphae. Beside others, Addy *et al*. (2005) included in their "Key to some dark septate fungi from roots" also ErM fungi [Variable White Taxon = *Meliniomyces variabilis* Hambleton & Sigler (Hambleton & Sigler, 2005), *Scytalidium vaccinii* Dalpé, Litten & Sigler (thus also its teleomorph *R. ericae*) and *O. maius*]. Logically, if DS hyphae of the listed ErM fungi were found in or around ericaceous root, they could be ascribed to DSE-association. The presence of DS hyphae on the root surface, however, may be reliably attributed to DSE-association if the hyphae form a dense parenchymatous mantle sensu Wurzburger and Bledsoe (2001) because this structure was to date never observed in connection with ErM fungi. Typical ErM fungi (*O. maius*, *R. ericae*) never formed similar mantle in our previous re-synthesis experiments. In FER samples (Fig. 4), a net of parenchymatous cells developed under a layer of non-colonized rhizodermal cells; hypothetically, it could serve as an exchange point between plant and fungus, analogically to the Hartig net. This hypothesis, however, needs to be further investigated (see also discussion in Rains *et al*. 2003).

Judging DSE-association by the presence of microsclerotia, which are likely physiologically inactive in nutrient transport between DSE and the colonized plant, is in contradiction with AM (arbuscules), ErM (coils, loops) and EcM (Hartig net), where exactly these kinds of structures with active plant/fungus interfaces (Smith & Smith, 1990) are traced. Such structures in DSE-association may be hyaline intraradical hyphae (Barrow & Aaltonen, 2001; Barrow, 2003), which are usually overlooked, or the parenchymatous mantle or net, as discussed above. There is also a possibility that intracellular coils formed by *P. fortinii* in ericaceous roots may play role in possible nutrient transport between DSE and the host plant. Similarly to parenchymatous mantle, also this hypothesis needs further investigation.

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References

Addy HD, Hambleton S, Currah RS. 2000. Distribution and molecular characterization of the root endophyte *Phialocephala fortinii* along an environmental gradient in the boreal forest of Alberta. *Mycological Research* 104: 1213-1221.

Addy HD, Piercey MM, Currah RS. 2005. Microfungal endophytes in roots. *Canadian Journal of Botany* 83: 1-13.

Ahlich K, Sieber TN. 1996. The profusion of dark septate endophytic fungi in non-ectomycorrhizal fine roots of forest trees and shrubs. *New Phytologist* 132: 259-270.

Allen TR, Millar T, Berch SM, Berbee ML. 2003. Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. *New Phytologist* 160: 255-272.

Barrow JR, Aaltonen RE. 2001. Evaluation of the internal colonization of *Atriplex canescens* (Pursh) Nutt. roots by dark septate fungi and the influence of host physiological activity. *Mycorrhiza* 11: 199-205.

Barrow JR. 2003. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in arid southwestern USA rangelands. *Mycorrhiza* 13: 239-247.

Bougoure DS, Cairney JWG. 2005a. Fungi associated with hair roots of *Rhododendron lochiae* (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture-independent molecular methods. *Environmental Microbiology* 7(11): 1743-1754.

Bougoure DS, Cairney JWG. 2005b. Assemblages of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (Ericaceae) as determined by culturing and direct DNA extraction from roots. *Environmental Microbiology* 7(6): 819-827.

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N. 1996. *Working with mycorrhizas in forestry and agriculture*. ACIAR Monograph 32, Canberra. 374 pp.

Cairney JW, Ashford AE. 2002. Biology of mycorrhizal associations of epacrids (*Ericaceae*). *New Phytologist* 154: 305-326.

Cairney JWG, Meharg AA. 2003. Ericoid mycorrhiza: a partnership that expoits harsh edaphic conditions. *European Journal of Soil Science* 54: 735-740.

Cázares E, Trappe JM, Jumpponen A. 2005. Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. *Mycorrhiza* 15: 405-416.

Chaurasia B, Pandey A, Palni LMS. 2005. Distribution, colonization and diversity of arbuscular mycorrhizal fungi associated with central Himalayan rhododendrons. *Forest Ecology and Management* 207: 315-324.

Currah RS, Tsuneda A, Murakami S. 1993a. Conidiogenesis in *Oidiodendron periconioides* and ultrastructure of ericoid mycorrhizas formed with *Rhododendron brachycarpum. Canadian Journal of Botany* 71: 1481-1485.

Currah RS, Tsuneda A, Murakami S. 1993b. Morphology and ecology of *Phialocephala fortinii* in roots of *Rhododendron brachycarpum. Canadian Journal of Botany* 71: 1639-1644.

Dighton J, Coleman DC. 1992. Phosphorus relations of roots and mycorrhizas of *Rhododendron maximum* L. in the southern Appalachians, North Carolina. *Mycorrhiza* 1: 175-184.

Domsch KH, Gams W, Anderson, T. 1980. Compendium of Soil Fungi. Academic Press, New York.

Dostálková A. 1981. *Rododendrony (Rhododendrons).* Academia, Prague. 155 pp.

Douglas GC, Heslin MC, Reid C. 1989. Isolation of *Oidiodendron maius* from *Rhododendron* and ultrastructural characterization of synthesized mycorrhizas. *Canadian Journal of Botany* 67: 2206-2212.

Duddridge JA, Read DJ. 1982. Ultrastructural analysis of the development of mycorrhizas in *Rhododendron ponticum. Canadian Journal of Botany* 60: 2345-2356.

Hambleton S, Currah RS. 1997. Fungal endophytes from the roots of alpine and boreal Ericaceae. *Canadian Journal Botany* 75: 1570-1581.

Hambleton S, Sigler L. 2005. *Meliniomyces*, a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (= *Hymenoscyphus ericae*), *Leotiomycetes. Studies in Mycology* 53: 1-27

Haselwandter K, Read DJ. 1980. Fungal associations of roots of dominant and sub-dominant plants in high-alpine vegetation systems with special reference to mycorrhiza. *Oecologia* 45: 57-62.

Hutton BJ, Dixon KW, Sivasithamparam K. 1994. Ericoid endophytes of Western Australian heaths (Epacridaceae). *New Phytologist* 127: 557-566.

Jumpponen A, Trappe JM. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytologist* 140: 295-310.

Jumpponen A. 2001. Dark septate endophytes – are they mycorrhizal? *Mycorrhiza* 11: 207-211.

Mandyam K, Jumpponen A. 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology* 53: 173-189.

Mejías JA, Arroyo J, Ojeda F. 2002. Reproductive ecology of *Rhododendron ponticum* (Ericaceae) in relict Mediterranean populations. *Botanical Journal of the Linnean Society* 140: 297-311.

Midgley DJ, Chambers SM, Cairney WG. 2004. Distribution of ericoid mycorrhizal endophytes and root-associated fungi in neighbouring Ericaceae plants in the field. *Plant and Soil* 259: 137-151.

Moore-Pankhurst S, Englander L. 1981. A method for the synthesis of mycorrhizal association between *Pezizella ericae* and *Rhododendron maximum* seedlings growing in a defined medium. *Mycologia* 73: 994-997.

Ohki T, Masuya H, Yonezawa M, Usuki F, Narisawa K, Hashiba T. 2002. Colonization process of the root endophytic fungus *Heteroconium chaetospira* in roots of Chinese cabbage. *Mycoscience* 43: 191-194.

Pearson V, Read DJ. 1973. The biology of mycorrhiza in the *Ericaceae*. I. The isolation of the endophyte and synthesis of mycorrhiza in aseptic culture. *New Phytologist* 72: 371-379

Peterson RL, Massicotte, HB, Melville, LH. 2004. *Mycorrhizas: Anatomy and Cell Biology*. NRC Research Press, Ottawa.173 pp.

Rains KC, Nadkarni NM, Bledsoe CS. 2003. Epiphytic and terrestrial mycorrhizas in a lower montane Costa Rican cloud forest. *Mycorrhiza* 13: 257-264.

Read DJ, Haselwandter K. 1981. Observations on the mycorrhizal status of some alpine plant communities. *New Phytologist* 88: 341-352.

Read DJ, Perez-Moreno J. 2003. Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytologist* 157: 475-492.

Ruotsalainen AL, Väre H, Vestberg M. 2002. Seasonality of root fungal colonization in low-alpine herbs. *Mycorrhiza* 12: 29-36.

Schadt CW, Mullen RB, Schmidt SK. 2001. Isolation and phylogenetic identification of a dark-septate fungus associated with the alpine plant *Ranunculus adoneus*. *New Phytologist* 150: 747-755.

Smith, SE, Smith FA. 1990. Tansley Review No. 20: Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. *New Phytologist* 114: 1-38.

Smith SE, Read DJ. 1997. *Mycorrhizal Symbiosis*. Second Edition. Academic Press. London. 605 pp.

Stoyke G, Currah RS. 1991. Endophytic fungi from the mycorrhizae of alpine ericoid plants. *Canadian Journal of Botany* 69: 347-352.

Stoyke G, Egger KN, Currah RS. 1992. Characterization of sterile endophytic fungi from the mycorrhizae of subalpine plants. *Canadian Journal of Botany* 70: 2009-2016.

Urcelay C. 2002. Co-occurrence of three fungal root symbionts in *Gaultheria poeppiggi* DC in Central Argentina. *Mycorrhiza* 12: 89-92.

Usuki F, Junichi AP, Kakishima M. 2003. Diversity of ericoid mycorrhizal fungi isolated from hair roots of *Rhododendron obtusum* var. *kaempferi* in a Japanese red pine forest. *Mycoscience* 44: 97-102.

Usuki F, Narisawa K. 2005. Formation of structures resembling ericoid mycorrhizas by the root endophytic fungus *Heteroconium chaetospira* within roots of *Rhododendron obsutum* var. *kaempferi*. *Mycorrhiza* 15: 61-64.

Vohník M, Lukančič S, Bahor E, Regvar M, Vosátka M, Vodnik D. 2003. Inoculation of *Rhododendron* cv. Belle-Heller with two strains of *Phialocephala fortinii* in two different substrates. *Folia Geobotanica* 38: 191-200.

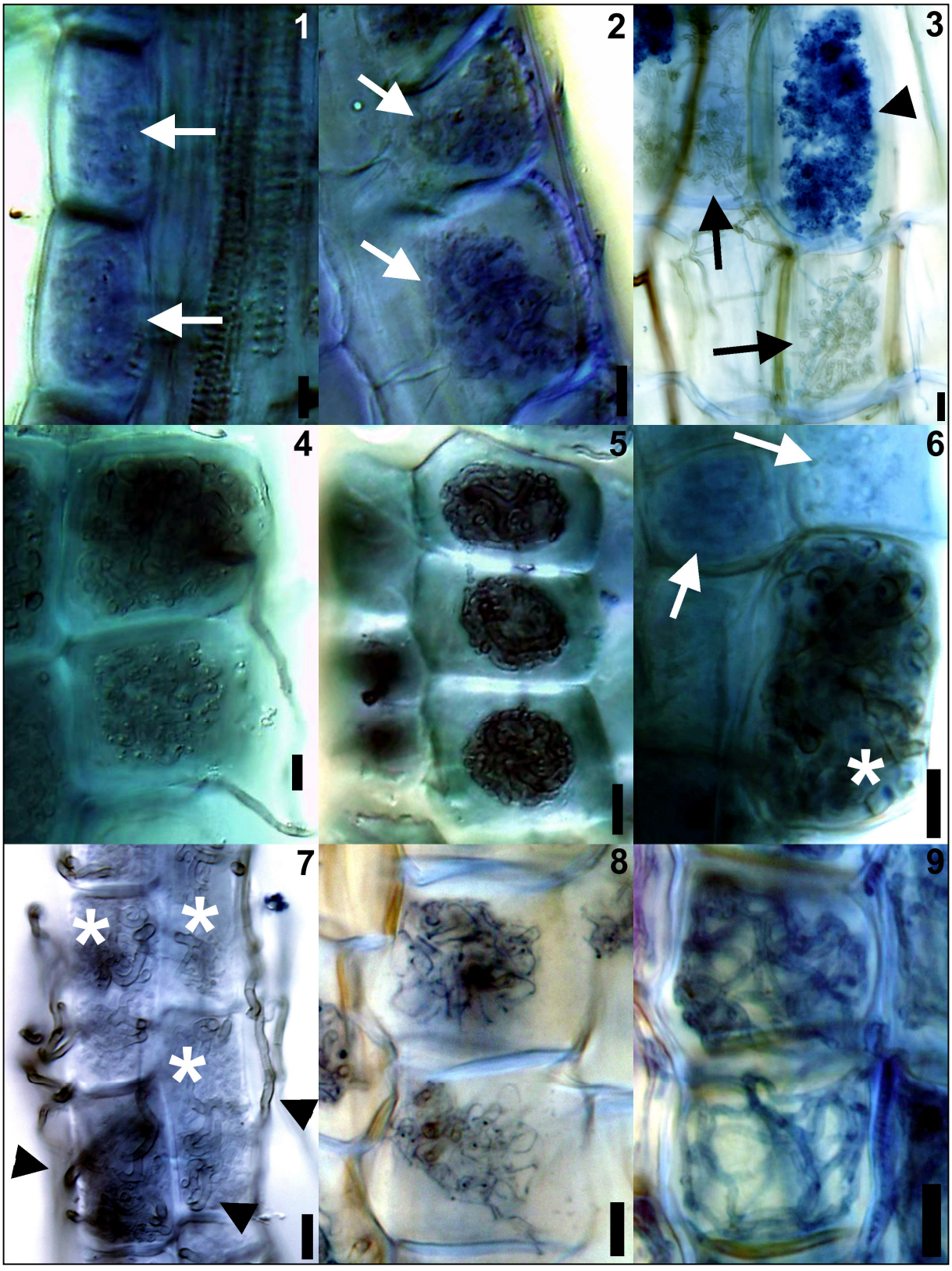
Vohník M, Albrechtová J, Vosátka M. 2005. The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro. *Symbiosis* 40: 87-96.

Wurzburger N, Bledsoe CS. 2001. Comparison of ericoid and ectomycorrhizal colonization and ectomycorrhizal morphotypes in mixed conifer and pygmy forests on the northern California coast. *Canadian Journal of Botany* 79: 1202-1210.

Yu T, Nassuth A, Peterson RL. 2001. Characterization of the interaction between the dark septate fungus *Phialocephala fortinii* and *Asparagus officinalis* roots. *Canadian Journal of Microbiology* 47: 741-753.

FIGURES

Figure 1: Typical ericoid mycorrhizal (ErM) colonization: fine blue-stained intracellular coils or loops (arrows); PON. **Figure 2**: Typical ErM colonization: fine blue-stained intracellular coils or loops (arrows); FER-Aut. **Figure 3:** Typical ErM colonization: fine blue-stained (arrowhead) or hyaline (arrows) intracellular coils or loops; LUT. **Figure 4:** Typical ErM colonization: fine hyaline to dark intracellular coils or loops; PON. **Figure 5:** Typical ErM colonization: dense black intracellular coils or loops; PON. **Figure 6:** Two distinctive ErM colonization patterns: fine blue-stained coils (arrows) and thicker dark brown coils (asterisk); FER-Esp. **Figure 7:** Typical ErM colonization: dense hyaline coils (asterisks) connected with dark, septate extraradical hyphae (arrowheads); LUT. **Figure 8:** Typical ErM colonization: blue-stained thin hyphae forming fine coils; LUT. **Figure 9:** Typical ErM colonization: thick, blue-stained hyphae forming loose coils; HIR. **Figure 10:** Typical DSE colonization: thick dark septate (DS) hypha (arrow) enters the rhizodermal cell and forms hyaline or blue stained or dark brown microsclerotia (m). The same thick DS hypha changes its diameter and forms fine loose loops (h; = the intermediate structures) in other cells; FER-Aut. **Figure 11:** Typical DSE colonization: a parenchymatous net (asterisk) formed by DS hyphae (arrows); KOT. **Figure 12:** A detail of the parenchymatous net; KOT. **Figure 13:** Typical DSE colonization: thick DS hyphae (arrows) forming hyaline hyphae (arrowheads), which explore the surface of the hair root; PON. **Figure 14:** Typical DSE colonization: DS hypha (arrowheads) enters the cell and forms thick, hyaline or blue-stained hyphae with septa (h). These hyphae remain narrow or become coiled; LUT. **Figures 15a, b, c:** A sequence of three pictures of the same hair root, showing morphology of atypical colonization, formed in the roots of KOT (bars = 50µm). **Figure 15a:** DS hyphae form loose coils (c; = the intermediate structures) in the rhizodermal cells. **Figure 15b:** The cells below the rhizodermis, which is colonized by the intermediate structures (c), are filled with dense blue-stained ErM-like coils (arrows). **Figure 15c:** Most of the rhizodermal cells are colonized by the intermediate structures (c). ErM-like, blue stained dense coils (arrows) are situated in the second layer of the root cells. **Figure 16:** Atypical colonization formed in the roots of HIR: loose coils formed by thick hyaline hyphae (arrowheads). **Figure 17:** Atypical colonization formed in the roots of FER-Fra: vesicles (v) resembling DSE microsclerotia are surrounded by blue-stained ErM (asterisks). **Figure 18:** Typical morphology of the intermediate structures: DS hyphae forming intracellular loose coils (arrows); FER-Fra. **Figure 19:** Typical morphology of the intermediate structures: DS hyphae forming intracellular loose coils (c); KOT. **Figure 20:** Typical morphology of the intermediate structures: DS hyphae (arrowheads) connected with intracellular loose coils (c); HIR. **Figures 21a – e:** A sequence of five pictures of the same hair root, showing morphology of atypical colonization, formed in the roots of FER-Aut, FER-Fra and KOT (bars = 50µm). **Figure 21a:** Dorsal view of a parenchymatous net (p) formed by DS hyphae under the layer of empty rhizodermal cells. A layer of cells filled with dense blue-stained ErM-like coils (asterisks) lies below this net; KOT. **Figure 21b:** A detail of the layer of the cells filled with dense blue-stained ErM-like coils (asterisks), which is formed below the parenchymatous net (p); KOT. **Figure 21c:** A cross view of the parenchymatous net (arrowheads), formed below the layer of empty rhizodermal cells (ec). The layer of cells filled with dense blue-stained ErM-like coils (asterisks) lies below this net. The ErM-like coils are connected with hyphae, forming the parenchymous net (arrow); KOT. **Figure 21d:** Hyphal connections of the parenchymatous net with the ErM-like coils (arrows); KOT. **Figure 21e:** Ventral view of the parenchymatous net (p); KOT. All bars correspond to 10µm unless other value is given.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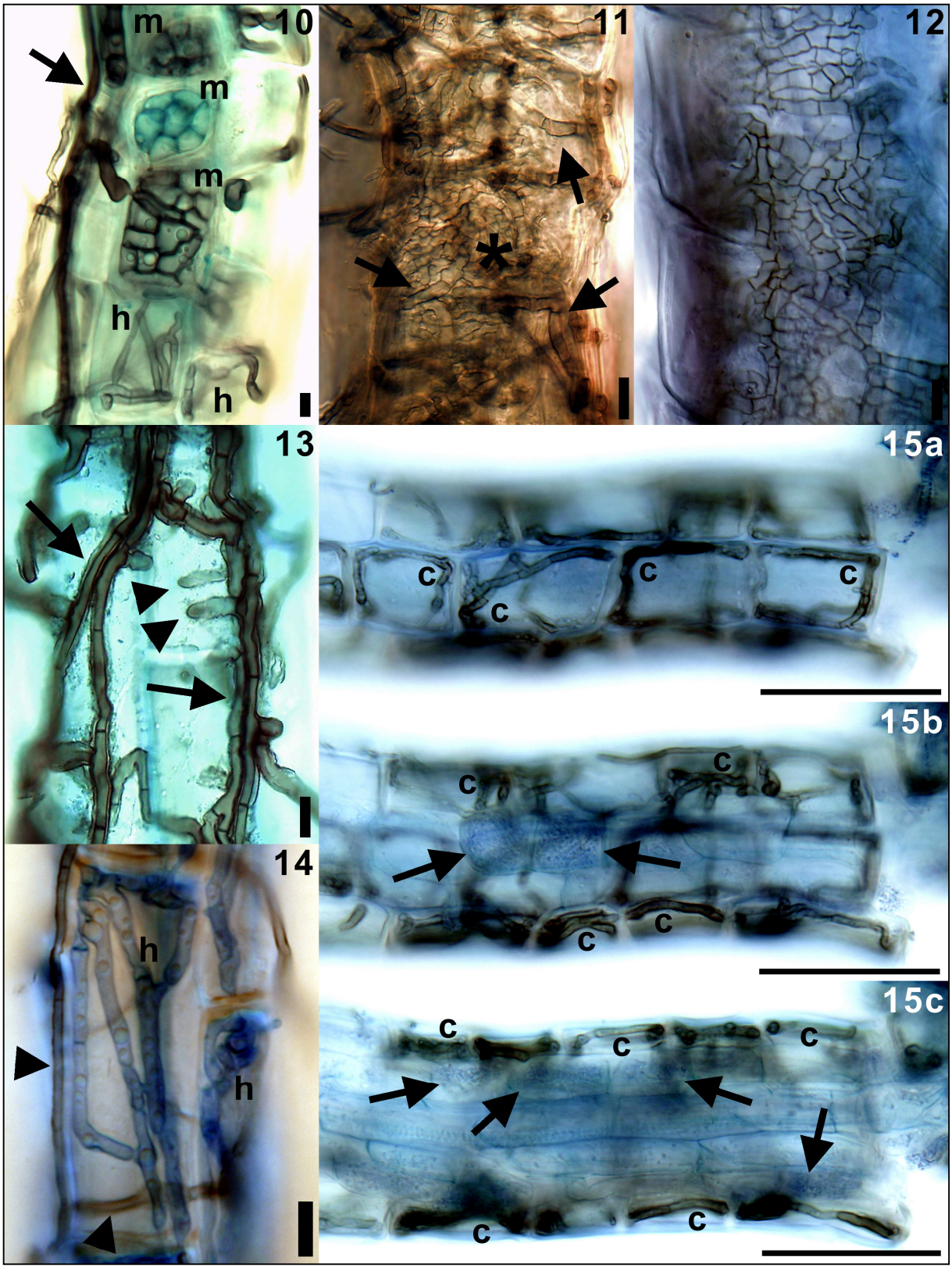
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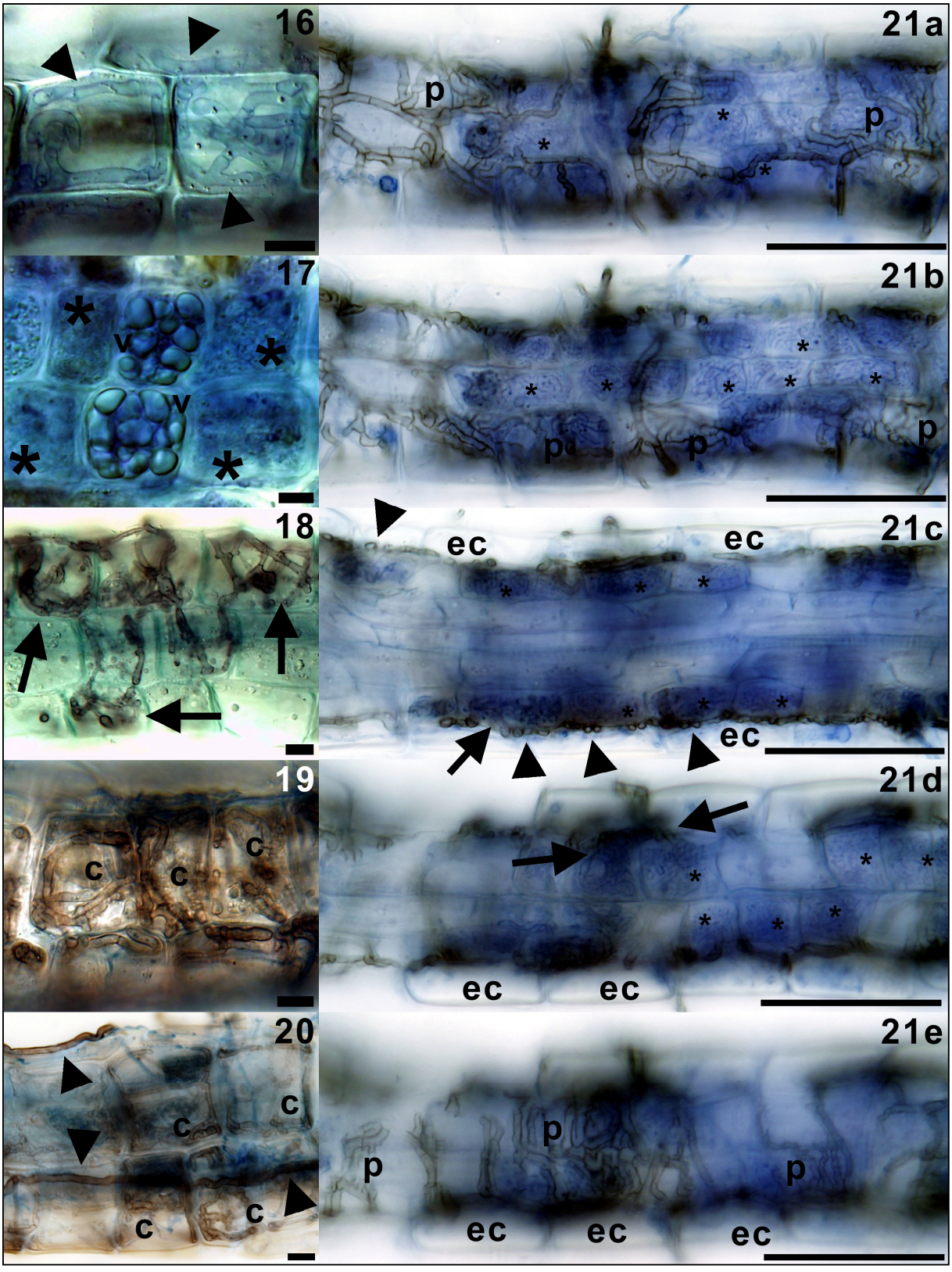
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Table 1: The list of *Rhododendron* species screened in this study, with their localities and short descriptions of observed root-fungus associations

	DSE/ErM/INT/ Total (%)	Ericoid mycorrhiza features	DSE-association features	Other mycelium in the rhizosphere / remarks
FER-Aut: *R. ferrugineum* (Austria, Gross Venediger Massif); 1693 – 2375 m a. s. l.; August 2005	n. a.	blue-stained, brown and hyaline hyphal coils	microsclerotia (brown, blue), brown hyphal coils, the parenchymatous net	trypan blue-stained basidiomycetous hyphae
FER-Fra: *R. ferrugineum* (France, Mt. Blanc Massif); 1459 – 2325 m a. s. l.; August 2005	n. a.	blue-stained, brown and hyaline hyphal coils	microsclerotia (brown, hyaline), brown hyphal coils, the parenchymatous net	samples from higher sites dominated by DSE
FER-Esp: *R. ferrugineum* (Spain, Central Pyrenees); 1792 – 2145 m a. s. l.; November 2005	6.8 / 41.6 / 6.2/ 54.6	blue-stained and hyaline hyphal coils	occasional microsclerotia (brown), occasionally the parenchymatous net	samples from highest site dominated by ErM
HIR: *R. hirsutum* (Slovenia, Velika Planina); ~1400 m a. s. l.; June 2005	5.6 / 58.8 / 10.5/ 74.9	blue-stained, brown and hyaline hyphal coils	microsclerotia (brown), brown and hyaline hyphal coils	trypan blue-stained basidiomycetous hyphae, atypical thick hyaline intracellular hyphal loops
KOT: *R. kotschyi* (Romania, Carpathian Mts.); 1724 - 2505 m a. s. l.; September 2005	32.4 / 30.2 / 7.6/ 70.2	blue-stained and hyaline hyphal coils	loose net of brown DSE hyphae over some roots, brown coils, the parenchymatous net	roots of two individuals totally dominated by DSE, ErM present only sporadically
LAP: *R. lapponicum* (Finland, Kilpisjärvi); ~480 m a. s. l.; –2.3°C; 69°03'N; precipitations 25 – 65 mm; November 2005	55.0 / 8.5 / 22.4/ 85.9	blue-stained, brown and hyaline hyphal coils	microsclerotia (brown), loose adhering net of thick dark brown hyphae over some roots, brown coils	no other hyphae present
LUT: *R. luteum* (Slovenia, Boštanj); ~220 m a. s. l.; September 2005	22.7 / 16.8 / 7.5/ 47.0	blue-stained and brown hyphal coils	microsclerotia (brown), brown hyphal coils	brown and hyaline basidiomycetous hyphae
PON: *R. ponticum* (Spain, Puerto de Galis); ~420 m a. s. l.; 14 - 16°C; 36°08'N; precipitations 800 – 1400 mm; April 2005	8.6 / 52.0 / 3.5/ 64.1	blue-stained, brown, black and hyaline hyphal coils	brown to hyaline coils, brown and blue-stained microsclerotia; thick DS hyphae on the root surface	trypan blue-stained AM hyphae, samples dominated by black to hyaline ErM coils

Table 2: Ratios between ErM-colonized, DSE-colonized, INT-colonized and non-colonized cells of hair roots of six screened *Rhododendron* species. ErM = ericoid mycorrhiza; DSE = dark septate endophytes; non = non-colonized cells; INT = the intermediate structures; Col = cells colonized by ErM + DSE + INT. The values are mean ± SD. Different letters indicate significantly different groups of data using Kruskal-Wallis ANOVA and Kolmogorov-Smirnov two-sample test (p = 0.05).

	ErM : DSE	**DSE : non**	**ErM : non**	**INT : non**	**Col : non**
R. ferrugineum (n= 50)	53.231 ± 73.87 **c**	0.165 ± 0.21 **a**	1.126 ± 0.87 **c**	0.170 ± 0.19 **b**	1.461 ± 0.96 **b**
R. hirsutum (50)	82.007 ± 82.61 **c**	0.280 ± 0.45 **a**	3.000 ± 2.67 **e**	0.491 ± 0.50 **c**	3.771 ± 2.73 **c**
R. kotschyi (50)	1.708 ± 2.24 **b**	1.828 ± 1.84 **c**	1.596 ± 1.67 **cd**	0.444 ± 0.60 **c**	3.869 ± 3.67 **c**
R. lapponicum (50)	0.168 ± 0.14 **a**	5.761 ± 4.08 **d**	1.054 ± 1.56 **b**	2.502 ± 1.99 **d**	9.318 ± 7.11 **d**
R. luteum (50)	3.126 ± 9.54 **b**	0.557 ± 0.57 **b**	0.355 ± 0.30 **a**	0.221 ± 0.36 **ab**	1.134 ± 0.93 **a**
R. ponticum (50)	44.176 ± 58.52 **c**	0.358 ± 0.59 **a**	1.902 ± 1.32 **d**	0.095 ± 0.12 **a**	2.356 ± 1.64 **c**

1. 3. ČLÁNEK 2

In vitro interaction between dark septate endophytic *Phialocephala fortinii* and ericoid mycorrhizal *Rhizoscyphus ericae* and its effect on the growth and colonization rates of *Vaccinium myrtillus*

(manuskript)

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In vitro interaction between dark septate endophytic *Phialocephala fortinii* and ericoid mycorrhizal *Rhizoscyphus ericae* and its effect on the growth and colonization rates of *Vaccinium myrtillus*

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Abstract

In the present study, we investigated the effect of an *in vitro* interaction between an ericoid mycorrhizal (ErM) fungus *Rhizoscyphus ericae* (= RER-1) and two strains of a dark septate endophytic (DSE) fungus *Phialocephala fortinii* (= PFO-F and PFO-H) on their growth and coexistence in a mixed culture and on the growth and ErM/DSE colonization rates of their common host plants. The interaction between *P. fortinii* and *R. ericae* in a mixed culture was strain-specific. The prevailing mode of the interaction between PFO-F and RER-1 was coexistence, whereas the prevailing mode of the interaction between PFO-H and RER-1 was dominance of one fungus over the other. The growth of *V. myrtillus* seedlings was significantly influenced by different proportions of *P. fortinii* and *R. ericae* mycelia in their rhizosphere, which was connected with differences in the DSE and ErM colonization rates in their roots. The growth of the seedlings was positively correlated with increased ErM colonization and decreased DSE colonization. This is the first comparison of the effects of DSE-association and ErM on the growth of their common host plants. The effect of DSE-association on *V. myrtilllus* seedlings was relatively negative to neutral in comparison with the effect of ErM. One of the components of the positive effect of *R. ericae* could be moderation of the levels of DSE colonization.

Introduction

Ericoid mycorrhiza (ErM) represents the main root-fungus association in *Ericaceae* (Read, 1996). However, dark septate endophytic (DSE) fungi also inhabit ericaceous roots forming so-called DSE-association. DSE-association often co-occurs with other types of mycorrhizae (Mandyam and Jumpponen, 2005). Its co-occurrence with ErM was reported in studies using microscopic (Urcelay, 2002; Rains et al., 2003; Peterson et al., 2004; Cázares et al., 2005) and/or molecular methods (Hambleton and Currah, 1997; Midgley et al., 2004; Bougoure & Cairney, 2005).

The importance of ErM fungi for their host plants is well established (Smith and Read, 1997). In contrast, only little is known about DSE-association (Jumpponen and Trappe, 1998;

Jumpponen, 2001; Mandyam and Jumpponen, 2005). Considering the beneficial effect of *Phialocephala fortinii* Wang & Wilcox on their host plants, Jumpponen (2001) characterized the relationship between DSE fungi and their host plants as mycorrhizal. The eco-physiological significance of the co-occurrence of ErM and DSE-association in a common host plant is unknown. A wide range of interactions between these two types of root-fungus associations can be expected, with a wide range of effects on their host plants. Hypothetically, the host plants colonized simultaneously by ErM and DSE-association might gain an advantage over plants colonized by only one type of association, for example, by accessing a broader spectrum of nutrient sources.

In our previous study (Vohník et al., 2005), we separately or simultaneously inoculated *Rhododendron* cv. Azurro with *Oidiodendron maius* Barron and two strains of *P. fortinii* in a split root system and traced the effects of the (co-)inoculation on the host plants. Inoculation with single *O. maius* was the most effective in terms of phosphorus and nitrogen uptake by the host plants, but also the co-inoculation with *O. maius* and *P. fortinii* H led to increased uptake of both nutrients in comparison with non-inoculated plants. However, no synergic effect was observed when host plants were simultaneously inoculated with both *O. maius* and *P. fortinii* H. Inoculation with single *P. fortinii* H led to increased phosphorus uptake, but nitrogen uptake was not affected in comparison with the non-inoculated plants. Thus, the beneficial effect of the *O. maius* + *P. fortinii* H co-inoculation on nutrient uptake by the host plants could be attributed either to both interacting fungi or only to *O. maius*. *P. fortinii* H would then represent a neutral root associate.

In the present study, we investigated the interactions between the typical ErM fungus *Rhizoscyphus ericae* Zhuang & Korf and the typical DSE fungus *P. fortinii in vitro*. In Experiment 1, we screened their growth and the mode of their co-existence in a mixed culture. In Experiment 2, we followed the effects of their interaction on the growth of *Vaccinium myrtillus* L. seedlings and the DSE/ErM colonization rates in their roots.

Materials and methods

Fungal isolates

We tested *in vitro* interactions between two strains of the DSE fungus *P. fortinii* (= PFO-F and PFO-H) and one strain of the ErM fungus *R. ericae* (= RER-1). The PFO strains were previously isolated from surface-sterilized roots of *V. myrtillus* (PFO-F) and *Rhododendron* sp. (PFO-H) by Jansa and Vosátka (2000) and were identified as strains of *P. fortinii* using both phenotype and genotype analysis (Vohník et al., 2003). Both strains are deposited in the Culture Collection of Fungi (Department of Botany, Faculty of Science, Charles University in Prague, CZ) under the accession numbers CCF 3586 (PFO-F) and CCF 3587 (PFO-H). RER-1 is the culture derived from the strain UAMH 6735 (= GenBank AJ319078) isolated from the roots of *Calluna vulgaris* Hull. by Pearson and Read (1973).

Experiment 1

Each of six Petri dishes with MMN was inoculated with six agar plugs (diam. 5 mm) overgrown by either PFO mycelium or RER-1 mycelium. Three dishes contained a combination of three plugs with PFO-F and three plugs with RER-1; the remaining dishes contained a combination of three plugs with PFO-H and three plugs with RER-1. Figure 1 illustrates the positioning of the plugs in each dish. The inoculated dishes were incubated in the dark at room temperature for one month. During this period, the PFO and the RER-1 mycelia radiating from the plugs came into contact and subsequently, PFO hyphae started to grow around and through RER-1 colonies (Fig. 1). At this moment, we extracted new agar plugs from the area where the PFO mycelium overlapped with the RER-1 mycelium (= the overlapping area) and from the area where either PFO (= the PFO-dominated area) or RER-1 (= the RER-1-dominated area) dominated (Fig. 1) and transferred them into new Petri dishes with MMN. The new plugs from the overlapping area were extracted in a way that one half of each plug contained a majority of the PFO mycelium and the other half a majority of the RER-1 mycelium. Each new dish contained five new plugs: one from the PFO-dominated area, one from the RER-1-dominated area, and three from the overlapping area. Figure 2 illustrates the positioning of the new plugs in the new dishes. There were 15 dishes containing the PFO-F + RER-1 combination and 15 dishes containing the PFO-H + RER-1 combination. The dishes with the new plugs were incubated in the dark at room temperature. Two types of mycelium started to radiate from the new plugs, either separately or simultaneously from one plug. One type corresponded to PFO and the other corresponded to RER-1. Both types formed dense delimited colonies with progressing time (Fig. 2).

After one week of cultivation, we determined the dominance of PFO and/or RER-1 in these colonies. This was done by macroscopic evaluation of the proportions of PFO/RER-1 mycelium in each colony with respect to the different origins of the plugs, and by comparing the observed frequencies of both mycelia with expected frequencies using a χ^2 test. We expected that the colonies radiating from the plugs from the PFO-dominated area would be formed only by the PFO mycelium, the colonies radiating from the plugs from the RER-1-dominated area would be formed only by the RER-1 mycelium, and that the colonies radiating from the plugs from the overlapping area would be formed by two approximately equal PFO and RER-1 sub-colonies. Thus, the expected frequency derived from the number of dishes for each PFO + RER-1 combination (= 15) was: i) in the plugs from the PFO-dominated area: 15 for both PFO strains, 0 for RER-1; ii) in the plugs from the RER-1-dominated area: 15 for RER-1, 0 for both PFO strains; and iii) in the overlapping area: 15 for both PFO strains and for RER-1. The same evaluation was performed after 10 weeks from the beginning of the experiment.

After one week from the beginning of the experiment, we also measured the diameters of three colonies in each dish, each colony representing one of the three areas described above. From the three colonies representing the overlapping area, only the middle was measured. The obtained

values were tested with Levene's test for homogeneity of variances and with Kolmogorov-Smirnov's test for normal distribution. Because the assumptions of ANOVA were met, they could be analyzed using 3-way ANOVA, where the PFO strain (PFO-F and PFO-H), the origin of the plug (the PFO-dominated, the RER-1-dominated, and the overlapping area), and the dish (1 to 15 for both PFO + RER-1 combinations) were chosen as independent factors and the colony diameter as a dependent variable. The effect of the dish was nested in the effect of the PFO strain. Statistically significant differences between the variants (= the PFO + RER-1 combinations) and between their sub-variants (= the different areas of the PFO/RER-1 dominance) were evaluated using a HSD test ($p=0.05$).

Experiment 2

Each of twelve Petri dishes with MMN was inoculated with five agar plugs, one plug from the PFO-dominated area, one from the RER-1-dominated area, and three plugs from the overlapping area. The plugs were obtained and arranged in the dishes in the same way as in Experiment 1. This design was chosen to ensure that the seedlings from the different sub-variants would develop under similar environmental conditions, especially under similar humidity and similar CO_2 concentration. The same design as Experiment 1 also enabled a comparison of results of both experiments. Six of the dishes contained the PFO-F + RER-1 combination and six dishes contained the PFO-H + RER-1 combination. The dishes with the plugs were incubated one week at room temperature in the dark. Then, six-week-old axenic seedlings of *V. myrtillus* were inserted into the central part of each plug. The dishes with the seedlings were sealed with Parafilm™ and with a protective adhesive tape and cultivated in a growth chamber (16/8h day/night, 23°C, 150 µmol m^{-2} s^{-1}). There were five seedlings in each dish, one seedling in the plug from the PFO-dominated area, three seedlings in the plugs from the overlapping area and one seedling in the plug from the RER-1-dominated area. Figure 3 illustrates the positioning of the plugs with the seedlings in the dishes.

After two months, the seedlings were carefully extracted from the dishes and weighed. From the seedlings representing the overlapping area, only the middle was weighed. The seedlings were too small to measure their dry weight, especially when their roots were separated for an estimation of colonization rates. Thus, only their total fresh weight (TFW) could be recorded. The use of TFW as an indicator of biomass production is, in our opinion, justified by the fact that the seedlings from all three sub-variants were cultivated together under the same water and CO_2 regime. Two seedlings from the PFO-dominated area died back during cultivation, one in the PFO-F + RER-1 variant and one in the PFO-H + RER-1 variant. To maintain the same number of dishes in each sub-variant, we included the TFW of the seedlings from the dishes with the withered seedlings, considering the TFW of both withered seedlings as zero, in the statistical analysis. The obtained values were tested with Levene's test for homogeneity of variances and with Kolmogorov-Smirnov's test for normal distribution. Because the assumptions of ANOVA were met, they could

be analyzed using 3-way ANOVA, where the PFO strain, the origin of the plug, and the dish (1 to 6 for both PFO + RER-1 combinations) were chosen as independent factors and TFW of the *V. myrtillus* seedlings as a dependent variable. The effect of the dish was nested in the effect of the PFO strain. Statistically significant differences between the variants and their sub-variants were evaluated using a LSD test ($p = 0.05$).

After weighing, the roots of the seedlings were separated from the shoots, cleared in 10% KOH, acidified in 3% HCl, and stained with trypan blue. All stained roots were then screened at high magnifications (400x or 1000x) for the presence of ErM and DSE structures using a light microscope equipped with DIC. We considered intracellular dense hyaline coils or loops as indicators of ErM and microsclerotia and/or thick dark or hyaline septate hyphae as indicators of DSE-association. In addition to these characteristic structures, we also found loose intracellular loops formed by fine hyaline hyphae (Fig. 4), which were morphologically intermediate between the ErM and the DSE colonization patterns.

After this preliminary screening, we selected the seedlings with the root systems formed by at least four lateral roots and recorded their colonization by DSE-association, ErM, and intermediate structures (IS). The colonization was recorded on a cell-by-cell basis in at least four lateral roots per screened seedling. At least 400 rhizodermal cells (100 cells per lateral root) were evaluated in the smallest root systems, but in average 750 cells were evaluated per root system. Then, for each screened seedling we calculated the average colonization by DSE-association, ErM, and IS as well as the ratios (1) between the DSE-colonized and the non-colonized cells, (2) between the ErM-colonized and the non-colonized cells, (3) between the colonized (= DSE + ErM + IS) and the non-colonized cells, and (4) between the DSE-colonized and the ErM-colonized cells. When the root system of a seedling was colonized by only one type of root-fungus association, we enabled the calculation of the ratios by considering that just one cell of such seedling was colonized by the other type. The obtained values were tested with Levene's test for homogeneity of variances and with Kolmogorov-Smirnov's test for normal distribution. The ratios between the DSE- and the ErM-colonized cells were log-transformed. After that, the assumptions of ANOVA were met and we analyzed the ratios using 3-way ANOVA, where the PFO strain, the origin of the plug, and the dish were chosen as independent factors and the four ratios as dependent variables. The effect of the dish was nested in the effect of the PFO strain. Statistically significant differences between the variants were evaluated using a LSD test ($p = 0.05$).

Results

Experiment 1

After one week from the beginning of the experiment, the observed frequencies of all three fungi did not significantly differ from the expected frequencies. In the PFO-F + RER-1 variant, the PFO-F mycelium occupied at least one half of the colony in 30 plugs (= 15 plugs from the PFO-F-

dominated area + 15 plugs from the overlapping area) vs. the expected 30 plugs ($\chi^2 = 0.00$, d.f. = 2, $p < 1.00$) and the RER-1 mycelium occupied at least one half of the colony in 27 plugs (= 15 plugs from the RER-1-dominated area + 12 plugs from the overlapping area) vs. the expected 30 plugs ($\chi 2 = 0.60$, d. f. = 2, $p < 0.74$). In the PFO-H + RER-1 variant, the PFO-H mycelium occupied at least one half of the colony in 30 plugs (= 15 plugs from the PFO-H-dominated area + 15 plugs from the overlapping area) vs. the expected 30 plugs ($\chi^2 = 0.00$, d. f. = 2, $p < 1.00$) and the RER-1 mycelium occupied at least one half of the colony in 29 plugs (= 15 plugs from the RER-1-dominated area + 14 plugs from the overlapping area) vs. the expected 30 plugs ($\chi^2 = 0.07$, d. f. = 2, $p < 0.97$).

After 10 weeks however, the observed frequencies of the RER-1 mycelium significantly differed from the expected frequencies. In the PFO-F + RER-1 variant, the RER-1 mycelium occupied at least one half of the colony in 13 plugs (= 12 plugs from the RER-1-dominated area + 1 plug from the overlapping area) vs. the expected 30 plugs ($\chi^2 = 13.67$, d. f. = 2, $p < 0.001$). In the PFO-H + RER-1 variant, the RER-1 mycelium occupied at least one half of the colony in 8 plugs (= 8 plugs from the RER-1-dominated area + 0 plugs from the overlapping area) vs. the expected 30 plugs ($\chi^2 = 18.27$, d. f. = 2, $p < 0.0001$). The observed frequencies of the mycelium of both PFO strains did not differ from the expected frequencies after 10 weeks (in both cases $\chi^2 = 0.00$, d. f. = 2, $p < 1.00$). The PFO-F mycelium occupied at least one half of the colony in 44 plugs (= 15 plugs from the PFO-F-dominated area + 15 plugs from the overlapping area + 14 plugs from the RER-1-dominated area) vs. the expected 30 plugs and the PFO-H mycelium occupied at least one half of the colony in 38 plugs (= 15 plugs from the PFO-H-dominated area + 15 plugs from the overlapping area + 8 plugs from the RER-1-dominated area) vs. the expected 30 plugs.

There were significant effects of the PFO strain ($F = 11.9$, $p = 0.0009$), the origin of the plug ($F = 147.5$, $p = 0.0000$), and the interaction between the PFO strain and the origin of the plug ($F = 18.9$, $p = 0.0000$) on the size of the colonies radiating from the plugs. The effect of the dish was not significant ($F = 1.4$, $p = 0.25$). The colonies from the PFO-F + RER-1 variant radiating from the plugs from the PFO-F-dominated area had an average diameter of 1.60 ± 0.03 cm (mean ± SE; significantly different group d), from the overlapping area an average diameter of 1.53 ± 0.03 cm (cd), and from the RER-1-dominated area an average diameter of 1.37 ± 0.03 cm (b). The colonies from the PFO-H + RER-1 variant radiating from the plugs from the PFO-H-dominated area had an average diameter of 1.63 ± 0.03 cm (d), from the overlapping area an average diameter of 1.47 ± 0.02 cm (bc), and from the RER-1-dominated area an average diameter of 1.14 ± 0.04 cm (a) (Table 1).

Experiment 2

Beside the characteristic DSE and ErM structures (see Materials and Methods), the *V. myrtillus* roots were colonized by loose intracellular loops formed by fine hyaline hyphae (Fig. 4). This colonization pattern was morphologically intermediate between the DSE and the ErM

colonization patterns and was therefore distinguished as a separate colonization category. It was more prominent in the PHO-H + RER-1 variant than in the PFO-F + RER-1 variant, reaching up to 7% and 1% of the screened root cells, respectively.

There was a significant effect of the origin of the plug ($F = 6.4$, $p = 0.017$) on TFW of the *V. myrtillus* seedlings. There was no significant effect of the PFO strain ($F = 0.7$, $p = 0.42$), the dish ($F = 1.7$, $p = 0.19$), or the interaction between the PFO strain and the origin of the plug ($F = 0.3$, $p = 0.61$). The seedlings from the PFO-F + RER-1 variant growing from the plugs from the PFO-F-dominated area had an average weight of 10.5 ± 2.3 mg (ab) and an average 23% colonization by PFO-F and 5% colonization by RER-1. The seedlings from the overlapping area had an average weight of 12.7 ± 1.8 mg (b) and an average 15% colonization by PFO-F and 20% colonization by RER-1. The seedlings from the RER-1-dominated area had an average weight of 14.9 ± 2.3 mg (b) and an average 14% colonization by PFO-F, 27% colonization by RER-1, and 1% colonization by IS. The seedlings from the PFO-H + RER-1 variant growing from the plugs from the PFO-H-dominated area had an average weight of 5.8 ± 2.0 mg (a) and an average 43% colonization by PFO-H and 7% colonization by IS. The seedlings from the overlapping area had an average weight of 15.4 ± 1.8 mg (b) and an average 28% colonization by PFO-H, 15% colonization by RER-1, and 7% colonization by IS. The seedlings from the RER-1-dominated area had an average weight of 12.5 ± 2.4 mg (b) and an average 11% colonization by PFO-H, 36% colonization by RER-1, and 2% colonization by IS (Table 1).

There was a significant effect of the PFO strain ($F = 8.6$, $p = 0.009$) and the origin of the plug ($F = 3.6$, $p = 0.050$) on the ratio between the DSE-colonized and the non-colonized root cells of *V. myrtillus* seedlings. In the seedlings from the PFO-F + RER-1 variant growing from the plugs from the PFO-F-dominated area the ratio was 0.35 ± 0.11 (ab), in the seedlings growing from the overlapping area the ratio was 0.25 ± 0.09 (a), and in the seedlings growing from the RER-1-dominated area the ratio was 0.21 ± 0.09 (a). In the seedlings from the PFO-H + RER-1 variant growing from the plugs from the PFO-H-dominated area the ratio was 0.86 ± 0.06 (c), in the seedlings growing from the overlapping area the ratio was 0.64 ± 0.17 (bc), and in the seedlings growing from the RER-1-dominated area the ratio was 0.25 ± 0.11 (a) (Table 1). There were no effects of the dish ($F = 0.11$, $p = 0.88$) and the interaction between the PFO strain and the origin of the plug ($F = 1.8$, $p = 0.19$).

There was a significant effect of the origin of the plug ($F = 10.1$, $p = 0.001$) on the ratio between the ErM-colonized and the non-colonized root cells of the *V. myrtillus* seedlings. In the seedlings from the PFO-F + RER-1 variant growing from the plugs from the PFO-F-dominated area the ratio was 0.09 ± 0.07 (ab), in the seedlings growing from the overlapping area the ratio was 0.32 ± 0.08 (abc), and in the seedlings growing from the RER-1-dominated area the ratio was 0.39 ± 0.11 (c). In the seedlings from the PFO-H + RER-1 variant growing from the plugs from the PFO-H-dominated area the ratio was 0.01 ± 0.01 (a), in the seedlings growing from the overlapping area the

ratio was 0.33 ± 0.13 (bc), and in the seedlings growing from the RER-1-dominated area the ratio was 0.72 ± 0.08 (d) (Table 1). There were no significant effects of the PFO strain ($F = 1.6$, $p = 0.22$), the dish ($F = 0.2$, $p = 0.82$), and the interaction between the PFO strain and the origin of the plug ($F = 2.1$, $p = 0.15$).

There were no significant effects of the origin of the plug ($F = 1.5$, $p = 0.25$) and the dish ($F = 1.0$, $p = 0.38$) on the ratio between the colonized and the non-colonized rhizodermal cells of the *V. myrtillus* seedlings. Also, the effect of the interaction between the PFO strain and the origin of the plug was not significant ($F = 0.33$, $p = 0.72$). The effect of the PFO strain was only marginally significant ($F = 3.4$, $p = 0.079$) (Table 1).

There was a significant effect of the origin of the plug ($F = 14.9$, $p = 0.0001$) on the ratio between the DSE-colonized and the ErM-colonized rhizodermal cells of the *V. myrtillus* seedlings. In the seedlings from the PFO-F + RER-1 variant growing from the plugs from the PFO-F-dominated area the ratio was 64.6 ± 38.8 (cd), in the seedlings growing from the overlapping area the ratio was 1.05 ± 0.46 (ab), and in the seedlings growing from the RER-1-dominated area the ratio was 0.44 ± 0.17 (a). In the seedlings from the PFO-H + RER-1 variant growing from the plugs from the PFO-H-dominated area the ratio between the DSE-colonized and the ErM-colonized cells was 151.8 ± 64.7 (d), in the seedlings growing from the overlapping area the ratio was 28.91 ± 27.1 (bc), and in the seedlings growing from the RER-1-dominated area the ratio was 0.34 ± 0.14 (a) (Table 1). There were no significant effects of the PFO strain ($F = 1.0$, $p = 0.32$), the dish ($F = 0.1$, $p = 0.92$), and the interaction between the PFO strain and the origin of the plug ($F = 1.27$, $p = 0.31$).

Discussion

The mycorrhizal character of the typical ErM fungus *R. ericae* is well acknowledged (Read, 1996; Cairney and Meharg, 2003). In contrast with *R. ericae,* the mycorrhizal status of the typical DSE fungus *P. fortinii* is less clear, also due to the considerably lower number of investigations (Mandyam and Jumpponen, 2005). Jumpponen (2001) concluded that because of the beneficial effects caused by inoculation with *P. fortinii*, its relationship with host plants was mycorrhizal, "at least under some conditions".

However, beneficial effects of root-inhabiting fungi are usually assessed by comparing the fitness of the plants inoculated with single fungal strains with the fitness of non-inoculated plants. This approach poorly reflects the situation at natural sites where ericaceous plants can be simultaneously colonized by both ErM and DSE fungi (Hambleton and Currah, 1997; Urcelay, 2002; Rains et al., 2003; Midgley et al., 2004; Peterson et al., 2004; Bougoure & Cairney, 2005; Cázares et al., 2005) and individual plants differ in the levels of ErM and DSE colonization rather than in the presence or total absence of these associations (M. Vohník, unpublished data). In this study, we focused on the mode of *P. fortinii*/*R. ericae* co-existence and the interaction between these two fungi in a common culture *in vitro*. We also attempted to trace the effects of the

interaction between *P. fortinii* and *R. ericae* on their common host plants. Similarly to the situation at natural sites, the experimental plants were simultaneously colonized by both types of fungi and differed mainly in the levels of colonization by DSE and ErM. We asked whether this difference would be reflected in the growth of the experimental plants.

The results from Experiment 1 illustrate the behavior of *P. fortinii* and *R. ericae* in a common culture *in vitro*. After one week of cultivation, there were significant effects of the PFO strain, the origin of the plugs, and the interaction between the PFO strain and the origin of the plugs on the diameter of the colonies emerging from the plugs. Both *P. fortinii* strains had similar growth rates in the *P. fortinii*-dominated areas, but the average diameter of the RER-1 colonies radiating from the RER-1-dominated areas was significantly higher in the PFO-F + RER-1 variant than in the PFO-H + RER-1 variant. This means that already after one week of co-cultivation, *P. fortinii* strain-specifically influenced the growth of the RER-1 dominated colonies. Towards RER-1, PFO-H was more suppressive than (or less compatible with) PFO-F.

In contrast with the situation after one week of cultivation, after 10 weeks of cultivation, both PFO strains dominated all except one plug from the overlapping area (this plug was co-dominated by PFO-F and RER-1). Both PFO strains apparently had the ability to dominate over RER-1 in a common *in vitro* culture where the ratio between PFO and RER-1 mycelium was 1 : 1 at the beginning of the cultivation, as in the case of the plugs from the overlapping area.

RER-1 mycelium dominated one plug and co-dominated 11 plugs from the RER-1-dominated area in the PFO-F + RER-1 variant and dominated seven plugs and co-dominated one plug from the RER-1-dominated area in the PFO-H + RER-1 variant. Thus, in the colonies radiating from the plugs from the RER-1-dominated area, the prevailing mode of the PFO-F vs. RER-1 interaction was a coexistence of their mycelia, whereas it was the dominance of one mycelium over the other in the case of the PFO-H vs. RER-1 interaction. These results support the hypothesis that PFO-H is less compatible with RER-1 than PFO-F.

Based on the results of Experiment 1, we can presume that the main difference between the variants in Experiment 2 was the mode of interaction between *P. fortinii* and *R. ericae* and the main difference between the sub-variants within these variants were proportions of PFO and RER-1 mycelium in the rhizosphere of the *V. myrtillus* seedlings. In Experiment 2, we investigated whether these differences would affect the growth and DSE/ErM colonization rates of *V. myrtillus* seedlings.

There was a significant effect of the origin of the plug (= the area from which the plug was extracted at the beginning of Experiment 2) on TFW of the *V. myrtillus* seedlings. In general, the seedlings growing from the plugs from the RER-1-dominated and the overlapping areas had higher TFW than the seedlings growing from the PFO-dominated areas. This was notable especially in the case of the PFO-H + RER-1 variant, where TFW of the seedlings from the RER-1-dominated area and from the overlapping area were significantly higher than TFW of the seedlings from the PFO-H-dominated area.

Considering the results of Experiment 1 and the fact that both *P. fortinii* and *R. ericae* affect their host plants primarily through formation of an intracellular association with their roots, the differences in the growth of the *V. myrtillus* seedlings could be explained by looking at the DSE/ErM colonization levels in their roots. Therefore in Experiment 2, we in parallel investigated whether the differences in behavior of both PFO strains towards RER-1 would influence the intracellular colonization rates of *V. myrtillus* roots and whether different DSE/ErM colonization rates would be connected with differences in the growth of *V. myrtillus* seedlings. The following ratios were considered: (1) the DSE-colonized : non-colonized cells, (2) the ErM-colonized : non-colonized cells, (3) the colonized : non-colonized cells, and (4) the DSE-colonized : ErM-colonized cells.

There was not a significant difference in the (1) ratio among the sub-variants in the PFO-F + RER-1 variant (the ratios from all three sub-variants were similar to the ratio in the RER-1-dominated area from the PFO-H + RER-1 variant), but there was a significant difference in this ratio among the sub-variants in the PFO-H + RER-1 variant, where the highest ratio was in the seedlings growing from the plugs from the PFO-H-dominated area and the lowest in the seedlings growing from the plugs from the RER-1 dominated area. It means that PFO-F had lower colonization potential than PFO-H and that this potential was only marginally affected by the interaction with RER-1. On the other hand, the colonization potential of PFO-H was higher than PFO-F but was suppressed in the seedlings growing from the plugs from the RER-1-dominated area to the PFO-F level. In both variants, the highest (2) ratio had the seedlings from the RER-1-dominated areas and the lowest ratio had the seedlings from the PFO-dominated areas. This trend was evident especially in the PFO-H + RER-1 variant. The (4) ratio was highest in the seedlings growing from the plugs from the PFO-dominated areas and lowest in the seedlings growing from the plugs from the RER-1-dominated areas. Again, this trend was especially evident in the PFO-H + RER-1 variant. As can be seen, the proportions of DSE and ErM colonization tended to be negatively correlated.

Similarly to our previous unpublished observations from both *in vitro* and *in field* conditions, we found structures intermediate between the DSE and the ErM colonization pattern in the roots of the *V. myrtillus* seedlings. Their incidence reached up to 7% of the root cells in two sub-variants in the PFO-H + RER-1 variant, but only 1% in one sub-variant in the PFO-F + RER-1 variant. This difference suggests that IS were strain-specifically formed by PFO-H and hence, they belonged to DSE-association. Post hoc inclusion of IS into the DSE-colonization category would probably accent the difference between the colonization potential of PFO-F and PFO-H in Experiment 2. However, we decided to keep the IS-colonization category to emphasize their existence in ericaceous roots. IS might be parallel to hyaline hyphae observed by Yu *et al*. (2001) or Barrow (2003) in non-ericaceous roots. Because of similarity with the ErM colonization pattern, IS

might hamper proper evaluation of DSE/ErM colonization levels in ericaceous roots from field samples.

In the PFO-F + RER-1 variant, the relationship between the DSE/ErM colonization rates and the growth of the *V. myrtillus* seedlings is unclear, partly because there are no significant differences in the growth of the seedlings and no sharp differences in the colonization levels as in the PFO-H + RER-1 variant. The only trend that can be deduced based on the results is a negative correlation between TFW and the (4) ratio (see Table 1).

In the PFO-H + RER-1 variant however, there was a significant difference between TFWs of the seedlings growing from the plugs from the PFO-H-dominated area and TFWs of the seedlings growing from the plugs from the overlapping and the RER-1-dominated areas. In this variant, we can see a positive correlation between TFW and the (2) ratio and a negative correlation between TFW and the (4) ratio.

Supposing that the interaction between the two PFO strains and RER-1 was similar in both experiments, we can conclude that the growth of the *V. myrtillus* seedlings expressed as their TFW was significantly influenced by different proportions of PFO and RER-1 mycelia in their rhizosphere (= the effect of the origin of the plug). This probably caused the differences in the DSE and the ErM colonization rates and in the ratio between them. The growth of the seedlings tended to be positively correlated with increased ErM colonization and decreased DSE colonization. The differences in the DSE/ErM colonization rates were more pronounced in the PFO-H + RER-1 variant. Considering the results of Experiment 1, this could be due to lower compatibility between PFO-H and RER-1 than between PFO-F and RER-1.

To our knowledge, this study is the first comparison of the effects of DSE-association and ErM on the growth of common host plants. In addition, we for the first time showed a strain-specific linkage of proportions of DSE and ErM mycelium in the rhizosphere with proportions of DSE and ErM colonization and the growth of host plants under *in vitro* conditions. Interpretation of results of *in vitro* studies with respect to natural situations is certainly very limited. However, results of Experiment 1 indicate strain-specific interactions between *P. fortinii* and *R. ericae*, which might influence their coexistence also in the rhizosphere of ericaceous plants at natural sites. Such interactions might influence ericaceous host plants in a similar way as in Experiment 2.

In our study, DSE colonization had a relatively neutral to negative effect and ErM colonization had a relatively positive effect on the growth of the host plants. The neutral effect of DSE colonization on the growth of host plants found in this study corresponds with results of our previous study (Vohník et al., 2003), where we found a neutral effect of inoculation with both PFO-F and PFO-H on the growth of *Rhododendron* cv. Belle-Heller. The positive effect of colonization by *R. ericae* corresponds with generally accepted positive influence of ErM fungi on their host plants (Smith and Read, 1997). Our study shows that besides the expectable improved nutrition of

its host plants, the positive effect of *R. ericae* could be seen in the moderation of the levels of DSE colonization.

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References

Barrow, J. R. 2003. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in arid southwestern USA rangelands. *Mycorrhiza* **13**: 239-247.

Bougoure, D. S., Cairney, J. W. G. 2005. Fungi associated with hair roots of *Rhododendron lochiae* (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture-independent molecular methods. *Environmental Microbiology* **7**: 1743-1754.

Cairney, J. W. G., Meharg, A. A. 2003. Ericoid mycorrhiza: a partnership that exploits harsh edaphic conditions. *European Journal of Soil Science* **54**: 735-740.

Cázares, E., Trappe, J. M., Jumpponen, A. 2005. Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. *Mycorrhiza* **15**: 405-416.

Hambleton, S., Currah, R. S. 1997. Fungal endophytes from the roots of alpine and boreal Ericaceae. *Canadian Journal Botany* **75**: 1570-1581.

Jansa, J., Vosátka, M. 2000. In vitro and post vitro inoculation of micropropagated Rhododendrons with ericoid mycorrhizal fungi. *Applied Soil Ecology* **15**: 125-136.

Jumpponen, A., Trappe, J. M. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytologist* **140**: 295-310.

Jumpponen, A. 2001. Dark septate endophytes – are they mycorrhizal? *Mycorrhiza* **11**: 207-211.

Mandyam, K., Jumpponen, A. 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology* **53**: 173-189.

Midgley, D. J., Chambers, S. M., Cairney, W. G. 2004. Distribution of ericoid mycorrhizal endophytes and root-associated fungi in neighboring *Ericaceae* plants in the field. *Plant and Soil* 259: 137-151.

Pearson, V., Read, D. J. 1973. The biology of mycorrhiza in the Ericaceae. I. The isolation of the endophyte and synthesis of mycorrhiza in aseptic culture. *New Phytologist* **72**: 371-379.

Peterson, R. L., Massicotte, H. B., Melville, L. H. 2004. Mycorrhizas: Anatomy and Cell Biology. NRC Research Press, Ottawa.173 pp.

Rains, K. C., Nadkarni, N. M., Bledsoe, C. S. 2003. Epiphytic and terrestrial mycorrhizas in a lower montane Costa Rican cloud forest. *Mycorrhiza* **13**: 257-264.

Read, D. J. 1996. The structure and function of the ericoid mycorrhizal root. *Annals of Botany* **77**: 365-374.

Smith, S. E., Read, D. J. 1997. Mycorrhizal Symbiosis. Second Edition. Academic Press. London. pp 323-345.

Urcelay, C. 2002. Co-occurrence of three fungal root symbionts in *Gaultheria poeppiggi* DC in Central Argentina. *Mycorrhiza* **12**: 89-92.

Vohník, M., Lukančič, S., Bahor, E., Regvar, M., Vosátka, M., Vodnik, D. 2003. Inoculation of *Rhododendron* cv. Belle-Heller with two strains of *Phialocephala fortinii* in two different substrates. *Folia Geobotanica* **38**: 191-200.

Vohník, M., Albrechtová, J., Vosátka, M. 2005. The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro. *Symbiosis* **40**: 87-96.

Yu, T., Nassuth, A., Peterson, R. L. 2001. Characterization of the interaction between the dark septate fungus *Phialocephala fortinii* and *Asparagus officinalis* roots. *Canadian Journal of Microbiology* **47**: 741-753.

FIGURES:

Figure 1: A dish with mycelium of *Phialocephala fortinii* and *Rhizoscyphus ericae*, from which the plugs for the experiments were taken (for details see Materials and Methods).

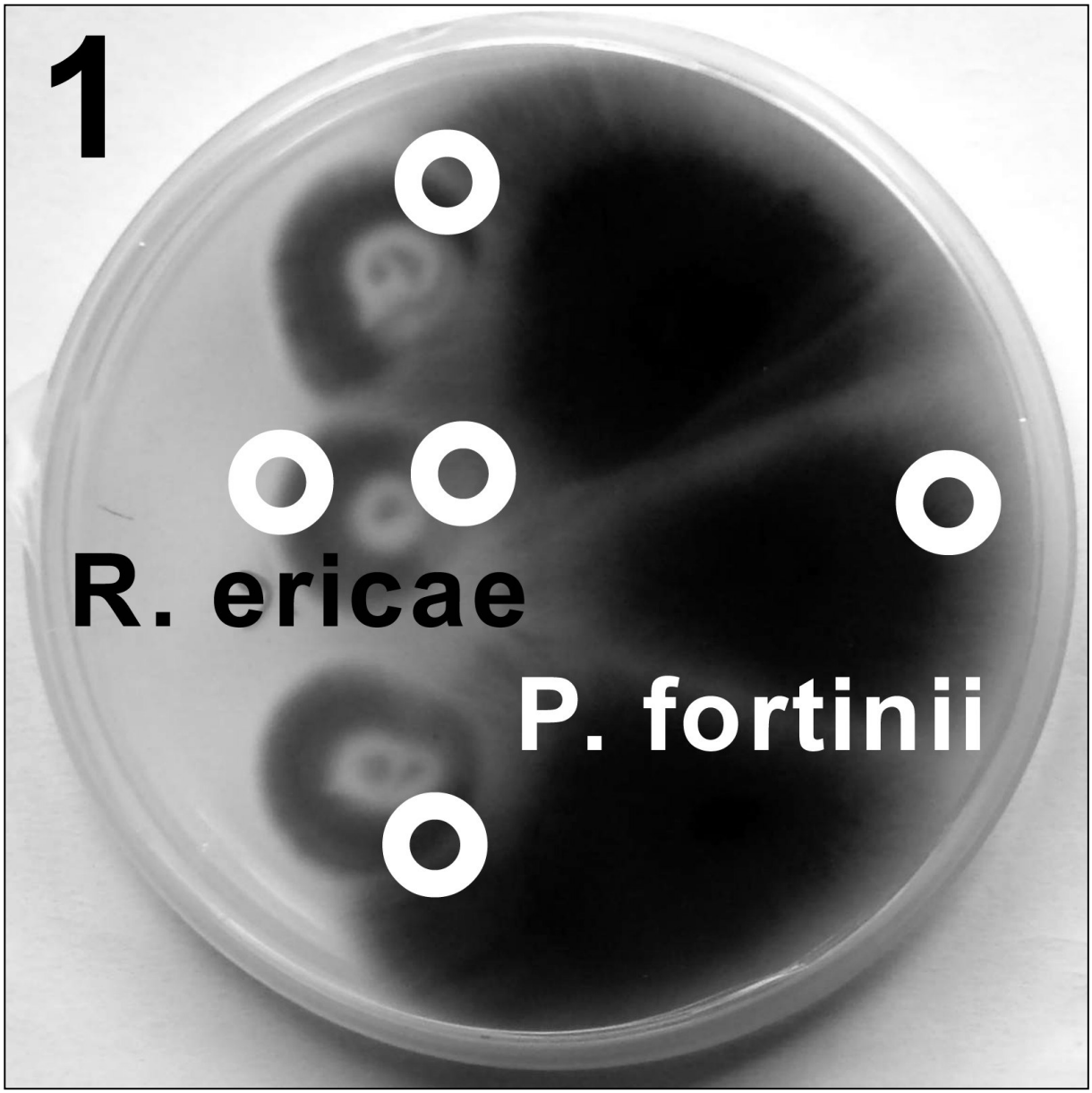

Fig 2: Positioning of the plugs with radiating *P. fortinii* and *R. ericae* mycelium in Experiment 1.

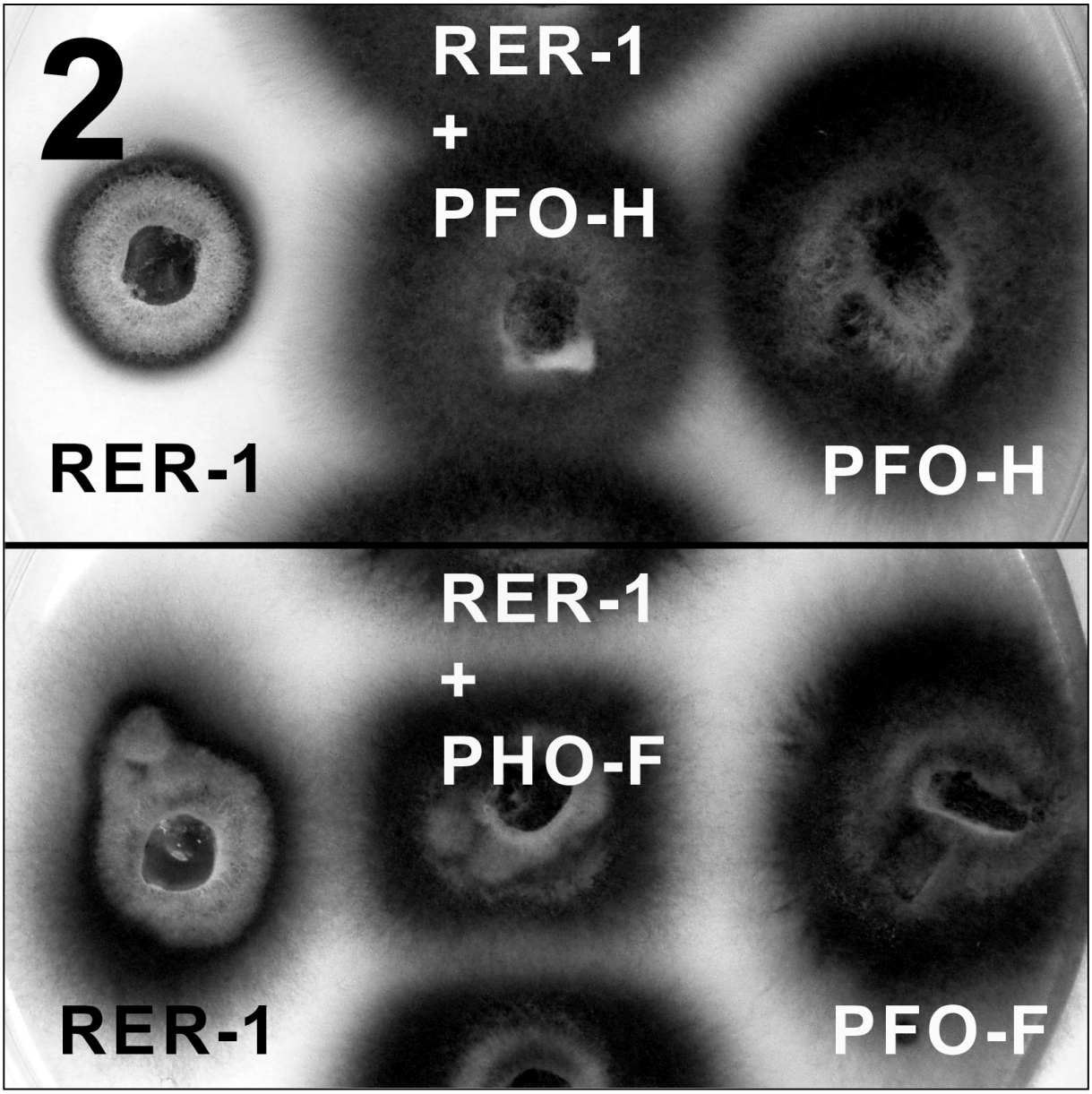

Fig 3: Positioning of the plugs with radiating *P. fortinii* and *R. ericae* mycelium and inserted *Vaccinium myrtillus* seedlings in Experiment 2;

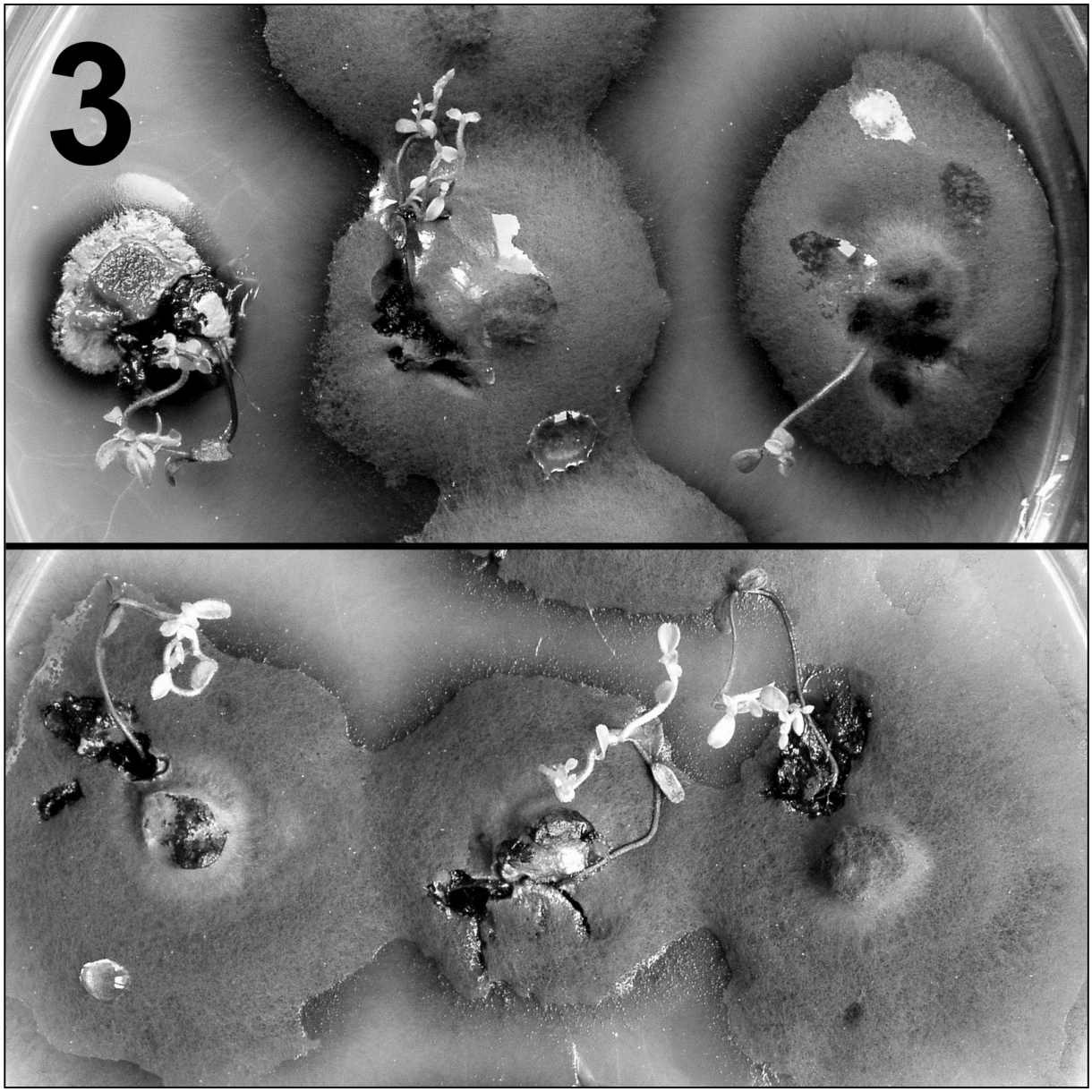

Fig 4: The three colonization categories found in the *V. myrtillus* roots: ***upper right picture*** = the DSE colonization pattern with intracellular microsclerotia (arrows); ***lower left picture*** = the ErM colonization pattern with dense intracellular coils (arrows); ***background picture*** = the structures intermediate between the DSE and the ErM colonization pattern (arrowheads). Bars correspond to 50µm.

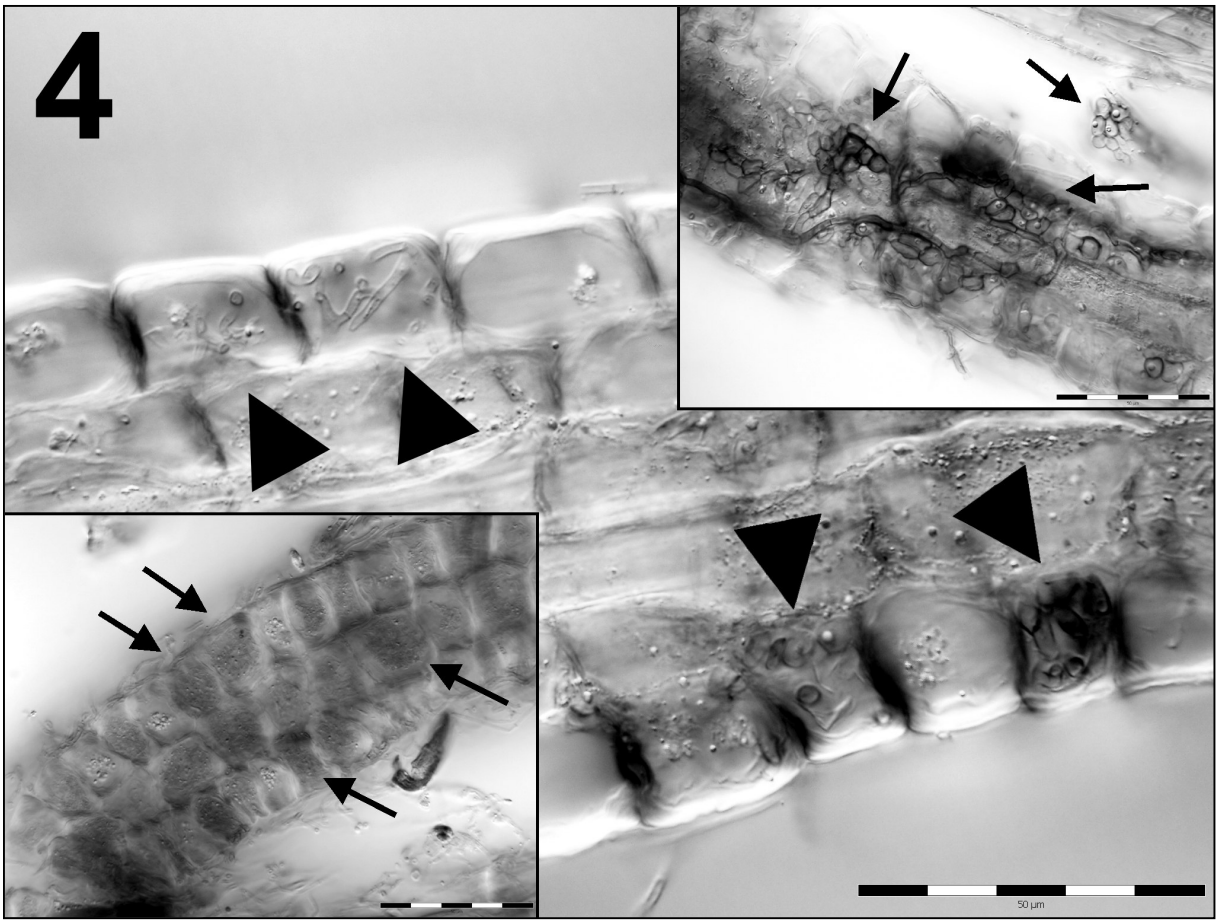

TABLE 1: I = Colony diameter (cm); **II** = Fresh weight of the *V. myrtillus* seedlings (mg); **III** = Percentual colonization of the *V. myrtillus* seedlings by DSE – ErM – intermediate structures (%); **IV** = The ratio between DSE-colonized and non-colonized root cells of the *V. myrtillus* seedlings; **V** = The ratio between ErM-colonized and non-colonized cells of the *V. myrtillus* seedlings; **VI** = The ratio between colonized (= DSE + ErM + intermediate structures) and non-colonized cells of the *V. myrtillus* seedlings; **PFO** = the *P. fortinii*-dominated area; **Overlap** = the overlapping area; **RER-1** = the *R. ericae*-dominated area; **PFO-F** = *P. fortinii* strain PFO-F; **PFO-H** = *P. fortinii* strain PFO-H. The first number in the brackets in the "Strain" column indicates the number of repetitions for I, the second number indicates the number of repetitions for II and the third number indicates the number of repetitions for III, IV, V and VI. The values are mean ± SD. The different letters indicate significantly different groups (a HSD test for I, a LSD test for II to VII; p = 0.05). For details see Materials and Methods.

Origin	**Strain**	**I**	**II**	**III**	**IV**	**V**	**VI**	**VII**
PFO	**PFO-F** (15; 6; 4)	1.60 ± 0.03 **d**	10.5 ± 2.3 **ab**	23-5-0	0.35 ± 0.11 **ab**	0.09 ± 0.07 **ab**	0.43 ± 0.16 **a**	64.6 ± 38.8 **cd**
	PFO-H (15; 6; 3)	1.63 ± 0.03 **d**	5.8 ± 2.0 **a**	43-0-7	0.86 ± 0.06 **c**	0.01 ± 0.01 **a**	1.00 ± 0.12 **a**	151.8 ± 64.7 **d**
Overlap	**PFO-F** (15; 6; 5)	1.53 ± 0.03 **cd**	12.7 ± 1.8 **b**	15-20-0	0.25 ± 0.09 **a**	0.32 ± 0.08 **abc**	0.58 ± 0.11 **a**	1.05 ± 0.46 **ab**
	PFO-H (15; 6; 5)	1.47 ± 0.02 **bc**	15.4 ± 1.8 **b**	28-15-7	0.64 ± 0.17 **bc**	0.33 ± 0.13 **bc**	1.13 ± 0.31 **a**	28.91 ± 27.1 **bc**
RER-1	**PFO-F** (15; 6; 5)	1.37 ± 0.03 **b**	14.9 ± 2.3 **b**	14-27-1	0.21 ± 0.09 **a**	0.39 ± 0.11 **c**	0.61 ± 0.19 **a**	0.44 ± 0.17 **a**
	PFO-H (15; 6; 4)	1.14 ± 0.04 **a**	12.5 ± 2.4 **b**	11-36-2	0.25 ± 0.11 **a**	0.72 ± 0.08 **d**	1.03 ± 22 **a**	0.34 ± 0.14 **a**

1. 4. ČLÁNEK 3

The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro

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The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro

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Abstract

A pioneering attempt to simultaneously introduce an ericoid mycorrhizal (ErM) *Oidiodendron maius* Barron (*O.m.*) and two strains of dark septate endophyte (DSE) *Phialocephala fortinii* Wang & Wilcox (strain *P.f.* F and *P.f.* H) into root systems of individual *Rhododendron* cv. Azurro plants was conducted in split root systems. The inoculation had no effect on total biomass of inoculated rhododendrons. However, plants accumulated more root biomass into compartments inoculated with *P.f.* H than to those non-inoculated or inoculated with *P.f.* F. Plants with the highest foliar P concentrations had been inoculated with *O.m.*, co-inoculated with *O.m.* and *P.f.* H and inoculated with *P.f.* H. Inoculation with *O.m.* and co-inoculation with *O.m.* and *P.f.* H also altered N uptake. Inoculation with *O.m.* and its co-inoculation with both *P. fortinii* strains decreased foliar C:N ratios. All fungi colonized host roots at low levels, *P.f.* F being the most successful colonizer. Contrary to the other fungi, *O.m.* also colonized *Rhododendron* microcuttings at low levels *in vitro,* and the colonization pattern was distinct from *Hymenoscyphus ericae*. Both *P. fortinii* strains exhibited a typical DSE colonization pattern *in vitro*. Our study indicates that *O. maius* and *P. fortinii* positively affect host plant nutrition and demonstrates interactions between separately developing ErM and DSE fungi, which significantly affect plant physiology.

Key words: ericoid mycorrhiza, dark septate endophytes, DSE-association, split root system, colonization level, pseudomycorrhiza, co-inoculation

Introduction

Members of *Ericaceae* dominate vast areas in both Northern and Southern hemisphere, which are often characterized by "harsh edaphic conditions" (Cairney and Meharg 2003). These conditions include low pH, low nutrient availability, high soil C:N ratio and high contents of phenolic compounds and toxic elements (Smith and Read 1997). The roots of members of *Ericaceae* are

colonized by ErM fungi, which are regarded as important mutualistic associates. ErM fungi positively influence growth, survival and competitiveness of their host species by enhancing nutrient uptake (Read 1996, Read and Perez-Moreno 2003) and alleviating heavy metal toxicity (Perotto et al., 2002).

The genus *Oidiodendron* contains a number of fungi, which inhabit the roots of ericaceous species (Couture et al., 1983, Dalpé 1986, Xiao and Berch 1995, Johansson 2001), including rhododendrons (Douglas et al., 1989, Currah et al., 1993a, Jansa and Vosátka 2000, Usuki et al., 2003). Among oidiodendrons, *O. maius* is frequently detected in roots of ericaceous species (Perotto et al., 2002). However, the ecophysiological role of oidiodendrons in the rhizosphere of ericaceous species has not been studied extensively: there is only little, if any, experimental work on nutrient uptake by plants inoculated with *Oidiodendron* species. This contrasts with their frequent occurrence and isolations from ericoid mycorrhizal roots from natural sites. Our general knowledge about the effects of oidiodendrons on the physiology of ericaceous plants is limited to reports about their heavy metal resistance (Martino et al., 2000) and saprotrophic capabilities (Rice and Currah 2001, Piercey et al., 2002). The majority of the data regarding nutrient uptake in ErM is based on experiments with *H. ericae* (Bajwa and Read 1985, Bajwa *et al* 1985, Kerley and Read 1995, Kerley and Read 1997, Kerley and Read 1998).

In addition to ErM fungi, root endophytes belonging to the miscellaneous group of DSE fungi are reported to colonize roots of ericaceous plants. The DSE comprise ascomycetous fungi with a ubiquitous distribution and wide range of host plants, yet their effects on host physiology are ambiguous (Jumpponen and Trappe 1998a). *P. fortinii*, the most prominent DSE fungus, was found in roots of several ericoid species (Stoyke et al., 1992, Currah et al., 1993b, Ahlich and Sieber 1996), including rhododendrons (for list see Jumpponen and Trappe 1998a). Similarly to oidiodendrons, our knowledge about the ecophysiological significance of DSE is scarce. Plant responses to DSE generally vary from negative to positive (Jumpponen and Trappe 1998a, Jumpponen 2001), and inoculation with DSE often causes no apparent effect on biomass production or nutrient uptake by host plants in pot cultures (Jumpponen and Trappe 1998b, Vohník et al., 2003). Nevertheless, stressing their potential to improve plant growth and nutrient uptake under certain conditions, Jumpponen (2001) qualified their effect on host plants as “mycorrhizal”.

It can be expected that at natural sites with ericaceous plant species, both ErM and DSE fungi occur together in the soil and interact. The nature of these interactions is, however, unknown. Direct observations have confirmed multiple colonization in roots of ericaceous species. Urcelay (2002) reported the simultaneous occurrence of ErM, DSE and even arbuscular mycorrhiza in roots of *Gaultheria poeppiggi* DC (*Ericaceae*). Also, molecular methods proved the simultaneous presence of ErM (*H. ericae*-like and oidiodendrons) and DSE (*P. fortinii*) fungi within the same root system (Midgley et al., 2004).

To contribute to the understanding of physiological processes in ericaceous host plants influenced by ericoid mycorrhizal oidiodendrons and DSE fungi, we focused on the effects of (co-

)inoculation with *O. maius* and two strains of *P. fortinii* on growth, nutrient uptake and root biomass distribution in *Rhododendron* cv. Azurro. Two experiments were conducted. The Fungal Compatibility Experiment, carried out in split Petri dishes with tissue culture derived cuttings, screened for compatibility of the fungal isolates with rhododendrons. A parallel Fungal Efficacy Experiment was conducted in split root systems to ensure spatial isolation of two fungi, inoculated into an individual root system, and was designed to screen the effects of inoculated fungi on their host plants.

Materials and Methods

Fungal Compatibility Experiment

Tissue culture derived *Rhododendron* sp. cuttings with newly emerging roots were introduced into split Petri dishes (Figs. 1a-4a) with perforated central septa and root compartments containing the medium (MMR throughout the following text) after Dalpé (1986), which is modified from Mitchell and Read (1981). Root compartments were inoculated with pieces of agar overgrown by mycelium of following fungal strains: *Oidiodendron maius* B, *Phialocephala fortinii* F, *Phialocephala fortinii* H and a strain of *Hymenoscyphus ericae,* included for the comparison of the colonization pattern of this prominent ericoid mycorrhizal fungus with *O. maius* B. The fungal strains were pre-cultivated two months prior to the experiment in the dark at room temperature on PDA media (39 $g.l^{-1}$, Difco). *O. maius* B was previously isolated from *Rhododendron* sp., *P. fortinii* F from *Vaccinium myrtillus* L. and *P. fortinii* H from *Rhododendron* sp. (Jansa and Vosátka 2000). These strains were identified both morphologically and using phylogenetic analysis (Vohník et al. 2003). *H. ericae* was the isolate from Leake and Read (1989), originally isolated from *Calluna vulgaris* Hull. One set of split dishes was left non-inoculated. After the inoculation all dishes were sealed with Parafilm™, and the root compartments were wrapped with aluminum foil to block light radiation. There were 5 cuttings in each treatment, including a non-inoculated control.

After 3 months from the inoculation, the roots of the cuttings were separated from the shoots, divided into halves and stained either with trypan blue or chlorazol black, respectively, according to the method described by Brundrett et al. (1996). Roots were then observed at high magnification (400-1000x) with DIC, using Olympus BX60 microscope. Pictures were taken with Olympus DP70 camera.

Fungal Efficacy Experiment

Rooted tissue culture derived cuttings of *Rhododendron* cv. Azurro of equal total and root system size were planted into split root systems composed of two Petri dishes. Prior to the introduction of experimental plants, all dishes were perforated at the side to allow insertion of roots (Fig. 5a). Dishes were then filled with peat:perlite (2:1) substrate amended with a slow release fertilizer (Osmocote, 5-6 months release time, $2g.l^{-1}$) and autoclaved twice in consecutive 24-hr periods at 121°C for 60 minutes.

Plants, 50 total, in the split root systems were cultivated in a growth chamber (16/8h, 25/20°C day/night, 150µmol $m^{-2}s^{-1}$, 85±5% relative humidity) and regularly watered two times per week with de-ionized water. After 50 days of cultivation, the split root system of each plant was visually checked to ensure the roots were distributed equally between both dishes. This procedure resulted in a selection of 42 plants of similar size and equal root distribution, which were then used for the experiment.

Mycelium of *O. maius* B, *P. fortinii* F and *P. fortinii* H was pre-cultivated for two months on PDA media (39g of PDA powder, Difco, per 1L of de-ionized water) at room temperature in the dark. Plants in the split root systems were inoculated i) separately with single fungal strains onto one dish (then the other dish remained non-inoculated) – treatments B=*O. maius* B, F=*P. fortinii* F and H=*P. fortinii* H or ii) simultaneously with two different fungal strains, each inoculated into one of the dishes – treatments BF=*O. maius* B+*P. fortinii* F and BH=*O. maius* B+*P. fortinii* H. At the beginning of the experiment, there were 7 plants in each treatment including a non-inoculated control. The inoculation was performed by pipetting the inoculum onto the substrate in the dishes. The inoculum was prepared by homogenizing the mycelium of the respective fungus (together with the agar growth medium from below the colony) from 3 Petri dishes with 300ml of autoclaved water. In the case of both *P. fortinii* strains, which in two months had overgrown the whole surface of medium in dishes, the total content of the dishes (the mycelium + the growth medium) was homogenized. For *O. maius* cultures, which did not overgrow the whole surface, we excised only the fungal colonies and the medium directly below them. 5ml of the suspension were used per each inoculation resulting in a total amount of 5ml added per plant in treatments B, F and H and 10ml in treatments BF and BH. Unequal inoculum volume as well as the presence of residues of the growth medium in the inoculum did not influence the analyzed parameters of inoculated plants (see Results). Both dishes of control plants remained non-inoculated. The control variant was set up to find whether the root biomass distribution at the end of the experiment would be equal between the two non-inoculated dishes of the same plant, thus the shift in the root biomass was due to the inoculation. After the inoculation, each dish was wrapped with aluminum foil and placed into a plastic sack to prevent cross-contamination between dishes (Fig. 5b). The experimental plants were randomly placed in the growth chamber and regularly watered two times per week with de-ionized water.

The roots were harvested 16 weeks after the inoculation. One of the rhododendrons inoculated with *O. maius* B developed extremely badly compared to the rest of plants and was excluded from the measurements. The shoots were separated from the roots, and the leaves were counted and their area measured (LI-3100 Area Meter, LI-COR Inc., USA). The shoots were then dried in an oven at 80°C for 10 hours and weighed to obtain the weight of the dried shoot biomass. The dried leaves were homogenized in a grinding mill and analyzed for N, P, and C content (methods after Ehrenberger and Gorbach, 1973). The concentration of P was expressed in µg/g of the dried leaf biomass; the content of N and C was given as percentage of the two substances in the

analyzed samples. C:N ratio was calculated using percent carbon and nitrogen contents in the dried leaves.

Roots from both dishes of one experimental plant were separated, washed under tap water, dried with filtration paper and weighed. This yielded the total weight of the fresh root biomass of the whole plant, and the total weights of the fresh root biomass from each of the two dishes, belonging to the same plant. About one half of the roots from each dish were separated, weighed again and the ratio between the weight of the separated part vs. the total weight of the fresh root biomass from the same dish was calculated. The separated part was dried in an oven for 4 hours at 80°C and weighed again. Supposing that all parts of the root system would change their weight during the drying the same way, we re-calculated the total weight of the dried roots from one dish using the weight of the dried separated part and the ratio mentioned above. Obtained values were used for the calculation of the total weight of the dried root biomass of the whole plant and for the distribution of root biomass within the single root system. The distribution of root biomass within the single root system was found by calculating the ratio between the weight of the dried root biomasses in inoculated vs. non-inoculated dishes (treatments B, F, and H) and between weights of the dried root biomasses from the dishes inoculated with *O. maius* B vs. inoculated with *P. fortinii* F or *P. fortinii* H (treatments BF and BH).

Remaining roots not used for weighing were used to assess mycorrhizal colonization. Roots were treated as in *Fungal Compatibility Experiment* and observed at high magnifications with DIC, using the equipment listed above. The gridline intersection method (Brundrett et al., 1996) proved unsuitable for evaluating colonization in the roots because of low colonization levels. As an alternative to percent colonization, we divided roots into two related classes. The first class represented poor colonization characterized by very scarce occurrence of both intra- and extracellular hyphae, and the second class involved roots colonized in the same way at higher level with occasional, but still scarce presence of hyphal clumps on the root surface.

Data were statistically analyzed for homogeneity of variances using Levene´s test and for normal distribution using Chi-Square test. The data that did not have homogenous variances or normal distribution were sqrt (foliar P content) or log transformed (foliar N and C content, the distribution of root biomass) to meet assumptions of ANOVA. One-way ANOVA was used to evaluate the effect of the inoculation on the weight of the dried shoot, root and total biomass, on foliar nutrient content, C:N ratio and root biomass distribution. Calculated ratios of root biomass distribution were divided into two groups: i) single fungus inoculation, the second dish non-inoculated (treatments B, F and H) and ii) co-inoculation with two fungi, one dish inoculated with *O. maius* B, the other with one of *P. fortinii* strains (treatments BF and BH) and the differences between treatments were calculated within these groups. Significant differences between treatments were evaluated using LSD test at $p \leq 0.05$. Statistica ^{TM} 5.1 software was used for the statistical analysis.

Results

Fungal Compatibility Experiment

There were no apparent differences in the growth and branching of the shoots among cuttings, and all grew well without any signs of nutrient deficiency. Only the plants inoculated with *O. maius* B seemed to exhibit less vigorous shoot growth compared to the others, but this observation was not statistically evaluated because of a low number of replications per variant. Plants from all treatments except those inoculated with *O. maius* B developed vigorous, branched root system (Figs. 2a-4a). All fungal isolates except *O. maius* B grew sufficiently on the media. In contrast, plants inoculated with *O. maius* B had reduced, dark pigmented and non-branched thick roots (Fig. 1a). *O. maius* B colonies grew very slowly reaching maximum diameter of 1.5 cm, compared to 5cm for *H. ericae* and 7 cm for both *P. fortinii* colonies, at the end of the experiment. *O. maius* B colonies also produced a lightly brown pigment of unknown nature in the medium. Screening of both trypan blue and chlorazol black stained roots revealed a typical DSE-colonization pattern in treatments inoculated with *P. fortinii* F and *P. fortinii* H: abundant dark septate hyphae surrounded the roots growing on its surface, penetrating rhizodermal cell walls and occasionally forming primordia of hyaline microsclerotia (Fig. 3b). *H. ericae* formed typical ericoid mycorrhizal colonization pattern: its hyphae grew along the root surface penetrating into rhizodermal cells and filling them with dense hyphal coils (Fig. 2b). Trypan blue completely failed to stain mycelium of *O. maius* B, and its intracellular hyphae were stained only lightly with chlorazol black. The colonization pattern of *O. maius* B was distinct from *H. ericae*: its hyphae also formed intracellular coils, but these had altered morphology and were looser compared to those of *H. ericae* (Fig. 1b). The intracellular hyphae of *O. maius* B never filled the cells to such extent as those of *H. ericae*. Cells colonized with hyphae of *O. maius* B in the described pattern, which resembled ErM, were very scarce, and overall colonization was much less prominent and developed than that of *H. ericae*.

Fungal Efficacy Experiment

The inoculation with either fungus had no significant effect on the weight of the dried shoot, root or total biomass, shoot:root ratio, leaf area or number of leaves of experimental plants. However, the inoculation had a significant effect on the distribution of root biomass within the root system of plants inoculated with single fungal strains ($F=3.28$, $p=0.042$). This was expressed by statistically different ratio between the weights of the dried root biomass found in inoculated vs. non-inoculated dishes. Experimental plants developed significantly more root biomass in the dishes inoculated with *P. fortinii* H than non-inoculated dishes ($p=0.03$) and dishes inoculated with *P. fortinii* F ($p=0.01$). A similar but non-significant ($p=0.097$) trend was observed for dishes inoculated with *O. maius* B, which appeared to favor root biomass distribution in comparison with *P. fortinii* F. The distribution of root biomass in the group of plants co-inoculated with *O. maius* B and one of the *P. fortinii* strains was not influenced by the inoculation ($F=1.74$, $p=0.217$).

The inoculation had significant effects on the foliar P content (F=7.05; p=0.0001). In comparison with the non-inoculated control, the separate inoculations with *O. maius B* and *P. fortinii* H increased the P content in the leaves 1.34 (p=0.00007) and 1.22 times (p=0.005), respectively. The co-inoculation with *O. maius* B and *P. fortinii* H increased the P content in leaves 1.25 times (p=0.001) compared to the control and 1.23 times (p=0.002) compared to the co-inoculation with *O. maius* B and *P. fortinii* F. The inoculation with *O. maius* B also increased the foliar P content in comparison with *P. fortinii* F (1.25 times, p=0.001) and with the co-inoculation with *O. maius* B and *P. fortinii* F (1.31 times, p=0.0002). *P. fortinii* H also increased the P content in comparison with *P. fortinii* F (1.14 times, p=0.052). The inoculation also effected the N foliar content, but this effect was only marginally significant (F=1.99; p=0.053). At the given level of significance, the inoculation with *O. maius* B increased the N content 1.24 times (p=0.004) compared to the control, 1.15 times (p=0.041) compared to the inoculation with *P. fortinii* F and 1.17 times (p=0.025) compared with *P. fortinii* H. Co-inoculation with *O. maius* B and *P. fortinii* H also increased the N content comparing to the control (1.16 times, p=0.034). The inoculation had no effect on the foliar C content but significantly influenced the foliar C:N ratio (F=2.79, p=0.032). Inoculation with *O. maius* B decreased the C:N ratio in comparison with the control (p=0.002) and with *P. fortinii* H (p=0.036). Additonally, the co-inoculation with either *O. maius* B and *P. fortinii* H or with *O. maius* B and *P. fortinii* F decreased the C:N ratio compared to the control (p=0.011 and p=0.032, respectively).

Screening of the mycorrhizal colonization revealed very low levels of intracellular colonization by fungal hyphae. If present, *O. maius* B hyphae grew around the roots and were attached to the root surface, only very rarely penetrating rhizodermal cells to form sparse intracellular coils. Hyphae of *O. maius* B failed to stain with trypan blue and were only lightly stained with chlorazol black. *P. fortinii* H failed to produce abundant DSE-colonization. Its hyphae grew mostly on the root surface, and if they penetrated the root, they grew along the central axis producing mostly lightly stained (better with chlorazol black than in trypan blue) hyaline hyphae. The hyphae of *P. fortinii* H scarcely formed microsclerotia. Relatively high colonization occurred in the dishes inoculated with *P. fortinii* F, where dark septate hyphae formed well-developed hyaline microsclerotia. However, even in the dishes inoculated with P. *fortinii* F, the colonization was low, and we estimate it did not reach more than 5% (counting both the presence of hyphae and microsclerotia) of the total root length colonized. The colonization level of *O. maius* B and *P. fortinii* H belonged to the first class, with that of *P. fortinii* F within the second class as described in Materials and methods.

Discussion

Fungal Compatibility Experiment

The Fungal Compatibility Experiment showed that the selected fungi were able to form both ErM and DSE in the roots of *Rhododendron* micro-cuttings. *O. maius* B proved to be the least efficient

colonizer, exhibiting poor intracellular colonization compared to the typical ErM fungus *H. ericae*. The presence of *O. maius* B also reduced the development of the root system of the inoculated plants compared to the non-inoculated and the inoculated with *H. ericae* or either of the two strains of *P. fortinii*. Similar root growth depression by *O. tenuissimum* (Peck) Hughes was observed by Dalpé (1986) in *Vaccinium angustifolium* Ait., where *O. tenuissimum* failed to form ErM.

The root growth depression in mycorrhizal symbioses can be attributed to the fact, that mycorrhizal plants, which receive mineral nutrients from substrate through the mycelium of symbiotic fungi, can invest less energy into the formation of the root system. However, the situation with *O. maius* B-inoculated plants, in which roots remained mostly non-mycorrhizal, sharply contrasted with *H. ericae*-inoculated plants, which were readily ericoid mycorrhizal and simultaneously produced more vigorous shoots and especially roots. The mechanisms of the reduction of root, and to some extent, also shoot development by *O. maius* B thus remain unknown. Except for sucrose, the nutrients in MMR are in mineral form and easily accessible to plants, as revealed by the vigorous growth of the rhododendrons in the control treatment. In contrast to *H. ericae* and both *P. fortinii* strains, the presence of *O. maius* B apparently altered the ability of rhododendrons to draw the nutrients from MMR, likely by producing unknown inhibitory compound(s), which reduced the development of the root system, and thus reducing its absorptive area. Decreased nutrient uptake then reduced shoot growth.

In contrast to *H. ericae* and both strains of *P. fortinii,* the growth of *O. maius* B itself was reduced on MMR. It was also reduced when compared to other cultivation media we ordinary use for the cultivation of *O. maius* B (Potato Dextrose Agar, Malt Extract Agar, Modified Melin Norkrans medium – data not shown). Reduced growth of *O. maius* on MMR, together with the production of a pigment of unknown nature in the media was connected with reduced growth of the roots and shoots of the inoculated plants – it appears that *O. maius* B, apparently growing under sub-optimal conditions, did not act as an ericoid mycorrhizal symbiont.

Dulcos and Fortin (1983) found that the addition of a small quantity of glucose (0.5 g/L) to the culture medium enhanced ErM formation on *V. angustifolium* and *Vaccinium corymbosum* L. seedlings with *H. ericae* compared to the seedlings cultivated in the control medium without glucose. A higher concentration of glucose (5 g/L) decreased the colonization rate as well as the height of inoculated *Vaccinium* seedlings compared to the control medium. These results indicate that the level of the colonization and the nature and effectiveness of the fungus-root association in *in vitro* experiments with ErM and DSE fungi can be determined by the quality of the media, which agrees with our results from *O. maius* B, although we cannot provide any direct evidence about the effect of the limited C-availability.

Fungal Efficacy Experiment

In different habitats, different fungi usually inhabit plant roots, as is the case with ericaceous species (Midgley et al. 2004), even though the dominant role of *H. ericae* is stressed. McLean and

Lawrie (1996) stated, on the base of the different colonization patterns, that more than one fungus was involved in the screened ericoid mycorrhizae in the roots of epacridaceous plants from different natural sites. Each fungus may make different contribution to the ecophysiology of its host plant depending on the large scale of the environmental factors. This was also revealed in Fungal Compatibility Experiment, where ErM fungi *O. maius* B and *H. ericae*, grown on the same media, exhibited different effects on host plants. In the Fungal Efficacy Experiment, the direct comparison of the effects of *O. maius* and both *P. fortinii* strains on the nutrient uptake by the host plants showed a higher efficiency of *O. maius* than *P. fortinii*. The results of our previous unpublished experiments, performed under the conditions similar to those presented here, repeatedly indicated that *O. maius* B was more efficient in the terms of improved plant growth of rhododendrons than *H. ericae*, which we had not expected. Unfortunately, studies employing *O. maius,* and especially both *O. maius* and *H. ericae* simultaneously are missing, and the effect of both fungi is therefore difficult to compare. Such studies would elucidate whether oidiodendrons could be as efficient in facilitating nutrient uptake by host plants as *H. ericae* and thus play the same or similar role in the environments dominated by ericaceous species. Results of our study indicate that oidiodendrons might have such potential.

Phosphorus and especially nitrogen are primarily transported to host plants by ericoid mycorrhizal fungi, according to experiments using *H. ericae* as a mycorrhizal partner of ericaceous plants (Smith and Read 1997). Our study shows positive effects of inoculation with *O. maius,* in particular, on P uptake but also indicates a positive trend in N uptake by inoculated plants. To our knowledge, this is the first report about such effects observed for ericaceous plants inoculated with *O. maius*.

The plants inoculated with *O. maius* B had higher foliar N content when compared to both control and either of two *P. fortinii* strains and also higher foliar P content when compared to control and *P. fortinii* F. On the other hand, *P. fortinii* H also increased foliar P content compared to the control, which confirms its beneficial effect for the inoculated plants and at the same time highlights the strain specificity, since *P. fortinii* F failed to facilitate P uptake by the inoculated plants. In regard to nutrient availability for plants inoculated with DSE, Jumpponen et al. (1998) reported enhanced phosphorus uptake by *Pinus contorta* when inoculated with *P. fortinii*. Studies addressing similar questions with ericaceous host plants are missing. In our previous study (Vohník et al. 2003) we reported no effect of two *P. fortinii* strains (one of them was *P. fortinii* F from this study) on the growth of *Rhododendron* cv. Belle-Heller in two different substrates, either sterilized or non-sterilized.

A few authors have reported the presence of both ErM and DSE fungi in the root system of a single ericaceous plant (Urcelay 2002, Midgley et al., 2004). However, there are no experimental data explaining the ecophysiological significance of these observations. Our experiment is, to our knowledge, the first attempt to artificially introduce both kinds of fungi into the root systems of

individual host plants and to trace the effect of the introduction on the physiology of the inoculated plants.

We show that in the terms of the nutrient uptake, the interaction among *O. maius* and *P. fortinii* is highly strain-specific: co-inoculation with *O. maius* B and *P. fortinii* H significantly increased foliar P and N content compared to the control and increased foliar P content when compared to co-inoculation with *O. maius* B and *P. fortinii* F. Co-inoculation with *O. maius* B and *P. fortinii* F did not influence P and N uptake. This indicates that *P. fortinii* F, contrary to *P. fortinii* H, negated the positive effect of *O. maius* B on P uptake by the host plants. It is difficult to explain this observation, since both fungi were spatially separated and the only communication among them could be realized through the host plant shoot. The question whether the strain specificity of the interaction is a result of the origin of the fungal strains ("positively" interacting *O. maius* B and *P. fortinii* H were isolated from the roots of rhododendrons, "neutral" *P. fortinii* F from the roots of *V. myrtillus* – see Materials and Methods) remains to be answered in an experiment using *V. myrtillus* as a host plant.

It should be emphasized that even if the co-inoculation with *O. maius* B and *P. fortinii* H increased P uptake compared to the control, co-inoculation with *O. maius* B and *P. fortinii* strains was never more efficient in the terms of nutrient uptake by host plant than the inoculation with single *O. maius* B. We can draw two conclusions from these observations. Firstly, under the experimental conditions, plants will benefit most from the presence of the single *O. maius* B without the need for other fungi in or around the root system (here two strains of *P. fortinii*); secondly, even the presence of fungi other than ErM in or around root system (here *P. fortinii* H) will still increase P and N uptake compared to the non-mycorrhizal control treatment, without negatively influencing the measured physiological parameters of host plants. Under different experimental conditions, plants with a combination of *O. maius* B+*P. fortinii* H in or around roots could have the advantage of the presence of an extra DSE fungus, which might improve access to soil nutrient sources other than peat, which was used in our experiment. Based on these conclusions, we can hypothesize that under our experimental conditions, *O. maius* B is the superior associate with roots of *Rhododendron* cv. Azurro compared to both *P. fortinii* strains. It is interesting to compare this hypothesis with the findings of Midgley et al. (2004) who reported that 75% of 327 fungal endophyte isolates from ericaceous *Woollsia pungens* Cav. (Muell.) and *Leucopogon parviflorus* (Andr.) Lindl. was represented by a single putative taxon with affinities to *Helotiales* ericoid mycorrhizal ascomycetes, which was spatially widespread in the root systems of both plants. Therefore, it seems that single fungal strains are able to dominate in root systems of ericaceous plants, depending on the environmental conditions. It would be interesting to simultaneously inoculate the root system of a single host plant with multiple fungal strains. Shifting experimental conditions could reveal the preferences of inoculated fungi (or their host plants) in relation to the physiological response of host plants.

Foliar C:N ratio is linked to soil C:N ratio and is an important parameter characterizing substrate nutrient availability (Cairney and Meharg 2003). Our results show that co-inoculation with *O. maius* B and both *P. fortinii* strains decreased foliar C:N ratio, which would decrease C:N ratio in the rhododendron litter. The significance of this intricate effect remains unclear, since ericoid mycorrhizal fungi are known to be able to draw nutrients directly from complex organic substrates and thus have a competitive advantage in the environments with high C:N ratios (Read and Perez-Moreno 2003). Decreased soil C:N ratios would likely result in the substitution of ericaceous plants by other species better adapted to this environmental condition (Read *et al*. 2004).

Differences in root biomass distribution revealed the tendency of the experimental plants to accumulate more root biomass (reflected by the weight of the dried root biomass) into dishes inoculated with *P. fortinii* H than to those non-inoculated or inoculated with *P. fortinii* F. A similar trend, however statistically non-significant, was observed for *O. maius* B compared to non-inoculated dishes. Since there was no influence of inoculation on the total weight of the dried biomass of the whole root systems, we conclude that *P. fortinii* H, and to a lesser extent *O. maius* B, altered the distribution but did not stimulate extra production of roots in the inoculated dishes. There is also a possibility that the root distribution could have been influenced by the nutrients supplied to the substrate through the inoculation procedure, whereby the inoculum may have included some nutrients from the fungal culture medium (see Materials and Methods). If this was true, the inoculated dishes would have been favored in terms of the distribution of the roots, which was however not the case (compare the distribution of the roots in the dishes inoculated with *P. fortinii* F and *P. fortinii* H).

Both *O. maius* B and *P. fortinii* H failed to form extensive intracellular root colonization in the Fungal Efficacy Experiment. Intracellular structures, such as ericoid mycorrhizal coils and loops and DSE intraradical hyaline hyphae, supposed to be the nutrient exchange sites (Smith and Read 1997, Barrow 2003), were missing in significant amounts. Similar to our observation, Piercey et al. (2002) reported a lack of ErM structures in *Rhododendron groenlandicum* inoculated with two strains of *O. maius* and attributed this observation to the saprobic abilities of *O. maius* strains. The authors stated that oidiodendrons were more efficient as free-living saprobes than in association with roots. In contrast, Usuki et al. (2003) reported that *O. maius* formed ErM with *Rhododendron obtusum* var. *kaempferi*. At low levels of mycorrhizal colonization, it is difficult to attribute the effects of the inoculation directly to the inoculated mycorrhizal fungi. However, it is accepted that in the case of ectomycorrhizal symbioses, even low colonization levels can produce significant effects on host plant performance (Smith and Read 1997). Unfortunately, similar studies with ericoid mycorrhiza or DSE associations are missing.

The screening of the stained roots revealed that the fungi inoculated into the dishes in the Fungal Efficacy Experiment were alive during our experiment. The inoculated fungi were apparently able to survive without carbon flow from the plants and thus, behave saprotrophically. The increased nutrient content of host plant tissues observed with fungal inoculation, despite a lack

of vigorous mycorrhizal structures, indicates the ability of the inoculated fungi to enhance nutrient uptake in their host plants. Combining the observations of increased nutrient uptake, altered root biomass distribution and low colonization rates, we deduce that *O. maius* B and *P. fortinii* H increased nutrient availability in the rhizosphere of the host plants rather than directly transported nutrients into the plant tissues. As a result, the inoculated plants tended to produce more root biomass in the compartments with higher nutrient availability, thus increasing the area for nutrient absorption. Our deduction emphasizes the saprotrophic capabilities of both *O. maius* B and *P. fortinii* H strains and highlights the significance of such features for plants in general, because they enable the fungi from the mycorrhizal/saprotropic boundary to support the nutrient uptake of plants without considerable intracellular colonization. Under some circumstances, strains of *O. maius* and *P. fortinii* certainly can form intracellular structures of the ErM and DSE patterns. Our study indicates that the absence of such structures in the roots does not imply the absence of effects of these fungi on the physiology of the host plants.

For example, we observed proliferating, vigorously growing non-mycorrhizal roots of rhododendrons in organic material (old homogenized tree bark) colonized by mycelium connected with fruitbodies of saprotrophic *Agrocybe* (unpublished data). The root system outside of the organic material was much less developed, even when containing intracellular structures resembling ErM. The need to study the fungal community not only in, but also around, the roots of ericaceous plants is evident.

To summarize, the results of our experiments revealed i) positive effect of *O. maius* on P, and to some extent also on N uptake, connected with lowered foliar C:N ratio; ii) strain-specific influence of *P. fortinii* on P and N uptake and the potential of *P. fortinii* to improve this uptake in inoculated plants; iii) highly strain-specific interaction of *O. maius* with *P. fortinii*, driving the effect of both fungi on N a P uptake when inoculated simultaneously; iv) the ability of *P. fortinii* H to influence the biomass distribution in the root system of its host plant, likely by releasing nutrients into the rhizosphere and v) the ability of *O. maius* B and *P. fortinii* H to influence nutrient uptake at very low colonization rates, likely by increasing nutrient availability in the rhizosphere.

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References

Ahlich, K., Sieber, T. N. 1996. The profusion of dark septate endophytic fungi in non-ectomycorrhizal fine roots of forest trees and shrubs. *New Phytologist* **132**: 259-270.

Bajwa, R., Read, D. J. 1985. The biology of mycorrhiza in the Ericaceae: IX. Peptides as nitrogen sources for mycorrhizal and non-mycorrhizal plants. *New Phytologist* **101**: 459-467.

Bajwa, R., Abuarghub, S., Read, D. J. 1985. The biology of mycorrhiza in the Ericaceae. X. The utilization of proteins and the production of proteolytic enzymes by mycorrhizal plants. *New Phytologist* **101**: 469-486.

Barrow, J. R. 2003. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in arid southwestern USA rangelands. *Mycorrhiza* **13**: 239-247.

Brundrett, M., Bougher, N., Dell, B., Grove, T., Malajczuk, N. 1996. *Working with mycorrhizas in forestry and agriculture*. ACIAR Monograph 32, Canberra, 374 pp.

Cairney, J. W. G., Meharg, A. A. 2003. Ericoid mycorrhiza: a partnership that expoits harsh edaphic conditions. *European Journal of Soil Science* **54**: 735-740.

Couture, M., Fortin, J. A., Dalpé, Y. 1983. *Oidiodendron griseum* Robak: An endophyte of ericoid mycorrhiza in *Vaccinium* spp. *New Phytologist* **95**: 375-380.

Currah, R. S., Tsuneda, A., Murakami, S. 1993a. Conidiogenesis in *Oidiodendron periconioides* and ultrastructure of ericoid mycorrhizas formed with *Rhododendron brachycarpum*. *Canadian Journal of Botany* 71: 1481-1485.

Currah, R. S., Tsuneda, A., Murakami, S. 1993b. Morphology and ecology of *Phialocephala fortinii* in roots of *Rhododendron brachycarpum*. *Canadian Journal of Botany* **71**:1639-1644.

Dalpé, Y. 1986. Axenic synthesis of ericoid mycorrhiza in *Vaccinium angustifolium* Ait. by *Oidiodendron* species. *New Phytologist* **103**: 391-396.

Douglas, G. C., Heslin, M. C., Reid, C. 1989. Isolation of *Oidiodendron maius* from *Rhododendron* and ultrastructural characterization of synthesized mycorrhizas. *Canadian Journal of Botany* **67**: 2206-2212.

Duclos, J. L., Fortin, J. A. 1983. Effect of glucose and active charcoal on *in vitro* synthesis of ericoid mycorrhiza with *Vaccinium* ssp. *New Phytologist* **94**: 95-102.

Ehrenberger, F., Gorbach, S. 1973. *Methoden der organischen Elementar- und Spurenanalyse*. Verlag Chemie, Weinheim.

Jansa, J. Vosátka, M. 2000. *In vitro* and *post vitro* inoculation of micropropagated rhododendrons with ericoid mycorrhizal fungi. *Applied Soil Ecology* **15**: 125-136.

Johansson, M. 2001. Fungal associations of Danish *Calluna vulgaris* roots with special reference to ericoid mycorrhiza. *Plant and Soil* **231**: 225-232.

Jumpponen, A., Trappe, J. M. 1998a. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytologist* **140**: 295-310.

Jumpponen, A., Trappe, J. M. 1998b. Performance of *Pinus concorta* inoculated with two strains of root endophytic fungus, *Phialocephala fortinii*: effects of synthesis system and glucose concentration. *Canadian Journal of Botany* **76**: 1205-1213.

Jumpponen, A., Mattson, K. G., Trappe, J. M. 1998. Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. *Mycorrhiza* **7**: 261-265.

Jumpponen A. 2001. Dark septate endophytes – are they mycorrhizal? *Mycorrhiza* **11**: 207-211.

Kerley, S. J., Read, D. J. 1995. The biology of mycorrhiza in the Ericaceae. XVIII. Chitin degradation by *Hymenoscyphus ericae* and transfer of chitin-nitrogen to the host plant. *New Phytologist* **131**: 369-375.

Kerley, S. J., Read, D. J. 1997. The biology of mycorrhiza in the Ericaceae. XIX. Fungal mycelium as a nitrogen source for the ericoid mycorrhizal fungus *Hymenoscyphus ericae* and its host plant. *New Phytologist* **136**: 691-701.

Kerley, S. J., Read, D. J. 1998. The biology of mycorrhiza in the Ericaceae. XX. Plant and mycorrhizal necromass as nitrogenous substrates for ericoid mycorrhizal fungus *Hymenoscyphus ericae* and its host. *New Phytologist* **139**: 353-360.

Leake, J. R., Read, D. J. 1989. The biology of mycorrhiza in the Ericaceae: XIII. Some characteristics of the extracellular proteinase activity of the ericoid endophyte *Hymenoscyphus ericae*. *New Phytologist* **112**: 69-76.

Martino, E., Turnau, K., Girlanda, M., Bonfante, P., Perotto, S. 2000. Ericoid mycorrhizal fungi from heavy metal polluted soils: their identification and growth in the presence of zinc ions. *Mycological Research* **104**: 338-344.

McLean, C. B., Lawrie, A. C. 1996. Patterns of root colonization in epacridaceous plants collected from different sites. *Annals of Botany* **77**: 405-411.

Midgley, D. J., Chambers, S. M., Cairney, W. G. 2004. Distribution of ericoid mycorrhizal endophytes and root-associated fungi in neighbouring *Ericaceae* plants in the field. *Plant and Soil* **259**: 137-151.

Mitchell, D. T., Read, D. J. 1981. Utilization of inorganic and organic phosphates by the mycorrhizal endophytes of *Vaccinium macrocarpon* and *Rhododendron ponticum*. *Transactions of the British Mycological Society* **76**: 255-260.

Perotto, S., Girlanda, M., Martino, E. 2002. Ericoid mycorrhizal fungi: some new perspectives on old acquaintances. *Plant and Soil* **244**: 41-53.

Piercey, M. M., Thormann, M. N., Currah, R. S. 2002. Saprobic characteristics of three fungal taxa from ericalean roots and their association with the roots of *Rhododendron groenlandicum* and *Picea mariana* in culture. *Mycorrhiza* **12**: 175-180.

Read, D. J. 1996. The structure and function of the ericoid mycorrhizal root. *Annals of Botany* **77**: 365-374.

Read, D. J., Perez-Moreno, J. 2003. Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytologist* **157**: 475-492

Read, D. J., Leake, J. R., Perez-Moreno, J. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. *Canadian Journal of Botany* **82**: 1243-1263.

Rice, A. V., Currah, R. S. 2001. Physiological and morphological variation in *Oidiodendron maius*. *Mycotaxon* **79**: 383-396.

Smith, S. E., Read, D. J. 1997. *Mycorrhizal Symbiosis*. Second Edition. Academic Press. London. 605 pp.

Stoyke, G., Egger, K. N., Currah, R. S. 1992. Characterization of sterile endophytic fungi from the mycorrhizae of subalpine plants. *Canadian Journal of Botany* **70**: 2009-2016.

Urcelay, C. 2002. Co-occurrence of three fungal root symbionts in *Gaultheria poeppiggi* DC in Central Argentina. *Mycorrhiza* **12**: 89-92.

Usuki, F., Junichi A. P., Kakishima, M. 2003. Diversity of ericoid mycorrhizal fungi isolated from hair roots of *Rhododendron obtusum* var. *kaempferi* in a Japanese red pine forest. *Mycoscience* **44**: 97-102.

Vohník, M., Lukančič, S., Bahor, E., Regvar, M., Vosátka, M., Vodnik, D. 2003. Inoculation of *Rhododendron* cv. Belle-Heller with two strains of *Phialocephala fortinii* in two different substrates. *Folia Geobotanica* **38**: 191-200.

Xiao, G., Berch, S. M. 1995. The ability of known ericoid mycorrhizal fungi to form mycorrhizae with *Gaultheria shallon*. *Mycologia* **87**: 467-470.

	Total weight of the dried biomass (g) NS	Weight of the dried shoot biomass (g) NS	Weight of the dried root bimass (g) NS	P (µg/g) p=0.00011	N (%) p=0.053	C:N ratio p=0.032
B (n=6)	1.24 (0.91-1.57)	1.13 (0.85-1.41)	0.11 (0.06-0.17)	1288 (913-1662)**c**	1.39 (1.19-1.57)**c**	34.6 (29.7-39.6)**a**
F (7)	1.51 (1.25-1.77)	1.36 (1.15-1.57)	0.15 (0.10-0.20)	818 (725-913)**ab**	1.21 (1.13-1.29)**ab**	39.4 (36.4-42.4)**abc**
H (7)	1.55 (1.28-1.81)	1.40 (1.17-1.63)	0.15 (0.10-0.19)	1060 (849-1272)**bc**	1.19 (1.06-1.32)**ab**	40.5 (36.0-44.9)**bc**
BF (7)	1.36 (1.13-1.58)	1.23 (0.85-1.41)	0.12 (0.09-0.16)	745 (609-882)**a**	1.26 (1.16-1.36)**abc**	37.8 (34.6-41.1)**ab**
BH (7)	1.29 (1.04-1.53)	1.15 (0.91-1.40)	0.13 (0.08-0.19)	1138 (815-1460)**c**	1.30 (1.16-1.44)**bc**	36.7 (32.8-40.4)**ab**
Control (7)	1.50 (1.15-1.84)	1.35 (1.06-1.64)	0.15 (0.09-0.20)	720 (552-888)**a**	1.12 (0.93-1.32)**a**	43.6 (36.8-50.3)**c**

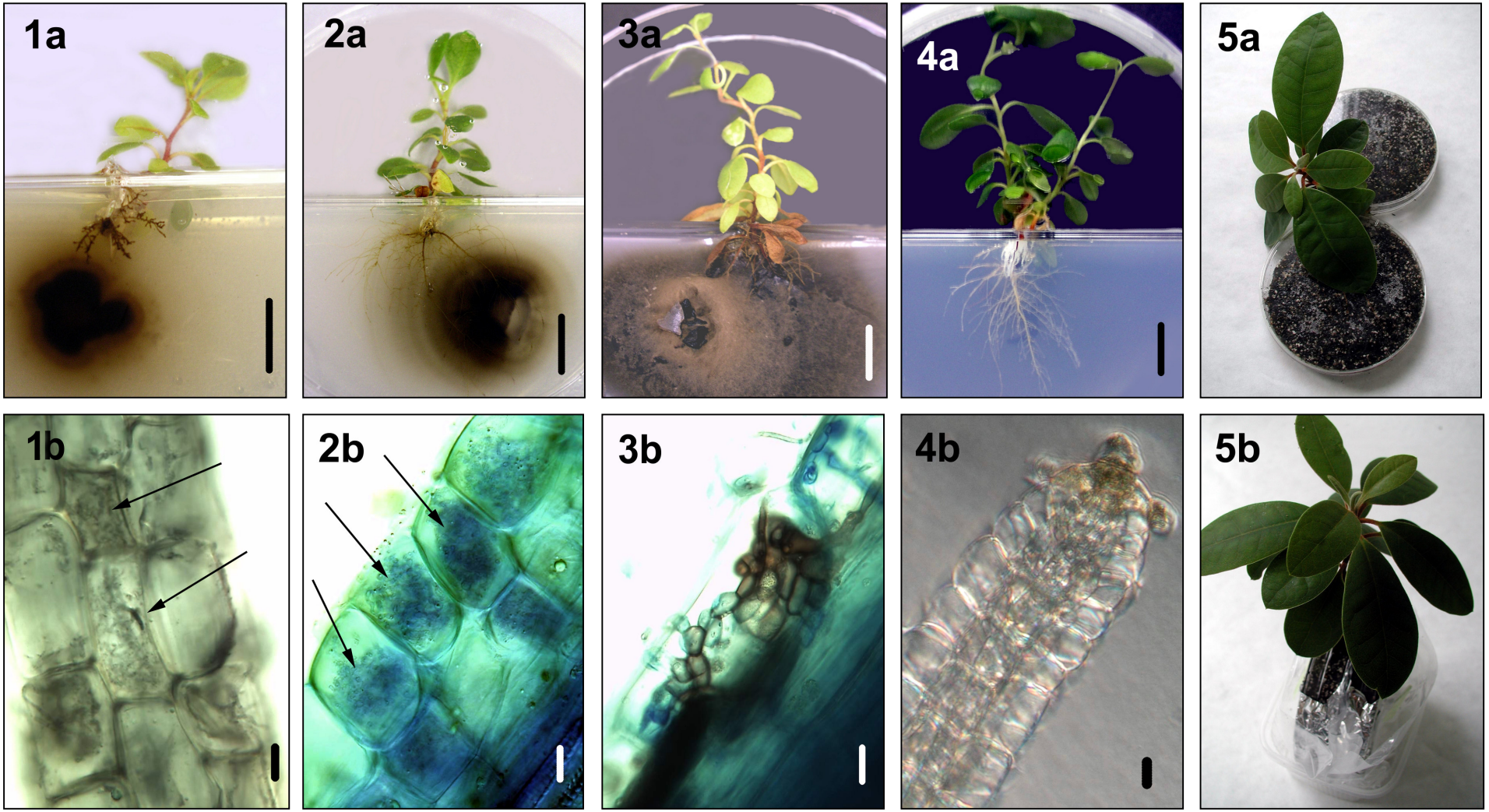
Table 1: The effects of the inoculation on the total weight of the dried biomass, the weight of the dried shoot and root biomass, foliar P content, percentual foliar N content and leaf C:N ratio. "**B**" corresponds to the treatment inoculated with *Oidiodendron maius* B; "**F**" with *Phialocephala fortinii* F; "**H**" with *Phialocephala fortinii* H; "**BF**" corresponds with the treatment co-inoculated with *O. maius* B and *P. fortinii* F; "**BH**" with the treatment co-inoculated with *O. maius* B and *P. fortinii* H. NS – non-significant effect of the inoculation. The numbers in the table are means of the measured parameters and the 95% Confidence Intervals. The different letters show significantly different groups at $p \leq 0.05$.

	0 - 0		B - 0		F - 0		H - 0		B - F		B - H	
WDR (mg)	73.9±32a	71.8±33a	64.2±33a	49.9±23a	74.1±28a	75.7±25a	81.4±22a	65.4±32a	64.7± 31a	58.7±23a	61.9±21a	73.0±45a
RATIO	1.04 (0.92-1.16)**a**		1.34 (0.81-1.86)**ab**		0.99 (0.77-1.22)**a**		1.61 (0.62-2.60)**b**		1.22 (0.67-1,77)a		0.99 (0.64-1.35)a	

Table 2: The effect of the inoculation on the distribution of root biomass between inoculated and non-inoculated dishes. The treatment **0-0** represents control with both dishes non-inoculated, **B-0** represents the treatment with one dish inoculated with *O. maius* B and the other non-inoculated, **F-0** is the treatment with one dish inoculated with *P. fortinii* F and the other non-inoculated, **H-0** is the treatment with one dish inoculated with *P. fortinii* H and the other non-inoculated. The treatment **B-F** had one dish inoculated with *O. maius* B and the other with *P. fortinii* F; **B-H** had analogically the other dish inoculated with *P. fortinii* H. **WDR** refers to the weight of the dried root biomass from a respective dish. **RATIO** expresses the ratio between the weight of the dried root biomass from the inoculated vs. non-inoculated dish (treatments B-0, F-0 and H-0), two non-inoculated dishes (treatment 0-0) or the dish inoculated with *O. maius* B vs. one of the *P. fortinii* strains (treatments B-F and B-H). The data were divided into two groups (in the white cells: treatments B-0, F-0 and H-0; in the gray cells: treatments B-F and B-H) for the statistical analysis (see Materials and Methods). The numbers in the table are means of the measured parameters ± SD (WDR) or the 95%Confidence Intervals (RATIO). The different letters show significantly different groups at $p \leq 0.05$.

Figures 1a-5b:

1a: Inoculation of *Rhododendron* cuttings with *O. maius* B resulted in reduction of the root development; **1b:** Intracellular structures (arrows) formed by *O. maius* B in *Rhododendron* root; **2a**: Rhododendrons inoculated with *H. ericae* developed branched root systems; **2b**: Intracellular structures (arrows) formed by *H. ericae* in *Rhododendron* roots; **3a**: Rhododendrons inoculated with both strains of *P. fortinii* developed branched root systems; **3b**: Intracellular microsclerotium formed by *P. fortinii* H; **4a**: Also non-inoculated rhododendrons had branched, well developed root systems; **4b**: a non-colonized root of a non-inoculated control plant; **5**: A *Rhododendron* cv. Azurro rooted cutting in the split root system; **5a**: before the inoculation, **5b**: after the inoculation. The fungal structures were stained with chorazol black (Fig. 1b) or trypan blue (Figs. 2b and 3b). Bars in the upper row correspond to 1cm, in the lower row correspond to10µm.


1a
2a
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1b
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3b
4b
5b

1. 5. Část I: Diskuse

Mykorhizní typy u alpínských rostlinných komunit, jejichž habitat se podobá habitatu některých rododendronů, zkoumaných v Článku 1 (*R. hirsutum*, *R. ferrugineum*, *R. kotschyi*), zkoumali zejména Haselwandter (1978), Haselwandter a Read (1980) a Read a Haselwandter (1981). Velmi zajímavá práce Haselwandter (1978) zjišťuje, že vyšší intenzita houbové kolonizace u vřesovcovitých souvisí s lepší fitness (*host vigour*) zkoumaných rostlin, byť je zde tato veličina definována jako podíl sušiny společenstva zkoumaného druhu na celkové rostlinné sušině dané lokality. Autor také uvádí, že míra mykorhizní kolonizace zkoumaných vřesovcovitých rostlin klesá se vzrůstající nadmořskou výškou. Míru mykorhizní kolonizace však autor určil pomocí chemického stanovení houbového chitinu (resp. jeho derivátu chitosanu) v kořenech, a proto není možné rozhodnout, do jaké míry výsledné hodnoty reprezentují mykorhizní houby (ať již ErM nebo DSE) a do jaké míry jsou ovlivněny přítomností jiných, parazitických či saprotrofních hub. Poslední dvě práce uvedené výše se vřesovcovitými zabývají pouze okrajově, zaměřily se mimo jiné i dva druhy rododendronů (*R. hirsutum* a *R. ferrugineum*). Autoři však u *R. ferrugineum* uvádějí pouze přítomnost ErM, u *R. hirsutum* pak neuvádějí žádnou kolonizaci, byť u ostatních rostlin berou v úvahu kolonizaci tmavými přepážkovanými hyfami (*dark septate hyphae*).

Článek 1 naproti tomu ukazuje, že všech šest zkoumaných druhů evropských rododendronů tvořilo vedle ErM také DSE-asociaci, byť proporce obou asociací se lišily v závislosti na druhu hostitelské rostliny, resp. na lokalitě, na které byly odebrány její kořeny. Tento rozpor může mít několik příčin: (i) samotný termín DSE-asociace se ustálil až s prací Jumpponena (2001), identita DSE hub byla objasněna až s rozvojem molekulárních technik. Je možné, že někteří autoři přítomnost DSE hub ignorovali, zejména pokud si uvědomovali převážně negativní vliv DSE hub (tedy MRA, viz úvod této části DP); (ii) zkoumání vřesovcovitých rostlin bylo ve většině prací pouze doplňkové, většina z nich byla primárně zaměřena na typicky AM rostliny; (iii) vřesovcovité rostliny byly tradičně považovány za ErM, přičemž i typičtí ErM mykobionti mají přepážkované a často i tmavé hyfy. DSE-asociace tak mohla být považována za ErM. To naznačují i výsledky Článků 1 a 2, které ukazují, že DSE mohou tvořit vnitrobuněčné struktury, připomínající morfologicky ErM.

Článek 1 a okrajově také Článek 2 popisují několik typů morfologických struktur, tvořených DSE. K těm atypickým patří zejména tzv. přechodné útvary (*intermediate structures*; viz výsledky, diskuse a obrázky k Článkům 1 a 2), tedy vnitrobuněčné smyčky, tvořené hyalinními až tmavými přepážkovanými hyfami, které morfologicky stojí mezi ErM a DSE-asociací, a tzv. parenchymatózní síť. Podobná struktura byla popsána např. v práci Wurzburger a Bledsoe (2001) ("*a partial DSE mantle*", viz Článek 1), ovšem na kořenech borovice a s odlišnou morfologií. Domnívám se, že obě tyto struktury, tedy přechodné útvary a parenchymatózní síť, by mohly být oněmi stále chybějícími (ve smyslu nepopsanými nebo neidentifikovanými) rozhraními, na kterých probíhá výměna látek mezi hostitelskou rostlinou a DSE houbou.

Z hlediska morfologie mykorhiz je zajímavý i popis atypické kolonizace u *R. kotschyi*, kdy je vrstva epidermálních buněk kolonizována přechodnými útvary a vrstva buněk primární kůry je ErM (viz obrázky 15a – 15c, Článek 1), a u *R. hirsutum*, *R. ferrugineum* a *R. kotschyi*, kdy se parenchymatózní síť tvoří mezi vrstvou nekolonizovaných rhizodermálních buněk a vrstvou ErM buněk primární kůry (viz obrázky 21a – 21e, Článek 1). Je možné, že se jedná o dosud nepopsaný morfologický typ mykorhizní asociace u vřesovcovitých, který (s výjimkou absence hyfového pláště) do určité míry může připomínat nedávno popsanou kavendišoidní ektendomykorhizu (*cavendishoid ectendomycorrhiza*, Setaro a kol. 2005), vyskytující se u andských vřesovcovitých rostlin. Aby tato hypotéza mohla být potvrzena nebo vyvrácena, je třeba identifikovat a izolovat mykobionta(-y), který(-ří) se této asociace účastní a tuto asociaci syntetizovat v kontrolovaných podmínkách. Doufám, že toto bude náplní mé příští práce.

Jak bylo uvedeno výše, rododendrony z různých lokalit se lišily proporcemi ErM a DSE-asociace (Článek 1). Jedním z faktorů, které mohou ovlivnit koexistenci ErM a DSE, by mohla být zeměpisná šířka. Read a Perez-Moreno (2003) uvádějí, že zvyšující se zeměpisná šířka se odráží v posunu ve složení mykorhizních společenstev (tedy společenstev rostlin, tvořících určité typy mykorhiz s určitými druhy mykorhizních hub). Zjednodušeně lze říci, že AM jsou postupně nahrazovány EcM, které jsou dále nahrazovány ErM, které jsou následně nahrazovány DSE. Dle tohoto schématu se předpokládá, že tropické oblasti jsou dominovány AM (ale viz např. Moyersoen a kol. 2001), oblast mírného pásma obsahuje především AM a EcM společenstva, boreální pásmo je pak dominováno EcM a ErM. Předpokládá se, že DSE nahrazují funkci ostatních mykorhizních hub v sub(-ant-)arktickém a (ant-)arktickém prostředí.

Je důležité se zamyslet, zda za tento posun mohou primárně mykorhizní houby, nebo hostitelské rostliny, nebo jejich vzájemná souběžná evoluce, nebo především jiné (a-)biotické charakteristiky prostředí. Pro první variantu hovoří např. skutečnost, že klíčivost spor AM hub je náchylnější k nízkým teplotám než klíčivost spor EcM hub, přičemž ErM a DSE houby se sporami většinou vůbec nešíří. V chladnějších oblastech pak může být tvorba AM omezena absencí nebo nízkou fitness AM hub. Je známo, že AM houby přímo ovlivňují druhovou variabilitu společenstva hostitelských rostlin (např. van der Heijden a kol. 1998). Hypoteticky tak mohou činit i EcM, ErM a DSE houby. V neposlední řadě pak některé možná "mykorhizocentrické" práce uvádějí možnost, že kořen vznikl během přechodu rostlin z vodního prostředí na souš jako samostatný orgán zejména (nebo alespoň také) proto, aby v něm mohly rostliny "ubytovat" (*to accomodate*, *to host*; česky snad *hostit*, *uchovávat*, avšak *ubytovat* se mi v tomto kontextu zdá velmi výstižné) mykorhizní houby (Brundrett 2002). Pro druhou variantu hovoří např. práce Ruotsalainen a Kytöviita (2004), ve které autorky zkoumaly efekt mykorhizní asociace s AM houbou *Glomus claroideum* a DSE *P. fortinii* u *Gnaphalium norvegicum* Gunnerus. Autorky dospěly k závěru, že mykorhizní prospěšnost (*mycorrhizal benefit*) pro hostitelskou rostlinu je u daných asociací při nižších teplotách nižší, tedy i úroveň mykotrofie *G. norvegicum* s teplotou klesá. Rostlina je tedy do jisté míry při nižších teplotách na mykorhizních houbách méně závislá, snad si tedy může i snáze

"vybírat" např. méně náročné či v dané situaci efektivnější mykobionty (EcM a zejména ErM houby mají v porovnání s AM houbami značné saprotrofní schopnosti, zejména ErM houby jsou v porovnání s AM houbami na hostitelské rostlině poměrně nezávislé apod.), nebo mykorhizní houby prostě "ignorovat". Myslím si, že nejpravděpodobnější je přechod mezi třetí a čtvrtou variantou, kdy za výslednou strukturu mykorhizních (rostlina + houba) komunit mohou jak rostliny, tak mykorhizní houby, ovlivňované navíc všemi faktory prostředí. Ostatně právě vnější prostředí musí nutně determinovat složení jak mykorhizních hub, tak jejich hostitelských rostlin. Z tohoto pohledu zajímavý výklad funkce mykorhizních hub jako hybatelů (*drivers*) ekosystémových procesů ve vřesovištních a boreálních lesních biomech podávají Read a kol. (2003). Autoři poukazují na skutečnost, že boreální biomy nejsou uniformní ani z hlediska geografického rozšíření rostlin, ani z hlediska mykorhizních typů, které tyto rostliny tvoří. Za prvotně důležitou autoři považují schopnost EcM a ErM hub rozkládat specifický opad (ten je zde brán jako primární faktor, ovlivňující biotu těchto biomů), který se v boreálním pásu tvoří, tím ovlivňovat cyklus uhlíku a dusíku v těchto biomech a zpřístupňovat živiny rostlinám na úkor např. půdních saprotrofních organizmů. Právě díky této schopnosti pak mohou mykorhizní houby systémem zpětných vazeb ovlivňovat produktivitu, druhové složení a produktivitu boreálních ekosystémů. Širší diskuse této problematiky nicméně přesahuje téma a rozsah této disertační práce.

Podobný vzorec, popisovaný např. v práci Read a Perez-Moreno (2003), byl nalezen i v kořenech evropských rododendronů (Článek 1), kdy nejvyšší míra DSE kolonizace byla nalezena u nejseverněji se vyskytujícího *R. lapponicum* a postupně klesala směrem k jižnějším lokalitám, přičemž míra DSE kolonizace byla u všech rododendronů negativně korelována s mírou ErM kolonizace. Zdá se tedy, že DSE-asociace opravdu dominuje kořeny rododendronů z chladnějších oblastí a že mezi DSE-asociací a ErM, resp. houbami, které ji tvoří, existuje určitá forma kompetice o kořeny hostitelských rostlin. Tyto úvahy jsou podpořeny výsledky Článku 2. Z nich je zřejmé, že DSE *P. fortinii* ve směsné in vitro kultuře postupně dominuje nad ErM houbou *R. ericae*. Je otázkou, do jaké míry jsou výsledky in vitro pokusů relevantní vzhledem k procesům, které probíhají na přirozených stanovištích. Na druhou stranu posun proporcí ErM a DSE-kolonizace v kořenech evropských rododendronů naznačuje, že určitá interakce mezi oběma druhy asociací pravděpodobně existuje.

Výsledky Článku 2 také ukazují, že míru kolonizace kořenů hostitelské rostliny alespoň v in vitro podmínkách určuje spíše interakce mezi ErM a DSE houbami, než rostlina sama. Zdá se, že záleží především na množství ErM/DSE mycelia, přítomného v rhizosféře hostitelské rostliny; různé proporce mycelia obou typů se odrážejí v různé míře ErM a DSE kolonizace kořenů hostitelské rostliny. Fyziologická odpověď rostliny na různou míru ErM/DSE kolonizace je plastická a zdá se býti negativně korelována s množstvím DSE mycelia v rhizosféře, tedy i s mírou DSE kolonizace. Samotná DSE-asociace má na růst rostliny neutrální nebo negativní vliv. Zdá se, že tento může být zmírněn nebo posunut současnou přítomností ErM. Podobný ochranný efekt

EcM hub proti MRA (tedy DSE) je zmiňován v prácích Richard a kol. (1971), Richard a Fortin (1975) nebo Hashimoto a Hyakumachi (2001).

Tento ochranný efekt může být důležitý i z jiného hlediska: ErM a DSE houby se jistě alespoň částečně liší ve svých fyziologických vlastnostech (není mi však známo, že by existovala publikovaná práce, která by toto porovnávala). Pro rostlinu proto může být současná přítomnost jak ErM, tak DSE-asociace výhodná, zvláště v heterogenním substrátu, kdy různé houby mohou čerpat živiny z různých zdrojů a tak rozšiřovat spektrum zdrojů i pro hostitelskou rostlinu. To je, alespoň částečně, zřejmé i z výsledků Článku 3. Z hlediska příjmu živin (fosfor, dusík) je pro rostlinu nejvýhodnější kolonizace pouze ErM houbou *Oidiodendron maius* Barron, na druhou stranu i současná kolonizace houbami *O. maius* a *P. fortinii* je rostlině v porovnání s nekolonizovanou variantou prospěšná. Je třeba zdůraznit, že efekt DSE-asociace, nalezený v Článcích 2 a 3, byl vždy kmenově specifický, a že se statisticky nepodařilo prokázat prospěšnost interakce mezi ErM a DSE houbami vzhledem ke zkoumaným fyziologickým procesům hostitelských rostlin. Negativní (resp. pozitivní) vliv jednoho kmenu *P. fortinii*, zjištěný v Článku 2 (resp. 3) byl v rozporu s pozitivním (resp. negativním) vlivem kmenu druhého. Z výsledků Článku 3 také vyplývá, že pozitivní vliv *P. fortinii* na hostitelskou rostlinu může být nepřímý, tj. nemykorhizní jak vysvětleno dříve nebo např. v pracích Piercey a kol. (2002) a Ruotsalainen a Kytöviita (2004). Izolát *P. fortinii* PFO-H totiž působil na hostitelskou rostlinu pozitivně ve srovnání s neinokulovanou kontrolou a s variantou inokulovanou izolátem *P. fortinii* PFO-F, přičemž kolonizační potenciál PFO-H byl nižší než PFO-F (viz Článek 3). Tato domněnka je podpořena i skutečností, že přítomnost PFO-H přes (nebo právě pro) nízkou schopnost kolonizovat kořeny hostitelské rostliny podpořila, pravděpodobně nepřímo, jejich růst v inokulovaném kompartmentu. To lze vysvětlit mimo jiné tak, že PFO-H netransportovala živiny získané mineralizací organického substrátu přímo do málo kolonizované hostitelské rostliny, ale uvolňovala je v minerální formě zpět do substrátu. Zvýšená nabídka dostupných živin pak stimulovala růst kořenů hostitelské rostliny. To svědčí právě o nemykorhizním efektu *P. fortinii*, protože u "pravé" mykorhizní houby obecně naopak dochází k relativnímu zmenšení kořenového systému hostitelské rostliny, protože funkci kořenů do jisté míry přebírá houbové mycelium.

Na závěr této kapitoly se chci krátce zaměřit na vliv DSE-asociace na hostitelskou rostlinu z opačné strany. Jak uvedeno výše, byl tento efekt neutrální nebo negativní, a to z hlediska růstu a/nebo příjmu živin kolonizovanou rostlinou. Je zajímavé porovnat rozměry dvou rododendronů, zkoumaných v Článku 1. Keře *R. ponticum* z nejjižnější lokality ve španělské Andalusii jsou vysoké několik metrů a nezřídka zasahují až k hranici stromového patra. Tento druh je kolonizován především ErM houbami, v menší míře pak DSE. Naproti tomu keříky *R. lapponicum* ze severního Finska jsou vysoké max. desítky centimetrů a jsou kolonizovány především DSE, v menší míře i ErM houbami.

Lze s určitou mírou nadsázky hypotetizovat, že kdyby byl určitý jedinec *R. lapponicum* na své severofinské lokalitě inokulován ErM houbami a v jeho kořenech by začala převažovat ErM

nad DSE-asociací, začal by ve srovnání s ostatními DSE-dominovanými jedinci rychleji tvořit větší množství biomasy s vyšším podílem biogenních prvků. Takový jedinec by pak s vyšší pravděpodobností vymrznul, nebo byl s vyšší pravděpodobností zkonzumován vyhládlými soby. Negativní efekt DSE-asociace, zjištěný v laboratorních podmínkách, by tak poskytl neinokulovaným jedincům nespornou evoluční výhodu.

2. ČÁST II:

Kolonizační potenciál *Meliniomyces variabilis* a vybraných ektomykorhizních a saprotrofních hub v kořenech typicky erikoidně mykorhizních a ektomykorhizních rostlin

Rhododendron luteum připravující se na zimu 2005 v rezervaci Wola Żarczycka, Polsko.

2. 1. Část II: Úvod

Kořeny prvních mykorhizních rostlin byly pravděpodobně kolonizovány předchůdci dnešních AM hub (Redecker a kol. 2000) a AM je považována za výchozí typ, ze kterého se vyvinuly ostatní typy mykorhizních symbióz (Redecker 2002), zejména EcM a evolučně nejmladší typy endomykorhiz, např. ErM (Cairney 2000). Jednotlivé typy mykorhiz jsou odlišné anatomicky, morfologicky i fyziologicky a většinou se nepřekrývá ani spektrum jejich hostitelských rostlin. Často se však na přirozených stanovištích rostliny a houby různých mykorhizních preferencí vyskytují pospolu a interagují. To může mít značné důsledky pro oba partnery, vstupující do mykorhizní symbiózy (Francis a Read 1994). Byla např. vyslovena domněnka, že ErM houby dokáží z prostředí eliminovat EcM houby a potažmo tak i jejich hostitelské rostliny (Walker a kol. 1999). Předpokládá se, že vysoká odolnost ErM hub vůči změnám prostředí a zejména jejich schopnost přežívat v půdě bez přítomnosti hostitele usnadnila expanzi ErM hostitelské rostliny *Gaultheria shallon* Pursh podél severozápadního pobřeží severní Ameriky na úkor původního EcM porostu (Xiao a Berch 1995). Naproti tomu se zdá, že AM hostitelské rostliny mohou efektivně vytěsňovat ErM rostliny (Hartley a Amos 1999). Je možné, že taková schopnost AM rostlin společně se zvýšenou depozicí dusíku urychluje ústup a zatravňování původních vřesovišť v severní Evropě (Johansson 2000).

Mykorhizní houby dokáží spojovat hostitelské rostliny systémem extraradikálního mycelia do vzájemně komunikujících celků, což zásadním způsobem ovlivňuje rostlinnou biodiverzitu, variabilitu a produktivitu ekosystémů (van der Heijden a kol. 1998). Zásadní význam tohoto fenoménu shrnul Read (1998), který pro síť mykorhizního mimokořenového mycelia v půdě použil výstižný (a vtipný - což bylo pravděpodobně pro jeho další užívání podstatnější) termín *wood wide web* (www). Lze předpokládat, že pomocí www jsou spojeny především rostliny stejných mykorhizních preferencí. Snad právě proto se studium funkčního propojení omezuje na různé EcM rostliny spojené myceliem EcM hub, různé AM rostliny spojené myceliem AM hub apod.

Přesto existuje mnoho dokladů o výskytu mykorhizních hub v kořenových systémech rostlin, které nejsou považovány za jejich hostitele, protože neodpovídají jejich mykorhizním preferencím. Horton a kol. (1998) například popsal současný výskyt EcM, AM a DSE-asociace u semenáčků typicky EcM *Pinus muricata*. Urcelay (2002) popsal současný výskyt AM, ErM a DSE-asociace v kořenech typicky ErM *G. poeppiggi*. Chaurasia a kol. (2005) popsal přítomnost AM u Himalájských rododendronů (viz úvod Část I). Je tedy teoreticky možné, že pomocí www mohou být propojeny i rostliny zdánlivě odlišných mykorhizních preferencí, přičemž se mykotrofně odlišná rostlina "přizpůsobí" a začne tvořit mykorhizní typ, který pro ni není typický. Osobně si však myslím, že přítomnost "netypických" mykorhiz u rostlin s jinou preferencí je spíše výjimečný a pravděpodobně nemá většího ekofyziologického významu.

Mykorhizní preference vřesovcovitých jsou spojeny s ErM houbami, ostatně název ErM hub je odvozen od názvu čeledi *Ericaceae*. Dále tvoří vřesovcovité za přirozených podmínek DSE-asociaci. Zřídka jsou v jejich kořenech nalezeny struktury (především vezikuly, zřídka arbuskuly),

typické pro AM. Tato skutečnost snad lze vysvětlit pomocí tzv. efektu chůvy okolních rostlin (Část I). Je třeba skepticky uvést, že údaje o přítomnosti AM v kořenech vřesovcovitých jsou založeny na mikroskopickém hodnocení kolonizace, a že případné arbuskuly mohou být těžce odlišitelné od ErM struktur (viz Obr. 3, Článek I). Ačkoliv se ErM a AM rostliny často vyskytují společně v rámci stejných habitatů (tedy "vedle sebe"), ze zkušenosti vím, že rostou většinou odděleně (tedy ne "mezi sebou"). Naproti tomu vřesovcovité rostliny pravidelně tvoří podrost EcM rostlin.

Houby tvořící EcM ve většině náležejí mezi bazidiomycety (ErM houby jsou askomycety). Tato skutečnost dává zajímavý podtext zprávám o výskytu bazidiomycetů uvnitř rhizodermálních a kortikálních buněk kořenů vřesovcovitých rostlin. Avšak, ačkoliv jsou první záznamy popisující podobná pozorování staré přes dvě desetiletí (Bonfante-Fasolo 1980) a zprávy o výskytu bazidiokarpů asociovaných s ErM vřesovcovitými rostlinami ještě starší (Seviour a kol. 1973), podstata tohoto fenoménu zůstává neobjasněna. Je možné, že se jedná o podobnou výjimku, jako v případě AM u vřesovcovitých. Na druhou stranu recentní zprávy naznačují, že bazidiomycety mohou kolonizovat kořeny vřesovcovitých podstatně častěji, než se předpokládalo. Výskyt příslušníků řádu *Sebacinales* (Heterobasidiomycetes) v kořenech vřesovcovitých mimo jiné shrnuje práce Weiß a kol. (2004), která zároveň zdůrazňuje široké spektrum mykorhizních typů, které mohou tyto houby tvořit. Dle dostupných pramenů se *Sebacinales* jako mykobionti účastní EcM, ErM i orchideoidní mykorhizy. Důvodem opomíjení přítomnosti těchto hub nejen v kořenech vřesovcovitých může být fakt, že se ve většině jedná o houby pouze obtížně (pokud vůbec) kultivovatelné. Detekce *Sebacinales* je tak možná povětšinou pouze sekvenováním jejich DNA, získané přímo z kolonizovaného kořene (např. Allen a kol. 2003). Zdá se, že houby tohoto řádu mohou kolonizovat jak EcM konifery (popř. i listnaté stromy), tak (ne-)zelené orchideje, rostoucí v jejich podrostu (viz shrnutí ve Weiß a kol. 2004), a mohou tak zajišťovat případné propojení obou typů rostlin. Doklad jejich přítomnosti u vřesovcovitých se však opírá hlavně o publikaci Allen a kol. (2003). Ekofyziologický význam výskytu *Sebacinales* u vřesovcovitých tak stále čeká na své ozřejmení. Přes hypotézy o nekompatibilnosti ErM a EcM hub (viz výše) a doposud nevyjasněný výskyt bazidiomycetů v kořenech vřesovcovitých však zůstává faktem, že typická kombinace EcM smrk (borovice, bříza apod.) + ErM borůvka (vřes, vřesovec, brusinka apod.) není alespoň v našich podmínkách ničím neobvyklým.

Není mi známo, že by existoval publikovaný doklad o výskytu ErM u typicky EcM rostlin. Toto však může být způsobeno používanou terminologií – ErM je definována především jako morfologicky charakteristická mykorhiza, tvořená typickým okruhem hub v kořenech zástupců čeledi *Ericaceae*. Vyskytují-li se typicky ErM houby na/v kořenech typicky EcM rostlin, o výskytu ErM se nehovoří, přestože tyto houby mohou tvořit útvary morfologicky podobné ErM. Pokud taková houba netvoří Hartigovu síť, není její přítomnosti většinou přikládán velký význam – předpokládá se, že je s kořeny asociována bez podstatného ekofyziologického významu. Obvykle je poukazováno na vysoký stupeň infektivity ErM hub, které mohou nespecificky kolonizovat i mykotrofně nekompatibilní rostliny. Pokud ovšem ErM houba v kořenech Hartigovu síť tvoří,

jedná se o EcM (pak i taková houba je považována za EcM houbu). Předpokládá se, že existují nejméně dvě houby, schopné tvořit jak ErM (typickou mykorhizu v kořenech vřesovcovitých), tak EcM (typickou mykorhizu s Hartigovou sítí). Obě náleží do tzv. *Rhizoscyphus ericae* – agregátu (Graf 1 na konci této kapitoly, viz dále). Toto úzké spektrum by mohlo být v budoucnosti rozšířeno o příslušníky *Sebacinales*.

Na druhou stranu existují publikované doklady o výskytu EcM u vřesovcovitých, tedy o výskytu Hartigovy sítě v kořenech typicky ErM rostlin. Nejsou příliš časté, hojně citovaná je např. práce Dighton a Coleman (1992). Vzhledem k tomu, že se jedná o publikaci z hlediska DP velmi relevantní (Část I i Část II), pokusím se ji detailněji popsat. Autoři zkoumali mykorhizy u *R. maximum* v jižní oblasti Apalačského pohoří, Jižní Karolina, USA. Odebírali 4.5 cm široké a 10 cm vysoké válcové vzorky (*core sampling*) rhizosférního substrátu uprostřed třech oblastí, charakterizovaných jako "*extensive, dense, monospecific stands of R. maximum*". Vzorky byly odebírány v těsné blízkosti kmenů *R. maximum*. Ze vzorků autoři ručně vybrali všechny (alespoň tak lze soudit z kapitoly Materiál a Metody) kořeny, roztřídili je do tří hlavních morfologických skupin a popsali vyskytující se mykorhizní asociace. Autoři pak porovnávali získané výsledky s kořeny téhož druhu, komerčně pěstovaného ve školce (popis pěstování ve školce je bohužel zcela kryptický). Tyto tři skupiny byly: **(i)** "*fine, hair-like roots consisting of stele and a single layer of cortical cells which had an outer lattice of thick, brown hyphae*"; jedná se pravděpodobně o popis kolonizovaných vlasových kořenů (*hair roots*), tedy orgánů typických pro vřesovcovité (např. Read 1996); **(ii)** "*beaded roots, containing a stele and a number of cortical cell layers*"; a **(iii)** "*roots which showed evidence of a fungal sheath*". Autoři uvádějí, že různé mykorhizy a kořenové morfotypy byly nacházeny v různých částech jednotlivých kořenových systémů, tedy vzájemně nesousedily. Neuvádějí však, jakým způsobem definovali jednotlivý kořenový systém, ani jakým způsobem určili, že kořeny opravdu náležely *R. maximum*. V rámci skupiny (i) identifikovali autoři na základě přítomnosti arbuskulí AM (tehdy VAM) a pravděpodobně i ErM [skupina (i) by měla být dominována ErM], v rámci skupiny (ii) identifikovali na základě přítomnosti arbuskulí AM, v rámci skupiny (iii) autoři rozlišili a popsali celkem devět (!) typů EcM. Jeden z nich na základě morfologie identifikovali jako *Cenococcum geophilum* (typicky EcM askomycet, viz Článek 5). Zdá se tedy, že *R. maximum* je schopen tvořit všechny nejrozšířenější mykorhizy (AM, EcM a ErM). Osobně, a tedy s nezbytnou dávkou subjektivity, si myslím, že způsob, jakým autoři odebírali kořeny zkoumaného rododendronu přímo vybízí k zamyšlení, nedocházelo-li k záměně kořenů rododendronů s jinými rostlinami. U evropských rododendronů jsem napříč kontinentem EcM ani AM nenašel (Článek I), zaměřil jsem se však pouze na vlasové kořeny, které by pravděpodobně spadaly do skupiny (i) výše uvedeného členění.

Zajímavou experimentální prací zkoumající přítomnost EcM u vřesovcovitých je publikace Smith a kol. (1995). Autoři v kultivačním experimentu zkoumali, zdali mohou vřesovcovité rostliny *G. shallon* a *Rhododendron macrophyllum* G. Don sdílet společné mykobionty s koniferami *Pseudotsuga menziesii* (Mirbel) Franco a *Tsuga heterophylla* (Raf.) Sarg. Jako

inokulum použili nesterilní substrát z lokality, kde se zmíněné rostliny vyskytovaly dohromady. Do tohoto substrátu autoři zasadili semenáčky zmíněných rostlin (vždy všechny 4 druhy do společného květináče) a po 8 – 10 měsících vyhodnocovali jejich EcM a ErM kolonizaci. EcM kolonizace byla hodnocena pomocí mikroskopického pozorování ručních řezů a kořenových roztlaků (*root squashes*), jejím indikátorem byla přítomnost Hartigovy sítě. Vzhledem k tématu DP se zaměřím pouze na výsledky, týkající se obou vřesovcovitých rostlin. U nich autoři nalezli dva EcM morfotypy a určili je jako "*Rhizopogon*-type" a "*Thelephora*-type". Typ *Rhizopogon* se vyskytoval u 19% a typ *Thelephora* u 7% semenáčků obou rostlin, ovšem vždy pouze ve stopových množstvích (*trace amounts*), definovaných jako méně než 1% kořenového systému. Oba morfotypy byly monopodiální, vyskytovaly se na terminálních kořenových špičkách. U typu *Rhizopogon* autoři pozorovali pro EcM netypickou intracelulární kolonizaci buněk kořene a Hartigovu síť mezi rhizodermálními buňkami, mykorhizy byly 1 – 1.5 mm dlouhé a měly průměr 0.2 – 0.3 mm. U typu *Thelephora* autoři pozorovali pouze ojedinělé intercelulární hyfy, Hartigova síť tedy nebyla typicky vyvinuta, mykorhizy byly 1 – 2 mm dlouhé a měly průměr 0.3 – 0.5 mm. Z uvedených výsledků je důležité zejména to, že EcM u obou vřesovcovitých rostlin byly přítomny pouze ve stopových množstvích. Navíc je třeba brát v úvahu efekt chůvy okolních EcM konifer, který mohl tvorbu EcM u netypických hostitelů podnítit. Autoři bohužel neilustrovali nalezené EcM morfotypy obrazovou přílohou, navíc identifikovali druhý z nich jako EcM i bez přítomnosti Hartigovy sítě. Troufám si také odhadnout, že autoři vzhledem k rozměrům obou morfotypů použili k jejich určení pouze kořenové roztlaky, nikoliv ruční řezy. V roztlacích se i atypická kolonizace, nalezená v kořenech evropských rododendronů (Článek 1) může jevit jako EcM. Doklady přítomnosti EcM u vřesovcovitých jsou proto z mého pohledu nejasné.

Uvažujeme-li však o případném napojení typicky ErM a typicky EcM hostitelských rostlin na společnou síť mimokořenového mycelia, nemusíme předpokládat, že v kořenech takto napojených rostlin bude zformován jenom jeden typ mykorhizy. Existují totiž mykorhizní houby, které dokáží tvořit ErM i EcM v závislosti na hostitelské rostlině (Bergero a kol. 2000, Vrålstad a kol. 2000 a 2002). Náleží do taxonomicky nelehce uchopitelného agregátu okolo typické ErM houby *Hymenoscyphus ericae* (Read) Korf & Kernan. Název *H. ericae* - agregát je dosud používán, přestože mykorhizní houba, která mu dala jméno, byla přeřazena do rodu *Rhizoscyphus* (viz Hambleton a Sigler 2005). V této práci budu používat označení *Rhizoscyphus ericae* – agregát (*R. ericae* – agregát), protože toto označení pokládám za logičtější. S rozvojem molekulární taxonomie se ukázalo, že se jedná o druhy *Cadophora* (dříve *Phialophora*) *finlandica* (Wang & Wilcox) Harr. & McNew a *Meliniomyces bicolor* Hambleton & Sigler (Hambleton a Sigler 2005). *C. finlandica* byla přitom před ustavením *R. ericae* – agregátu řazena mezi DSE a *M. variabilis* do okruhu *Variable White Taxon* (Summerbell 2005b).

Až práce Villarreal-Ruiz a kol. (2004) však ukázala, že *C. finlandica*, je schopná současně tvořit jak ErM, tak EcM. Autoři izolovali houbu z EcM morfotypu *Piceirhiza bicolorata* z kořenů *P. sylvestris* a pomocí sekvenování ITS regionů rDNA prokázali, že náleží do *R. ericae* - agregátu.

Tato houba při zpětné inokulaci tvořila EcM s *P. sylvestris* a ErM s *V. myrtillus* ve společném tripartitním systému. Je tedy možné, že právě askomycety *R. ericae* - agregátu jsou vhodnými kandidáty na poskytovatele hypotetické www, a že *C. finlandica* tvoří pomyslný most mezi EcM a ErM houbami (Vrålstad 2004).

Pokud je mi známo, nebyly ani teoreticky mezi houby tvořící www nikdy řazeny typické DSE houby, tedy především *P. fortinii*. To je v rozporu se skutečností, že kosmopolitně rozšířené DSE mají schopnost nespecificky kolonizovat kořeny pravděpodobně všech vyšších rostlin, a že jsou alespoň za určitých podmínek považovány za houby mykorhizní (Část I). Navíc Kaldorf a kol. (2004) uvádí, že *P. fortinii* tvořila EcM (hyfový plášť a Hartigovu síť) v kořenech několika linií transgenního topolu (*Populus tremula* L. x *P. tremuloides* Michx.), přičemž tento morfotyp byl nalezen u 14% zkoumaných kořenových špiček a představoval tak druhý nejrozšířenější morfotyp. Práce Kaldorf a kol. (2004) je z tohoto pohledu ojedinělá, např. Melin (1922) popsal asociaci hub, patřících do MRA (jejichž typickým představitelem je právě *P. fortinii*) jako pseudomykorhizu, tedy asociaci bez formování Hartigovy sítě (Část 1).

Zdá se, že jak houby *R. ericae* – agregátu, tak DSE mohou být na našem území pravidelně asociovány s kořeny semenáčků *Picea abies* (L.) Karst. V dosud nepublikované studii jsme se s kolegy (L. Mrnka, O. Koukol, M. Fendrych, J. Fehrer) pokoušeli izolovat a poté identifikovat EcM houby z kořenů semenáčků *P. abies* na několika lokalitách v NP Šumava. Drtivá většina získaných izolátů však náležela do tří taxonomických skupin, jejichž zástupci nejsou považováni za typicky EcM houby. Jednalo se o DSE houby (*Acephala applanata*, *P. fortinii*), houby *R. ericae* – agregátu (*C. finlandica*, *M. bicolor* a *Meliniomyces variabilis* – tato houba byla izolována nejhojněji) a houby z okruhu rodu *Gyoerffyella*. *C. finlandica* tvořila v in vitro experimentu s *P. abies* asociaci připomínající EcM (typická morfologie a anatomie včetně hyfového pláště a Hartigovy sítě, Obr. 3 na konci této kapitoly) a s *V. myrtillus* asociaci připomínající ErM (tvorba typických hyfových klubíček, Obr. 5). *M. bicolor* kolonizoval pouze kořeny *V. myrtillus* a tvořil v nich asociaci připomínající ErM (Obr. 9). DSE houby tvořily DSE-asociaci v kořenech obou typů rostlin (Obr. 15 a 17). *M. variabilis* také kolonizoval kořeny obou typů rostlin, když tvořil asociaci podobnou ErM s *V. myrtillus* (Obr. 13) a netypickou intracelulární asociaci s *P. abies* (Obr. 11).

Zbývá uvést, jaké výhody mykorhizním rostlinám napojení na www může přinášet. Je to zejména výměna látek mezi různými jedinci téhož, ale i rozdílných druhů. Např. Francis a Read (1994) prokázali transport uhlíku mezi rostlinami spojenými myceliem AM hub. Simard a kol. (1997) dokázali in situ, že mimokořenové mycelium EcM hub slouží také k výměně uhlíku mezi různými EcM hostitelskými rostlinami. To zpřesňuje náhled na koloběh uhlíku v ekosystémech a vrhá zcela nové světlo na tradiční schéma výměny minerálních a organických látek mezi hostitelskou rostlinou a mykorhizní houbou. Osobně si myslím, že houby *R. ericae* – agregátu, snad společně se některými *Sebacinales*, jsou při uvažování o www propojení typicky ErM a EcM rostlin nejžhavějšími kandidáty. Je však třeba zjistit, zda takové propojení v přirozených podmínkách skutečně existuje a zda má relevantní ekofyziologický význam.

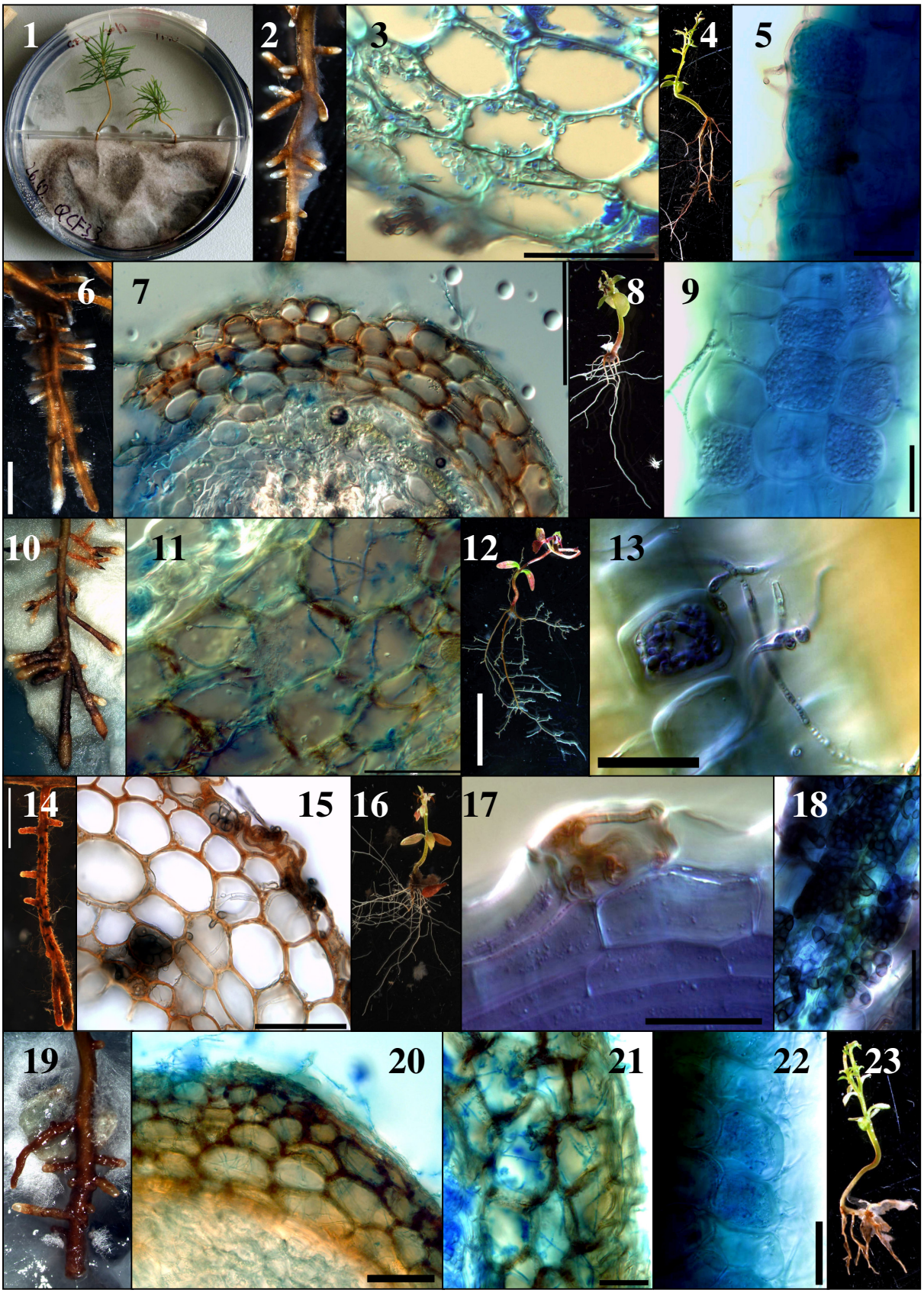
Přítomnost mykorhizních hub v rhizosféře, popř. přímo v kořenech rostlin s odlišnou mykorhizní preferencí může mít i jiné efekty, než napojení na hypotetickou www. Například tzv. hormonální teorie předpokládá aktivní účast auxinů, tvořených EcM houbou, při formování EcM jako morfologické struktury (např. Nylund 1988, Gay 1990, Rudawska a Kieliszewska-Rokicka 1997, Niemi a kol. 2002). Je logické, že auxiny tvořené takovými houbami by měly nespecificky ovlivňovat také houby a rostliny s odlišnou mykorhizní preferencí. Podobný efekt jsem v minulosti pozoroval u EcM houby *C. geophilum*, u půdní houby *Geomyces pannorum* a v menší míře také u *M. variabilis*. Část II této disertační práce si proto kladla následující cíle:

1. **Popsat kolonizační potenciál *Meliniomyces variabilis* v kořenech typicky ektomykorhizních a typicky erikoidně mykorhizních rostlin**
   Tento cíl je zpracován v Článku 4 této disertační práce.

2. **Porovnat kolonizační potenciál a mykorhizní efekt *Cenococcum geophilum*, *Geomyces pannorum* a *Meliniomyces variabilis* u typicky erikoidně mykorhizních rostlin**
   Tento cíl je zpracován v Článku 5 této disertační práce.

Obrázky k 2. 1. Část II: Úvod

Obr. 1: Schéma pokusu, zjišťujícího kompatibilitu izolovaných hub a typicky EcM a ErM hostitelských rostlin; ***Obr. 2***: Morfologie kořene *Picea abies*, kolonizovaného houbou *Cadophora finlandica*, připomínající EcM morfotyt *Piceirhiza bicolorata*; ***Obr. 3***: Hartigova síť, tvořená houbou *C. finlandica* v kořenech *P. abies*, úsečka = 50um; ***Obr. 4***: Morfologie kořenového systému *Vaccinium myrtillus*, kolonizovaného houbou *C. finlandica*; ***Obr. 5***: *C. finlandica* tvoří v kořenech *V. myrtillus* asociaci, připomínající ErM, úsečka = 20um; ***Obr. 6***: Morfologie kořene *P. abies*, kolonizovaného houbou *Meliniomyces bicolor*, úsečka = 2mm; ***Obr. 7***: Absence houbových struktur v kořeni *P. abies*, inokulovaném houbou *M. bicolor*, úsečka = 100um; ***Obr. 8***: Morfologie kořenového systému *V. myrtillus*, kolonizovaného houbou *M. bicolor*; ***Obr. 9***: *M. bicolor* tvoří v kořenech *V. myrtillus* asociaci, připomínající ErM, úsečka = 25um; ***Obr. 10***: Morfologie kořene *P. abies*, kolonizovaného houbou *Meliniomyces variabilis*; ***Obr. 11***: Intracelulární kolonizace kořene *P. abies* houbou *M. variabilis*, úsečka = 50um; ***Obr. 12***: Morfologie kořenového systému *V. myrtillus*, kolonizovaného houbou *M. variabilis*; ***Obr. 13***: *M. variabilis* tvoří v kořenech *V. myrtillus* asociaci, připomínající ErM, úsečka = 25um; ***Obr. 14***: Morfologie kořene *P. abies*, kolonizovaného houbou *Phialocephala fortinii*, připomínající pseudomykorhizu podle Melin (1922), úsečka = 2mm; ***Obr. 15***: Intracelulární kolonizace kořene *P. abies* houbou *P. fortinii*, patrné je tmavě hnědé mikrosklerocium a hyalinní až tmavě hnědé přepážkované hyfy, úsečka = 50um; ***Obr. 16***: Morfologie kořenového systému *V. myrtillus*, kolonizovaného houbou *P. fortinii*; **Obr. 17**: Intracelulární kolonizace rhizodermální buňky *V. myrtillus* houbou *P. fortinii*, připomínající intermediate structures (viz Část I a zejména Článek 1 této DP), úsečka = 25um; ***Obr. 18***: Typická morfologie DSE-asociace v kořeni *V. myrtillus*, kolonizovaném *P. fortinii*, zřejmá jsou četná mikrosklerocia, úsečka = 50um; ***Obr. 19***: Morfologie kořene *P. abies*, kolonizovaného typickou ErM houbou *Rhizoscyphus ericae*; **Obr. 20**: Intracelulární kolonizace kořene *P. abies* houbou *R. ericae*, úsečka = 100um; ***Obr. 21***: Intracelulární kolonizace kořene *P. abies* houbou *R. ericae*, úsečka = 25um; ***Obr. 22***: Intracelulární kolonizace kořenů *V. myrtillus* houbou *R. ericae*, úsečka = 25um; ***Obr. 23***: Morfologie kořenového systému *V. myrtillus*, kolonizovaného houbou *R. ericae*. Autoři fotografií jsou M. Fendrych a M. Vohník.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Graf 1 k 2. 1. Část II: Úvod

Kladogram agregátu *Rhizoscyphus ericae* (Bayesiánské *posterior probabilities* nad jednotlivými větvemi, podpora bootstrapů pod jednotlivými větvemi: parsimonie normálním písmem, distanční metoda kurzívou). *R. ericae* - agregát se v současné době skládá minimálně ze čtyř popsaných subkládů, tvořených houbami *Cadophora finlandica*, *Meliniomyces bicolor*, *M. variabilis* a *Rhizoscyphus ericae*. Pro vytvoření kladogramu byly použity sekvence hub, izolovaných z kořenů semenáčků *Picea abies* ze Šumavy (tučně) a sekvence z veřejných databází. Je zřejmé, že většina izolovaných hub náležela do subkládu *M. variabilis* (viz Část II: Úvod). Autorkou kladogramu je Dr. Judith Fehrer s přispěním Libora Mrnky (BÚ AVČR, Průhonice).

2. 2. ČLÁNEK 4

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

(manuskript)

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

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Abstract

Attempts to isolate ascomycetous *Hydnotrya tulasnei* Berk. & Br. from fresh hypogeous sporocarps into a pure culture yielded beside *H. tulasnei* also a strain of *Meliniomyces variabilis* Hambleton & Sigler (= MVA-2). Both phenotype and genotype analysis showed that MVA-2 was highly similar to another *M. variabilis* strain, isolated previously from a root tip of *Picea abies* (L.) Karst. The mycorrhizal potential of both *H. tulasnei* and *M. variabilis* is still dubious. Therefore, we attempted an in vitro synthesis of root-fungus association between *H. tulasnei*, both *M. variabilis* strains and typically ectomycorrhizal (*P. abies* and *Pinus sylvestris* L.) and typically ericoid mycorrhizal (*Vaccinium corymbosum* L.) host plants. For comparison a strain of *Rhizoscyphus ericae* (Read) Zhuang & Korf was also included. Both *M. variabilis* strains formed intracellular structures characteristic for ericoid mycorrhiza in *V. corymbosum* roots, and also colonized 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 colonization of the cortex. Reference strain of *R. ericae* showed similar behavior in *Picea*, *Pinus* and *Vaccinium* roots, only the intracellular colonization was more intensive and morphology of roots of both conifers was less changed when compared to *M. variabilis* and control. *H. tulasnei* failed to colonize the roots of *P. abies* and *V. corymbosum*, possibly due to sub-optimal conditions for its growth. To our knowledge, this is the first report about the isolation of a putative ErM/EcM fungus from inside a fresh sporocarp of a putative EcM fungus. The results of our study confirm that *M. variabilis* can form ericoid mycorrhiza and at the same time intracellularly colonize roots of coniferous plants, having no apparent negative effect on their growth. Further screening is

needed to elucidate whether the intracellular association between *M. variabilis* and roots of *Picea* and *Pinus* is under natural conditions regular or exceptional, and if it has a symbiotic character.

Keywords: ericoid mycorrhiza, ectomycorrhiza, colonization pattern, Variable White Taxon, *Hymenoscyphus ericae* – aggregate, *Rhizoscyphus ericae*

Introduction

The genus *Hydnotrya* (Pezizales, Discinaceae) comprises ascomycetous fungi with hypogeous sporocarps (Montecchi and Sarafini 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* (L.) Karst., *Tilia cordata* Miller, *Betula pendula* Roth., *Populus tremula* L.) in Estonia and described a putative *H. tulasnei* morphotype. Authors, however, did detect neither the exact host species (except beech in two localities in Denmark) nor isolated *H. tulasnei* into a pure culture. To our knowledge, reports about an in vitro re-synthesis of ectomycorrhiza (EcM) using *H. tulasnei* are missing.

Meliniomyces variabilis Hambleton & Sigler, formerly known as Variable White Taxon (Hambleton and Currah 1997), 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). On the base of re-synthesis trials 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).

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 a re-synthesis trial. However, each of the sporocarps yielded different mycelium. Thus, the aims of the reported study were: 1) to identify both fungal isolates obtained from *H. tulasnei* sporocarps, and 2) to elucidate their mycorrhizal potential by testing their ability to colonize roots of plant hosts typically ectomycorrhizal (*P. abies* and *Pinus sylvestris* L.) and ericoid mycorrhizal (*Vaccinium corymbosum* L.).

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 Táborsko region, southern Bohemia (CZ), in a forest dominated by *P.*

abies. The second was collected in August 2005 in a mixed forest between Držkov and Zásada u Jablonce nad Nisou municipalities, northern Bohemia (CZ). Both sporocarps were determined on the base of typical macro- and micro-morphological characteristics according to Montecchi and Sarafini (2000). Their specimens were deposited in Herbarium Mycologicum Musei Nationalis Pragae part Flora Bohemica. For additional information see Table 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 liter as growth regulators. The dishes were Parafilm^{TM}-sealed and cultivated at room temperature in the dark. Each sporocarp produced only one distinct type of fungal mycelium, differing in growth rates and morphology of the colonies. The isolate from the first sporocarp is assigned as MVA-2, from the second sporocarp as HTU-1 in the following text.

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 10 min. in 10% SAVO (household bleach containing active chlorine) and two washings in sterile water. The root tips were then placed on PDA (39 g 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 region's sequence was deposited in GenBank (Table 1). ITS region's 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 Leake and Read (1989).

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 Mo-Bio UltraClean^{TM} Microbial DNA Isolation Kit following manufacturer's instructions. 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 BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and ABI 3100 Genetic Analyser, following manufacturer's instructions. 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 relatively high content of glucose, MMN proved to be a suitable medium for ErM and EcM synthesis with members of *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 10x diluted MMN (1g of glucose per liter). 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 the manner 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 experimental plants were covered with a sterile moistened filter paper. The dishes were Parafilm^{TM}-sealed and placed in a vertical position into a growth chamber (16/8h day/night, 23°C, 150 µmol m^{-2} s^{-1}). After two (for *V. corymbosum*) or three (for *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 (20min 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. Stained roots/thin sections were observed using a microscope equipped by differential interference contrast optics at high magnifications (400x and 1000x). For *P. abies* and *P. sylvestris* thin sections, epifluorescent microscopy was employed according to Cudlín (1991).

Aseptic synthesis: Hydnotrya tulasnei

During sub-cultivation of 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 trial. After six weeks, HTU-1 did grow 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/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* L. seedlings directly onto the surface of the original HTU-1 colony formed at MS medium. The

original HTU-1 colony was still viable, because served as a source of viable mycelium for dishes without membranes/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). The second sporocarp yielded slowly growing, brown to orange submersed diffuse mycelium (= HTU-1). The ITS region's 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 region's sequence (735 bp) of HTU-1 showed 99% similarity with 731 bp of "Pezizales sp. B48" (= GenBank AJ534700 = *H. tulasnei*, Tedesoo et al. 2006). Thus, genotype analysis confirmed that HTU-1, isolated from the morphologically determined *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 region's sequences of HTU-1 and MVA-2 were deposited in GenBank (Table 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. 1a). Turgescent rhizodermal cells were filled with dense hyphal coils and colonized parts of the root system were embedded in hyphal wefts. RER-1 formed more intensive root colonization than both *M. variabilis* strains and its intracellular hyphae were thinner, resulting in more compact loops. Colonized micro-cuttings grew and performed well, however, there was no apparent macroscopic difference between the inoculated and non-inoculated plants.

MVA-1, MVA-2 and RER-1 also colonized *P. abies* and *P. sylvestris* roots. Root morphology of the colonized seedlings was changed in comparison with the non-inoculated seedlings (Fig. 1b). When a contact between *P. abies* primary root and the fungal colony was established, numerous short lateral roots without root hairs, resembling morphologically EcM root tips, were produced. In contrast, non-inoculated plants produced only few short lateral root tips, which were covered with root hairs. *P. sylvestris* lateral roots 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*, cross-sections revealed intracellular colonization of their cortex cells and absence of the Hartig net (Fig. 1c). Intracellular colonization was most intensive in the case of RER-1. However, the effect of RER-1 on the root morphology (the "EcM appearance") was weaker than in

the case of MVA-1 and MVA-2. The seedlings of *P. abies* colonized by MVA-1 and MVA-2 grew apparently better than the non-inoculated control seedlings, pointing at possible beneficial effect of *M. variabilis* on host plants. However, due to a low number of replicates (n=3) we did not perform 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 Hartig net or developed mantle were observed. Similar situation was 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 a putative ErM/EcM fungus *M. variabilis* from inside the fresh young sporocarp of another putative EcM fungus *H. tulasnei*. Significance of this finding together with possible *H. tulasnei – M. variabilis* co-existence could be resolved by screening of more *H. tulasnei* sporocarps. Future investigators should consider presence of endophytic fungi (e.g. *M. variabilis*) even in fresh, young *H. tulasnei* sporocarps.

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

M. variabilis is frequently isolated from EcM roots, namely from the "*Piceirhiza bicolorata*" morphotype (e.g. Vrålstad et al. 2000 and 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 trials (e.g. Vrålstad et al. 2002a, Piercey et al. 2002, this study).

The presence of MVA-1 and MVA-2 (and to limited extent also RER-1) changed superficial morphology of *P. abies* and *P. sylvestris* roots to the EcM appearance: *P. abies* lateral roots could resemble early stages of *P. 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 showed for example by Gay (1990) in IAA-affected lateral roots of *Pinus halapensis* Mill. It seems to be the case also in our study, because the Hartig net was missing in the lateral roots with the EcM appearance and instead, intracellular colonization occurring across the whole cortex was present.

Intracellular colonization 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 a potential to intracellularly colonize roots of typically ErM plants (Berch et al. 2002, Piercey et al.

2002, this study). While the significance of intracellular colonization in ericaceous roots can be deduced from the well-established ericoid mycorrhiza, the importance of intracellular colonization in coniferous roots is yet unknown.

However, the apparent ability of *M. variabilis* to colonize intracellularly both typically ErM and EcM host plants, and the fact that this colonization mode has at least no negative effect on colonized hosts (Piercey et al. 2002, this study) may have important eco-physiological consequences. Similarly to many boreal and temperate ecosystems, where ericaceous shrubs form understorey in coniferous forests, *Vaccinium* species frequently dominate understorey in *P. abies* stands in Southern Bohemia. Hypothesized connection between ErM and EcM plants via the mycelium of a common fungus is expected to play an important role in their life (Vrålstad 2004). *Cadophora finlandica* (Wang & Wilcox) Harr. & McNew is proposed to be a candidate for such 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 typically ErM plant *Rhododendron groenlandicum* (Oeder) Kron & Judd and typically EcM plant *Picea mariana* (Mill.) BSP. Similar re-synthesis was repeated 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 symbiotic character.

It can be questioned whether the three-months 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 the Hartig net development between *P. abies* and *C. finlandica*, another member of the *H. ericae* – aggregate, and *Cenococcum geophilum* Fr., a common ascomycetous EcM fungus, under identical scheme as used in this study (M. Vohník et al., unpublished data). It is also uncommon that EcM development would start with intracellular colonization of the cortex. Moreover, the intracellular colonization pattern of both *M. variabilis* strains in *P. abies* and *P. sylvestris* roots resembled this formed by the typical ErM fungus *R. ericae*, which have never formed EcM.

Alternatively, *M. variabilis* might be slower in the Hartig net formation than *C. finlandica* or *C. geophilum* and might include unusual intracellular phase in early stages of EcM development. To resolve this, prolonged cultivation period, more *M. variabilis* strains and possibly also other cultivation scheme reflecting natural conditions should be employed.

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determination of both *H. tulasnei* sporocarps, P. Cudlín (Laboratory of Forest Ecology, Institute of System Biology and Ecology, ASCR, České Budějovice) for help with epifluorescent technique.

References

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402

Berch SM, Allen TR, Berbee ML (2002) Molecular detection, community structure and phylogeny of ericoid mycorrhizal fungi. Plant Soil 244: 55-66

Cudlín P (1991) Epifluorescent microscopy for identification of ectomycorrhiza. Methods in Microbiology 23: 365-368

Gay G (1990) Effect of the ectomycorrhizal fungus *Hebeloma hiemale* on adventitious root formation in derooted *Pinus halepensis* shoot hypocotyls. Can J Bot 68: 1265-1270

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser 41:95-98

Hambleton S, Currah RS (1997) Fungal endophytes from the roots of alpine and boreal *Ericaceae*. Can J Bot 75: 1570-1581

Hambleton S, Sigler L (2005) *Meliniomyces*, a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (= *Hymenoscyphus ericae*), Leotiomycetes. Studies in Mycology 53: 1-27

Kolařík M, Kubátová A, Pažoutová S, Šrůtka P (2004) Morphological and molecular characterization of *Geosmithia putterillii*, *G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. Mycol Res 108: 1053-1069

Leake JR, Read DJ (1989) The biology of mycorrhiza in the Ericaceae: XIII. Some characteristics of the extracellular proteinase activity of the ericoid endophyte *Hymenoscyphus ericae*. New Phytol 112: 69-76

Montecchi A, Sarafini M (2000) Funghi ipogei d´ Europa. Associazione Micologica Bresadola, Trento, 714 pp

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15: 473-497

Newton AC, Haigh JM (1998) Diversity of ectomycorrhizal fungi in Britain: a test of the species-area relationship, and the role of host specificity. New Phytol 138: 619-627

Piercey MM, Thormann MN, Currah RS (2002) Saprobic characteristics of three fungal taxa from ericalean roots and their association with the roots of *Rhododendron groenlandicum* and *Picea mariana* in culture. Mycorrhiza 12: 175-180

Schild DE, Kennedy A, Stuart MR (1988) Isolation of symbiont and associated fungi from ectomycorrhizas of Sitka Spruce. Eur J Forest Pathol 18: 51-61

Tedersoo L, Hansen K, Perry BA, Kjøller R (2006) Molecular and morphological diversity of pezizalean ectomycorrhiza. New Phytol 170: 581-596

Villarreal-Ruiz L, Anderson IC, Alexander IJ (2004) Interaction between an isolate from *Hymenoscyphus ericae* aggregate and roots of *Pinus* and *Vaccinium*. New Phytol 164: 183-192

Vrålstad T, Fossheim T, Schumacher T (2000) *Piceirhiza bicolorata* – the ectomycorrhizal expression of the *Hymenoscyphus ericae* aggregate? New Phytol 145: 549-563

Vrålstad T, Schumacher T, Taylor FS (2002a) Mycorrhizal synthesis between fungal strains of the *Hymenoscyphus ericae* aggregate and potential ectomycorrhizal and ericoid hosts. New Phytol 153: 143-152

Vrålstad T, Myhre E, Schumacher T (2002b) Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. New Phytol 155:131-148

Vrålstad T (2004) Are ericoid and ectomycorrhizal fungi part of a common guild? New Phytol 164:7-10

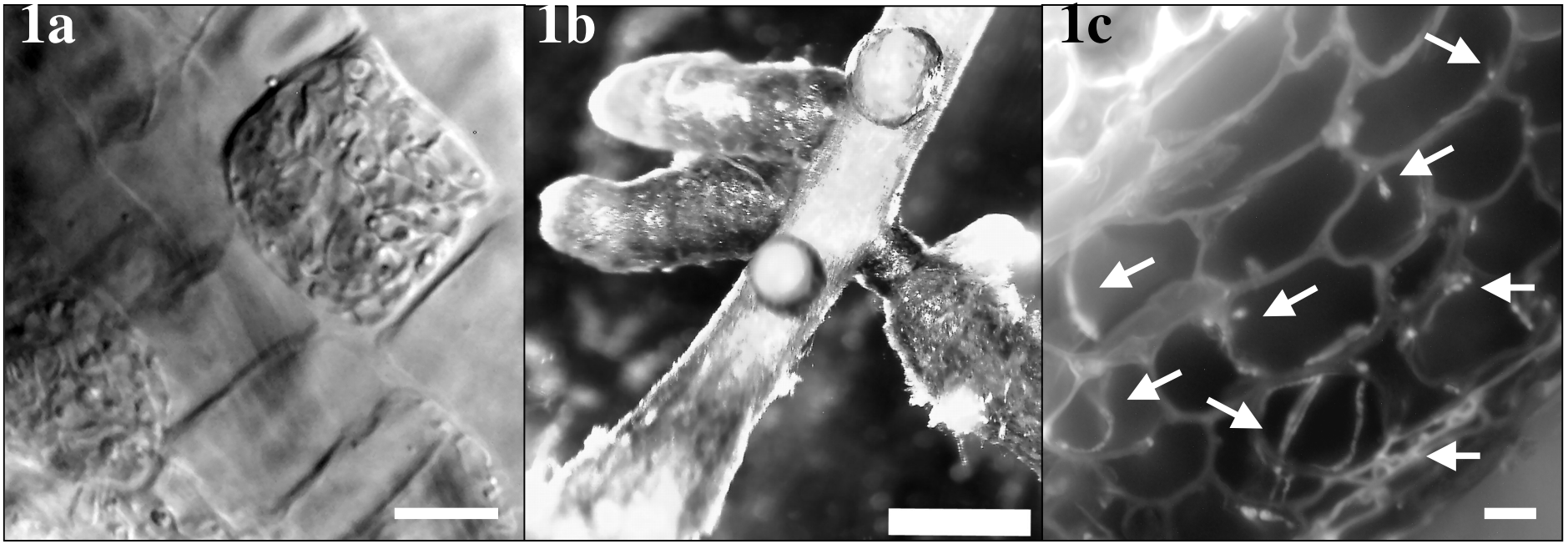

FIGURE 1a: Intracellular hyphal coils formed by MVA-1 and MVA-2 in the roots of *V. corymbosum* (bar = 10µm); **FIGURE 1b** Typical morphology of the *P. abies* lateral roots, inoculated with MVA-1 and MVA-2 (bar = 1mm); **FIGURE 1c** A thin section of a *P. abies* lateral root showing numerous *M. variabilis* hyphae (arrows) colonizing cortical cells (bar = 10µm).

Isolate	Source of the isolate	Date and location	Sporocarp specimen accession number	Culture accession	GenBank accession number	*Picea abies*	*Pinus sylvestris*	*Vaccinium corymbosum*
HTU-1	sporocarp of *H. tulasnei*	August 2004; *P. abies* - dominated forest, Táborsko region, S Bohemia (CZ)	PRM 902032	Available from M. Gryndler	AM261522	-	NT	-
MVA-1	root tip of a *P. abies* seedling	September 2003; *P. abies* stand, Modrava, Šumava NP, S Bohemia (CZ)	-	CCF 3583	AM261523	INT	INT	ErM
MVA-2	sporocarp of *H. tulasnei*	August 2005; mixed forest between Držkov and Zásada u Jablonce n. N., N Bohemia (CZ)	PRM 905514	Available from M. Gryndler	AM261524	INT	INT	ErM
RER-1	*C. vulgaris* hair root	Leake and Read, 1989	-	Leake and Read, 1989	Leake and Read, 1989	INT	INT	ErM

TABLE 1: Additional data about the fungal strains, used in this study, and their interaction with the roots of the 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 colonization; **ErM** = formation of ericoid mycorrhiza.

2. 3. ČLÁNEK 5

Interactions between *Cenococcum geophilum*, *Geomyces pannorum*, *Meliniomyces variabilis* and roots of *Rhododendron* and *Vaccinium*

(manuskript přijat k publikování v časopise *Folia Microbiologica*)

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Interactions between *Cenococcum geophilum*, *Geomyces pannorum*, *Meliniomyces variabilis* and roots of *Rhododendron* and *Vaccinium*

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ABSTRACT The roots of ericaceous plants are commonly inhabited by symbiotic ericoid mycorrhizal (ErM) fungi, which are known to improve fitness of their hosts. However, being underground absorptive organs, ericaceous roots come into contact and interact with a wider spectrum of fungi, including saprotrophic and ectomycorrhizal (EcM) ones. Such interactions may have an important impact on eco-physiology of ericaceous plants, yet their effects remain obscure. Among the representatives of non-ErM fungi which are occasionally isolated from ericaceous roots, we tested a potential of EcM *Cenococcum geophilum* Fr., saprotrophic *Geomyces pannorum* (Link) Sigler & Carmichael and a frequent root-associated, potentially ErM-forming *Meliniomyces variabilis* Hambleton & Sigler to colonize ericaceous roots and to support their development. Three *in vitro* experiments were set up to verify such abilities of these fungi, including also a typical ErM fungus, *Rhizoscyphus ericae* (Read) Zhuang & Korf, for comparison. All fungal strains intracellularly colonized rooted *Vaccinium* micro-cuttings: *G. pannorum* occasionally produced hyphal loops similar to ErM, *M. variabilis* and *R. ericae* exhibited typical ErM colonization pattern. *C. geophilum* hyphae grew vigorously on and around newly formed roots and rarely penetrated turgescent rhizodermal cells forming intracellular loose loops. Rooting of *Rhododendron* sp. micro-cuttings was not promoted by any fungal strain except *C. geophilum*. *C. geophilum* also promoted the most vigorous growth of *Rhododendron ponticum* L. seedlings. Our observations show that the widespread EcM fungus *C. geophilum* has a potential to colonize ericaceous roots and support their development, which may influence overall growth of ericaceous plants. As showed for *G. pannorum*, structures resembling ErM colonization may be formed by fungi, which are to date not regarded as ericoid mycorrhizal.

INTRODUCTION

Ericaceous plants form root-fungus associations mainly with ericoid mycorrhizal (ErM) fungi and dark septate endophytes (DSE), called ericoid mycorrhiza (ErM) in the former and DSE-association in the latter case. Extensive body of literature attests that ErM plays significant role in the life of ericaceous plants (Cairney and Meharg 2003; Read et al. 2004), yet the role of DSE remains to be uncovered (Jumpponen 2001; Mandyam and Jumpponen 2005). Similarly to DSE, the significance of ectomycorrhizal (EcM) fungi (Dighton and Coleman 1992; Stoyke and Currah

1993; Midgley et al. 2004), unknown basidiomycetous fungi (Seviour et al. 1973; Bonfante-Fasolo 1980; Bougoure and Cairney 2005a,b) or saprotrophic ascomycetous fungi (Allen et al. 2003), which are occasionally found in roots of ericaceous species, remains obscure.

Even though mycorrhizal fungi influence various components of host plant fitness (Jones and Smith 2004), their effect on the host plant nutrient uptake and enhanced growth is usually sought, putting other factors aside (Johnson et al. 1997; Jones and Smith 2004). In ericaceous species, primary effect of their root associated ErM fungi is in accessing nutrients (mainly N) from organic compounds together with detoxification of the substrate (Perotto et al. 2002; Cairney and Meharg 2003). Other factors are considerably less studied.

For example, fungal involvement in enhanced root development of ericaceous plants is yet an unresolved topic. Scarce reports about the influence of ErM fungi provide various results (e.g. Eccher and Noé 2002). Contrary to ErM fungi and their ericaceous hosts, considerably more work has been focused on the influence of EcM fungi on rooting and root development of EcM host plants (e.g. Gay 1990; Rudawska and Kieliszewska-Rokicka 1997; Niemi and Häggman 2002; Niemi et al. 2002), the experience and knowledge have however not been transferred into the field of ErM symbiosis.

During our past work with mycorrhizal and other soil-borne fungi, which was focused on aseptic syntheses with ericaceous host plants, several strains of different non-ErM fungi revealed a potential to positively interact with ericaceous roots, namely to form intracellular structures resembling ErM or positively influence root development of the inoculated micro-cuttings. Among the most unexpected interactions, *Geomyces pannorum*, a soil borne fungus and an occasional human skin pathogen (Domsch et al 1980, Gianni et al. 2003), colonized newly formed roots of *Rhododendron* and *Vaccinium* micro-cutings. An EcM fungus, *Cenococcum geophilum* and a frequent root-associated fungus, *Meliniomyces variabilis* supported the root development of *R. ponticum* seedlings, both fungi also colonizing newly emerged roots. All three fungi were recently detected in ericaceous roots by different authors (Lacourt et al. 2001, Midgley et al. 2004, for details about *M. variabilis* see Hambleton and Sigler 2005), however without determination of their interaction with the host roots.

To contribute to the knowledge on the interactions between non-ErM fungi and ericaceous roots, we aimed to simultaneously screen the ability of *C. geophilum*, *G. pannorum* and *M. variabilis* to colonize ericaceous roots and support their development in three *in vitro* trials. *Rhizoscyphus ericae* (Read) Zhuang & Korf, a representative of a typical ErM fungus, was also included in our study and screened for the same effects.

MATERIALS AND METHODS

Fungal isolates

Following fungal strains were tested for their ability to interact with roots of *Rhododendron* and *Vaccinium* seedlings/micro-cuttings: *C. geophilum* (= CGE-4 in the following text), *G. pannorun*

(= GPA-1), *M. variabilis* (= MVA-1) and *R. ericae* (= RER-1). CGE-4 is available from M. Vohník on request. GPA-1 was isolated from a contaminated *Rhododendron* sp. tissue culture and is deposited in the Culture Collection of Fungi (= CCF; Faculty of Science, Charles University, Prague) under the accession number CCF 3581. Its ITS1-5.8SrDNA-ITS2 sequence is deposited in GenBank under the accession number DQ494320. MVA-1 was originally isolated from *P. abies* roots, is deposited in CCF under the accession number CCF 3583 and its ITS1-5.8SrDNA-ITS2 sequence is deposited in GenBank under the accession number AM261523. RER-1 was originally isolated from *Calluna vulgaris* Hull. roots (Leake and Read 1989) and is available from M. Vohník on request.

Assessment of the fungal ability to colonize ericaceous roots

Rooted Vaccinium micro-cuttings

One compartment of each split Petri dish was filled with MMN and inoculated with agar plugs overgrown with mycelium of CGE-4, GPA-1, MVA-1 and RER-1. The other compartment was left without the medium. Suitability of the MMN medium for growth of *Rhododendron*/*Vaccinium* seedlings/cuttings and the tested fungal strains was screened in advance with positive results. Despite relatively high content of carbon in MMN, this medium showed to support ErM formation with *R. ericae* during the testing.

The dishes with the plugs were cultivated for one month at room temperature in the dark. After this period, one aseptic rooted *Vaccinium* cutting was inserted into each dish in a manner that its roots were placed on the surface of the fungal colonies and its shoot was accommodated in the empty compartment. There were three rooted micro-cuttings per each fungal strain including a non-inoculated control. Each dish was Parafilm^{TM}-sealed and placed in a growth chamber (16/8h, 25/20°C day/night, 150 µmol m^{-2} s^{-1}). After three months, plants were removed from the dishes, their roots were cleared with 10% KOH (20min 121°C), washed with tap water, acidified (1 min 3% HCl), washed with tap water, stained with trypan blue (1 hour 121°C) and de-stained overnight in lacto-glycerol. Stained roots were observed with DIC at high magnifications (400x and 1000x) using Olympus BX60 microscope. Photos were taken with Olympus DP70 camera.

. Additionally, the ability of GPA-1 to colonize *Vaccinium* roots was screened in a peat + perlite substrate. Three 300ml Erlenmayers´ flasks filled with moistened autoclaved peat : perlite (1:1) were pre-inoculated for one month with GPA-1 and three flasks were left non-inoculated. Three rooted micro-cuttings were inserted into each of the flasks, resulting in nine cuttings inoculated with GPA-1 and nine cuttings non-inoculated. After three months of cultivation in the growth chamber, the cuttings were harvested and their roots were treated as described above.

Rhododendron ponticum seedlings

Germinating surface-sterilized seeds of *R. ponticum* were placed onto margins of one-month-old fungal colonies (CGE-4, GPA-1, MVA-1 and RER-1), actively growing on MMN. There were

three Petri dishes each with four seedlings per fungus and control. After three months of cultivation in the growth chamber, the roots of the seedlings were stained, observed and documented as described above.

Assessment of the fungal ability to support development of ericaceous roots

Experiment 1

One compartment of each of 15 split Petri dishes was filled with the medium (= MMR) after Dalpé (1986). After solidification of MMR, a central septum of each dish was perforated to allow insertion of experimental plants. Aseptic *Rhododendron* sp. micro-cuttings were inserted in triplicates through perforated septum in a manner that approx. one fifth of their size was immersed into MMR. The dishes were inoculated with agar plugs collected from margins of actively growing colonies of all four tested fungi (CGE-4, GPA-1, MVA-1 and RER-1), representing separate variants. There were nine micro-cuttings in each variant including a non-inoculated control. The dishes were Parafilm^{TM}-sealed and placed in a vertical position into the growth chamber. After 15 weeks, the number of rooted micro-cuttings was counted and the average length of the roots was measured for each variant. Roots were excised and treated as described above.

Experiment 2

Aseptic *Rhododendron* micro-cuttings, the same origin as in the Experiment 1, were introduced into glass vessels (350 ml) with the standard MS medium without growth regulators. The vessels with 50 micro-cuttings were inoculated with CGE-4, GPA-1, MVA-1 or RER-1, Parafilm^{TM}-sealed and placed in the growth chamber. One vessel was left non-inoculated. After three months of cultivation, number of rooted micro-cuttings and colonization patterns in roots were assessed as described above.

RESULTS

Assessment of the fungal ability to colonize ericaceous roots

All fungal strains intracellularly colonized roots of either *Vaccinium* micro-cuttings or *R. ponticum* seedlings. Non-inoculated plants remained without any fungal colonization.

GPA-1 intracellularly colonized roots of *Vaccinium* micro-cuttings only in the peat - perlite substrate. Here it formed loose to dense trypan blue-stained coils in rhizodermal cells (Figs. 1 - 3), which resembled coils/loops formed by ericoid mycorrhizal fungi in ericaceous roots. On MMN medium, GPA-1 occasionally formed loose hyphal wefts around the roots of *Vaccinium* micro-cuttings, but without apparent intracellular colonization. The development of the roots of *R. ponticum* seedlings in the presence of GPA-1 was reduced (Fig. 8) and the reduced roots were without any fungal colonization.

Darkly brown septate hyphae of CGE-4 formed loose to dense mantle around the newly formed roots (Fig. 4) and also around the base of stems of the micro-cuttings. Often, hyphae

followed grooves between rhizodermal cells, occasionally penetrating turgescent cells with melanized or hyaline hyphae (Fig. 5), forming loose hyphal loops inside them (Fig. 6).

Both MVA-1 and RER-1 colonized rhizodermal cells of rooted *Vaccinium* micro-cuttings in a manner typical for ErM fungi. Rhizodermal cells were filled with hyphal coils/loops and the roots were often embedded in hyphal mantles. MVA-1 failed to colonize the roots of *R. ponticum* seedlings. Contrary to CGE-4 and RER-1 variants, the roots of the seedlings did not penetrate through MVA-1 fungal colonies into the agar medium, but developed extensively on the surface of the colonies.

RER-1 was more efficient than MVA-1 in terms of root colonization levels reaching approx. 15% and 25% of the total root length colonized in *Vaccinium* micro-cuttings and *R. ponticum* seedlings, respectively. Colonization level of GPA-1 was lower and reached approx. 5 %. The intracellular colonization by CGE-4 was lower than 1%.

There were no apparent differences in the size of the *Vaccinium* micro-cuttings among all inoculation variants. The growth of *R. ponticum* seedlings was positively influenced by CGE-4 and negatively by GPA-1. In the presence of CGE-4, the seedlings developed apparently better than in the rest of the variants. In contrast, the seedlings growing in the presence of GPA-1 had reduced roots and also their overall growth was reduced (Fig. 8).

Experiment 1

In the CGE-4 variant, five from the total nine micro-cuttings developed three or four roots >10 mm. In the GPA-1 variant, one micro-cutting developed one root <5 mm without any fungal colonization, the same situation was in the RER-1 variant. The non-inoculated micro-cuttings remained without any roots. There were no apparent differences in the growth of the cuttings between all variants.

Experiment 2

In the CGE-4 variant, 16 from 50 micro-cuttings produced abundant roots (Fig. 7), which were colonized in the same manner as in the Experiment 1. The micro-cuttings in the other variants remained without any roots. The rooted micro-cuttings inoculated with CGE-4 were approx. two-times bigger than the other, including the non-rooted micro-cuttings inoculated with CGE-4 (Fig. 7).

DISCUSSION

Even though *C. geophilum* is an EcM fungus, it is also occasionally detected in the surface-sterilized ericaceous roots (Midgley et al. 2004). However, its impact on the eco-physiology of ericaceous plants remains unclear. Stoyke and Currah (1993) found in a re-synthesis trial that hyphae of *C. geophilum* formed “loose wefts of hyphae on root surfaces, but rarely penetrated the root cortex” of ericaceous *Menziesia ferruginea* Smith in an aseptic culture. Similarly to Stoyke

and Currah (1993), *C. geophilum* reached very low colonization levels in our study. Stoyke and Currah (1993) stated that the association between *C. geophilum* and *M. ferruginea* appeared "potentially mycorrhizal", thus having potentially beneficial character. Also in our study, both extra- and intracellular *in vitro* presence of *C. geophilum* in ericaceous roots had a beneficial character, resulting in a stimulation of rooting/root development, which was connected with improved growth of the inoculated plants.

Despite the low colonization rate in the ericaceous roots, *C. geophilum* extraradical mycelium (ERM) appeared to play more important role than the intracellular structures in our study. This results in a situation where very low fungal intracellular colonization (but high presence of the hyphae in the rhizosphere) is connected with positive effect on host plant fitness. Such situation may occur under natural conditions, where ERM originating from *C. geophilum*-colonized EcM host plants occurring sympatrically with ericaceous plants comes into direct contact with roots of the latter. Then, *C. geophilum* can accidentally and at low levels colonize rhizodermal cells of its ericaceous hosts. This statement is supported i) by observations of Stoyke and Currah (1993) who observed low colonization levels of *C. geophilum* in *M. ferruginea* roots and noted that the colonization pattern "resembled associations observed in field samples", and ii) by infrequent detections of *C. geophilum* in ericaceous roots by molecular methods (Midgley et al. 2004). *C. geophilum* hyphal net in the rhizosphere of ericaceous plants then may positively interact with ericaceous roots, as showed in our study. Question however remains about the interaction between *C. geophilum* and ErM fungi, because most ericaceous roots are expected to be ErM under natural conditions (Perotto et al. 2002).

The essence of the rooting-stimulating effect of CGE-4 in this study was not determined and is an aim of our subsequent experiment. EcM fungi are known to produce phytohormones, especially auxins, which is together with the hormone theory and its validity discussed elsewhere (e.g. Nylund 1988; Gay 1990; Rudawska and Kieliszewska-Rokicka 1997; Niemi et al. 2002). Apparently, the effect of *C. geophilum* on the root development of its hosts is non-specific and may influence also non-EcM plants.

G. pannorum is a common air- and soil-borne fungus with cellulolytic and kerationolytic abilities, frequently isolated from various substrates and niches, including rhizosphere of peat bog plants (Domsch et al 1980). Lacourt et al. (2001) used a sequence of *G. pannorum* strain CLM 323.96 (GenBank AF307760) and noted that this strain had been originally isolated from roots of *Erica arborea* L. by Bergero et al. (2000). This study is to our knowledge the first attempt to describe the interaction between *G. pannorum* and ericaceous roots. GPA-1 formed intracellular coils in the rhizodermal cells of *Vaccinium* micro-cuttings, however only in the peat-based substrate. Similar structures formed by *Myxotrichum setosum* (Eidam) Orr, Kuehn & Plunkett, *Gymnascella dankaliensis* (Castellani) Currah (both with *Oidiodendron* anamorphs) and *Pseudogymnoascus roseus* Raillo (with *Geomyces* anamorph) were observed in *Vaccinium*

angustifolium Ait. roots by Dalpé (1989), who assigned them to ericoid mycorrhiza and declared *M. setosum*, *G. dankalienses* and *P. roseus* to be new ErM fungi.

In our study, the colonization potential of GPA-1 was low and the colonized *Vaccinium* micro-cuttings did not show any signs of improved fitness. On the other hand, also *M. setosum* and *P. roseus* reached low colonization levels (8 - 10% and 5 - 6%, respectively) in roots of *V. angustifolium*, without causing any apparent improvement of fitness of the host plants (Dalpé 1989). Commonly, fungal strains isolated from surface-sterilized ericaceous roots and/or forming intracellular coils in their rhizodermal cells are assigned as putative ErM fungi, regardless the colonization levels they reach [e.g. *M. setosum*, *G. dankalienses* and *P. roseus* by Dalpé (1989); saprotrophic *Capronia*-like fungi by Allen et al. (2003); dematiaceous hyphomycete *Heteroconium chaetospira* (Grove) Ellis by Usuki and Narisawa (2005)]. In our study, the root development and overall growth of *R. ponticum* seedlings growing in contact with GPA-1 cultures was depressed. On the other hand, *in vitro* growth of rooted *Rhododendron* micro-cuttings can be depressed by *Oidiodendron maius* Barron, which *ex vitro* forms ErM and improves nutrient uptake by *Rhododendron* cv. Azurro (Vohník et al. 2005). To conclude, the apparent ability of GPA-1 to colonize rhizodermal cells of ericaceous plants together with the relatedness of *G. pannorum* to *Oidiodendron* genera, which contains many ErM species (Dalpé 1986 and 1991; Hambleton et al. 1998; Lacourt et al. 2001) may indicate *G. pannorum* as a putative ErM fungus.

M. variabilis, formerly known as Variable White Taxon, is a fungus with yet not clear mycorrhizal status (Hambleton and Sigler 2005). In this study, MVA-1 did not influence rooting of *Rhododendron* micro-cuttings nor colonized the roots of *R. ponticum* seedlings nor apparently influenced their growth in comparison with non-inoculated control, but intracellularly colonized roots of *Vaccinium* micro-cuttings in a manner similar to ErM. On the other hand, the typical ErM fungus *R. ericae* colonized also the *R. ponticum* seedlings, indicating *R. ericae* as a more infective fungus that *M. variabilis* under chosen experimental conditions. These observations together with those reviewed by Hambleton and Sigler (2005) indicate that beside the morphology of its colonies, also the mycorrhizal potential of *M. variabilis* may be variable.

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REFERENCES

Allen T.R., Millar T., Berch S.M., Berbee M.L.: Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. *New Phytol.* **160**: 255-272 (2003).

Bergero R., Perotto S., Girlanda M., Vidano G., Luppi A. M.: Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (*Quercus ilex*). *Mol. Ecol.* **9**: 1639-1649 (2000).

Bonfante-Fasolo P.: Occurrence of a basidiomycete in living cells of mycorrhizal hair roots of *Calluna vulgaris*. *Trans. Br. Mycol. Soc.* **75**: 320-325 (1980).

Bougoure D.S., Cairney J.W.G.: Fungi associated with hair roots of *Rhododendron lochiae* (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture-independent molecular methods. *Environ. Microbiol.* **7**: 1743-1754 (2005a).

Bougoure D.S., Cairney J.W.G.: Assemblages of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (Ericaceae) as determined by culturing and direct DNA extraction from roots. *Environ. Microbiol.* **7**: 819-827 (2005b).

Cairney J.W.G., Meharg A.A.: Ericoid mycorrhiza: a partnership that expoits harsh edaphic conditions. *Eur. J. Soil. Sci.* **54**: 735-740 (2003).

Dalpé Y.: Axenic synthesis of ericoid mycorrhiza in *Vaccinium angustifolium* Ait. by *Oidiodendron* species. *New Phytol.* **103**: 391-396 (1986).

Dalpé Y.: Ericoid mycorrhizal fungi in the Myxotrichaceae and Gymnoascaceae. *New Phytol.* **113**: 523-527 (1989).

Dalpé Y.: Statut endomycorhizien du genre Oidiodendron. *Can. J. Bot.* **69**: 1712-1714 (1991).

Dighton J., Coleman D.C.: Phosphorus relations of roots and mycorrhizas of *Rhododendron maximum* L. in the southern Appalachians, North Carolina. *Mycorrhiza* **1**: 175-184 (1992).

Domsch K.H., Gams W., Anderson T.H.: Compendium of soil fungi. Vol. 1, Academic Press, London New York Toronto Sydney San Francisco 1980.

Eccher T., Noé N.: Influence of ericoid endomycorrhizae inoculated in vitro on rooting and early growth of micropropagated plants of *Vaccinium corymbosum*. *Acta Hort*. **574**: 373-378 (2002).

Gay G.: Effect of the ectomycorrhizal fungus *Hebeloma hiemale* on adventitious root formation in derooted Pinus halepensis shoot hypocotyls. *Can. J. Bot.* **68**: 1265-1270 (1990).

Gianni C., Caretta G., Romano C.: Skin infection due to *Geomyces pannorum* var. *pannorum*. *Mycoses* **46**: 430-432 (2003).

Hambleton S., Egger K.N., Currah R.S.: The genus *Oidiodendron*: species delimitation and phylogenetic relationship based on nuclear ribosomal DNA analysis. *Mycologia* **90**: 854-869 (1998).

Hambleton S., Sigler L.: *Meliniomyces*, a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (= *Hymenoscyphus ericae*), *Leotiomycetes*. *Stud. Mycol*. **53**: 1-27 (2005).

Johnson N.C., Graham J.H., Smith F.A.: Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytol*. **135**: 575-585 (1997).

Jones M.D., Smith S.E.: Exploring functional definitions of mycorrhizas – are mycorrhizas always mutualisms? *Can. J. Bot*. **82**: 1089-1109 (2004).

Jumpponen A.: Dark septate endophytes – are they mycorrhizal? *Mycorrhiza* **11**: 207-211 (2001).

Lacourt I., Girlanda M., Perotto S., Del Pero M., Zuccon D., Luppi A.M.: Nuclear ribosomal sequence analysis of *Oidiodendron*: towards a redefinition of ecologically relevant species. *New Phytol*. **149**: 565-576 (2001).

Leake J.R., Read D.J.: The biology of mycorrhiza in the Ericaceae: XIII. Some characteristics of the extracellular proteinase activity of the ericoid endophyte *Hymenoscyphus ericae*. *New Phytol*. **112**: 69-76 (1989).

Mandyam K., Jumpponen A.: Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology* **53**: 173-189 (2005).

Midgley D.J., Chambers S.M., Cairney W.G.: Distribution of ericoid mycorrhizal endophytes and root-associated fungi in neighbouring Ericaceae plants in the field. *Plant Soil* **259**: 137-151 (2005).

Niemi K., Häggman H.: *Pisolithus tinctorius* promotes germination and forms mycorrhizal structures in Scots pine somatic embryos in vitro. *Mycorrhiza* **12**: 263-267 (2002).

Niemi K., Vuorinen T., Ernstsen A., Häggman H.: Ectomycorrhizal fungi and exogenous auxins influence root and mycorrhiza formation of Scots pine hypocotyl cuttings in vitro. *Tree Physiol.* **22**: 1231-1239 (2002).

Nylund J.E.: The regulation of ectomycorrhiza formation – carbohydrate and hormone theories revised. *Scand. J. For. Res*. **3**: 465-470 (1988).

Perotto S., Girlanda M., Martino E.: Ericoid mycorrhizal fungi: some new perspectives on old acquaintances. *Plant Soil* **244**: 41-53 (2002).

Read D.J., Leake J.R., Perez-Moreno J.: Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. *Can. J. Bot*. **82**: 1243-1263 (2004).

Rudawska M.L., Kieliszewska-Rokicka B.: Mycorrhizal formation by *Paxillus involutus* strains in relation to their IAA-synthesizing activity. *New Phytol.* **137**: 509-517 (1997).

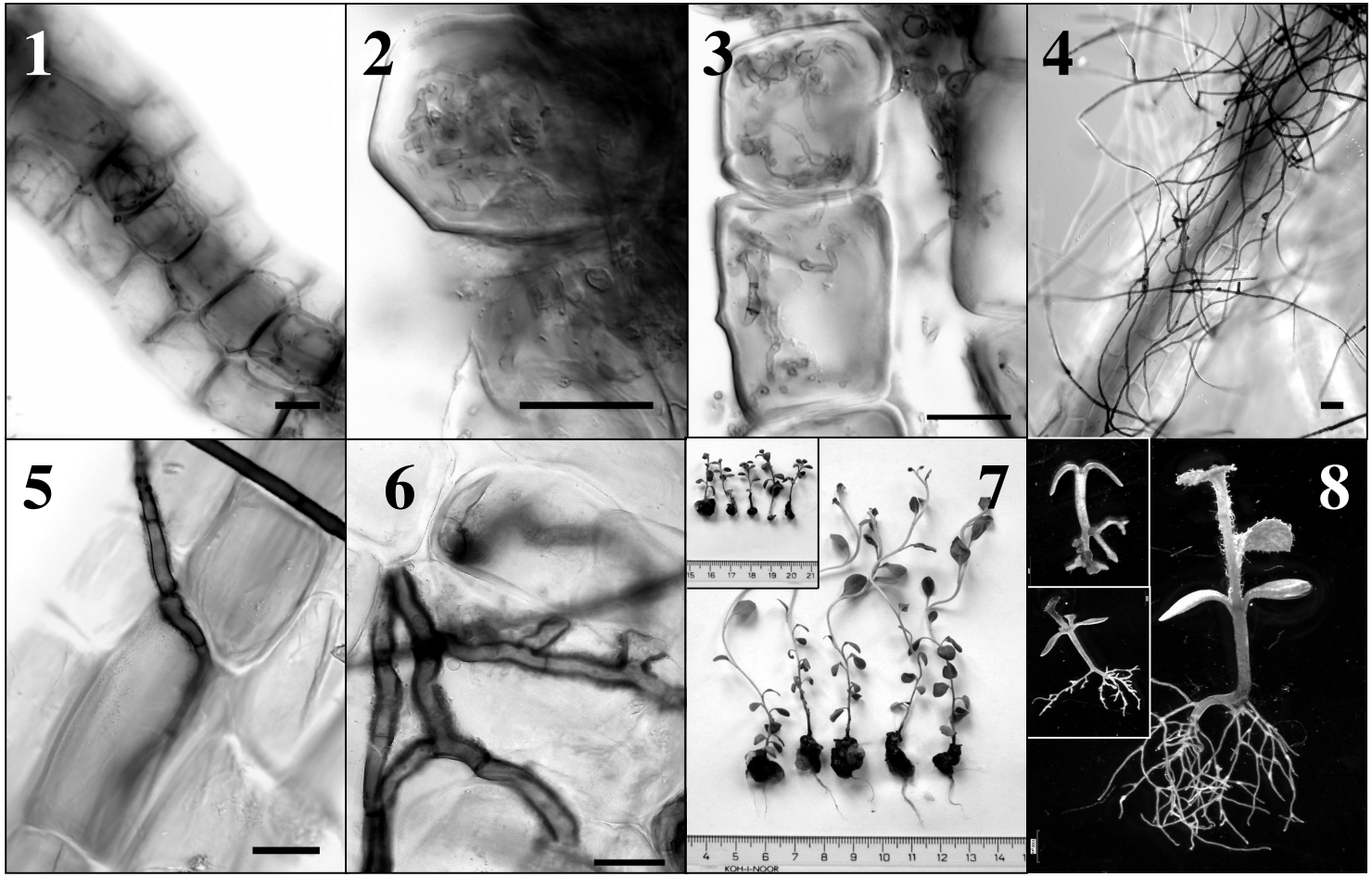
Seviour R.J., Willing R.R., Chilvers G.A.: Basidiocarps associated with ericoid mycorrhizas. *New Phytol*. **72**: 381-385 (1973).

Stoyke G., Currah R.S.: Resynthesis in pure culture of a common subalpine fungus-root association using *Phialocephala fotinii* and *Menziesia ferruginea* (Ericaceae). *Arct. Alp. Res.* **25**: 189-193 (1993).

Usuki F., Narisawa K.: Formation of structures resembling ericoid mycorrhizas by the root endophytic fungus *Heteroconium chaetospira* within roots of *Rhododendron obsutum* var. *kaempferi*. *Mycorrhiza* **15**: 61-64 (2005).

Vohník M., Albrechtová J., Vosátka M.: The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro. *Symbiosis* 40: 87-96 (2005).

Figures 1 & 2 & 3: hyphal loops formed by *Geomyces pannorum* in the roots of *Vaccinium corymbosum*; **Figure 4**: a loose hyphal weft formed by *Cenococcum geophilum* around the roots of *Rhododendron ponticum*; **Figure 5**: a dark septate hypha enters the rhizodermal cell of *R. ponticum*; **Figure 6**: extra- and intracellular structures formed by *C. geophilum* in the roots of *V. corymbosum*; **Figure 7**: a comparison of the stimulation effect of *C. geophilum* on *Rhododendron* sp. micro-cuttings (control cuttings in the smaller photo); **Figure 8**: a comparison of the effect of *C. geophilum* (big photo), *Geomyces pannorum* (upper smaller photo) and *M. variabilis* (lower smaller photo) on the development of *R. ponticum* seedlings. Bars represent 10um unless other indicated.



2. 4. ČÁST II: Diskuse

(Ektomyko-)rhizosféra, potažmo pak i rhizoplána ektomykorhizních rostlin, a zejména houby, které ji obývají, byly předmětem výzkumu ještě před vlastním objevem EcM symbiózy (Frank 1885). Specifický prostor vymezený dosahem mimokořenových hyf vyrůstajících z jednotlivých EcM kořenových špiček obvykle obsahuje odlišné spektrum hub, než okolní EcM hyfami nekolonizovaná půda. Toto spektrum je nezřídka dominované nemykorhizními houbami typu *Penicillium*, *Umbelopsis*, *Mortierella*, *Trichoderma*, *Cylindrocarpon* apod. (shrnuto např. v Summerbell 2005b). Ektomykorhizosféra je houbami (ale i ostatní půdní mikroflórou a mikrofaunou) obvykle kolonizována v hojnější míře, než okolní substrát; tento jev se nazývá "rhizosférní efekt" (*rhizosphere effect*) a byl dle Summerbell (2005b) pravděpodobně poprvé pozorován O. Hagemem již v roce 1910.

V ektomykorhizosféře jsou kromě výše uvedených hub již od počátků minulého století pravidelně a hojně nalézány houby, jejichž mykorhizní status je nejistý. Jedná se především o houby dříve řazené do komplexu MRA. Ten byl tradičně členěný na skupiny α a β. S rozvojem molekulárních technik bylo zjištěno, že hlavním představitelem MRA α je DSE-houba *P. fortinii*, taxonomický status MRA β zůstává nejasný. O významu MRA, tedy především *P. fortinii* a DSE-asociace, kterou tato houba v kořenech většiny vyšších rostlin tvoří, pojednává Část I této DP. Vedle MRA komplexu jsou s menší frekvencí v ektomykorhizosféře nalézány také houby, dříve řazené do komplexu VWT. Summerbell (2005b) uvádí jako jeden z prvních nálezů VWT některé houby, izolované z pseudomykorhizních kořenů *Picea mariana* (Mill.) BSP Richardem a Fortinem v roce 1974. VWT je také často asociován s mykorhizosférou vřesovcovitých rostlin (Hambleton a Currah 1997). S rozvojem molekulárních technik byla jako hlavní představitel VWT identifikována houba *M. variabilis* (Hambleton a Sigler 2005).

Výsledky Článku 4 a Článku 5 této DP ukazují, že *M. variabilis* vnitrobuněčně kolonizuje kořeny vřesovcovitých rostlin, přičemž v jejich rhizodermálních buňkách tvoří struktury typické pro ErM. Vzhledem k časté asociaci *M. variabilis* s kořeny vřesovcovitých za přirozených podmínek (viz Hambleton a Sigler 2005), k výsledkům našich in vitro syntetických pokusů a s přihlédnutím k prácím Piercey a kol. (2002) a Berch a kol. (2002) tak lze prohlásit, že *M. variabilis* má potenciál tvořit v kořenech vřesovcovitých ErM. Výsledky Článku 4 navíc ukazují, že oba zkoumané kmeny *M. variabilis* dokáží vnitrobuněčně kolonizovat typicky EcM hostitelské rostliny *P. abies* a *P. sylvestris*. To je v souladu s výsledky práce Schild a kol. (1988), která uvádí podobný způsob kolonizace u *Picea sitchensis* Carr., a s výsledky práce Piercey a kol. (2002), kde

M. variabilis podobným způsobem kolonizuje hostitelskou rostlinu *P. mariana*. Lze tedy prohlásit, že vnitrobuněčná kolonizace kořenů typicky EcM hostitelských rostlin je u *M. variabilis* alespoň v kontrolovaných podmínkách obvyklým jevem. Je hypoteticky možné, že by *M. variabilis* v přirozených podmínkách mohl plnit roli propojovatele rostlin různých mykorhizních preferencí, v tomto případě rostlin, typicky tvořících ErM a EcM, přičemž v kořenech typicky EcM rostlin by *M. variabilis* netvořil EcM, ale byly by kolonizovány způsobem popsaným výše. Taková role byla hypotetizována u dalšího zástupce *R. ericae* – agregátu, houby *C. finlandica* (Vrålstad 2004). *C. finlandica* však na rozdíl od *M. variabilis* tvoří v kořenech typicky EcM hostitelských rostlin Hartigovu síť, charakteristickou pro EcM a kořenové buňky EcM rostlin nekolonizuje vnitrobuněčně (viz Část II: Úvod, Obr. 3).

Přestože je *C. geophilum* typickou EcM houbou, její hyfy lze často nalézt i ve rhizosféře sousedících ErM rostlin, a byla také izolována z povrchově sterilizovaných kořenů vřesovcovitých rostlin (např. Midgley a kol. 2004). Dighton a Coleman (1992) identifikovali jeden z devíti EcM morfotypů, nalezených u *R. maximum*, jako *C. geophilum*. Stoyke a Currah (1993) inokulovali vřesovcovitou rostlinu *Menziesia ferruginea* Smith houbou *C. geophilum*. Nedocházelo k tvorbě EcM, mycelium tvořilo okolo kořenů volnou síť hyf a pouze vzácně hyfy penetrovaly vnitrobuněčně. Autoři uvádějí, že asociace pozorovaná v kontrolovaných podmínkách odpovídá asociaci, pozorované v přírodě. Tyto závěry odpovídají výsledkům, uvedeným v Článku 5. *C. geophilum* identickým způsobem kolonizovalo kořeny *Vaccinium* sp., *Rhododendron* sp. a *Rhododendron ponticum* L., přičemž nikdy nedocházelo k tvorbě EcM. Zdá se tedy, že *C. geophilum* nemá schopnost ve vlasových kořenech vřesovcovitých rostlin tvořit EcM. Přesto byl vliv *C. geophilum* na tvorbu a rozvoj kořenů všech zkoumaných rostlin pozitivní, a to jak ve srovnání s kontrolou, tak s typickou ErM houbou *R. ericae*. Velmi pravděpodobně za to může produkce auxinů zkoumaným kmenem *C. geophilum*, neboť koncentrace IAA v jeho myceliu dosahuje v průměru poměrně vysoké hodnoty 20 pmol/g čerstvé hmotnosti (Vohník a kol., nepublikováno). *C. geophilum* tak může stimulovat nejen rozvoj kořenů své typicky EcM hostitelské rostliny, ale i rostlin sousedních, přičemž právě vřesovcovité pravidelně tvoří podrost EcM porostu. Takováto podpora rozvoje kořenového systému se odráží i na růstu vřesovcovitých rostlin, jak ukazují výsledky Článku 5.

Je zřejmé, že typicky EcM *C. geophilum* může nemykorhizně ovlivňovat i rostliny jiných mykorhizních preferencí. Pozitivní efekt mykorhizní houby tedy nemusí být spjat s tvorbou mykorhizy. Vřesovcovité rostliny, zejména pokud rostou v přítomnosti EcM rostlin, tak hypoteticky mohou využívat přítomnosti EcM hub ve své rhizosféře, aniž by

jim za to přímo odváděly uhlíkaté sloučeniny, jak je pravidlem u mykorhizní symbiózy. Jednalo by se tedy o určitou formu komenzálního vztahu. Lze však předpokládat, že i *C. geophilum* v rámci tohoto hypotetického vztahu profituje, např. využíváním kořenových exudátů nebo odumřelých kořenů jako zdroje energie.

Mykorhizní status je u hub asociovaných s kořeny vřesovcovitých rostlin určován primárně pomocí resyntetických pokusů, přičemž za erikoidně mykorhizní je považována houba, tvořící v rhizodermálních buňkách vřesovcovitých útvary typické pro ErM (viz Článek 1). Na základě tohoto kritéria Dalpé (1989) prohlásila *Myxotrichum setosum* (Eidam) Orr, Kuehn & Plunkett, *Gymnascella dankaliensis* (Castellani) Currah (obě teleomorfní *Oidiodendron*) a *Pseudogymnoascus roseus* Raillo (anamorfní *Geomyces*) za ErM houby, když tvořily hyfové smyčky v kořenech *Vaccinium angustifolium* Ait. Výsledky Článku 5 ukazují, že podobnou schopnost má i půdní keratinolytická houba *G. pannorum*, příbuzná výše uvedeným. To může poukazovat na její potenciální schopnost tvořit ErM i v přirozených podmínkách. Na druhou stranu byl růst kolonizovaných rostlin v porovnání s kontrolními rostlinami ovlivněn negativně, což svědčí proti této domněnce. Případné mykorhizní vlastnosti této houby tak musí být posouzeny v experimentu, reflektujícím podmínky, panující na přirozených stanovištích vřesovcovitých rostlin.

3. ČÁST III:

Interakce mezi erikoidně mykorhizními a DSE houbami a krytenkami

Rhododendron hirsutum z náhorní planiny Velika Planina, Slovinsko.

3. 1. Část III: Úvod

Část III této DP je z hlediska rozsahu textu nejmenší, opírá se o jeden původní článek. Přestože si myslím, že je z určitého pohledu nejzajímavější. Pojednává o interakci půdních prvoků (*Protozoa*), resp. protist (*Protista*) (v textu se budu přidržovat termínu "prvoci" jako překladu v současné době stále používaného *protozoans*), mykorhizních hub a jejich hostitelských rostlin, tedy organizmů, lišících se na úrovni říší. Problematika v ní zkoumaná má bohužel i jiné superlativum – je ze všech tří částí nejhůře metodicky/experimentálně uchopitelná.

Prvoci představují významnou část půdní bioty zejména proto, že hrají klíčovou roli při dekompozici organických složek půdy, čímž se významně podílejí na koloběhu živin v přírodě (např. Vargas 1990 nebo Bonkowski 2004). Krytenky (někdy také kryténky, zast. thékaméby, angl. *testate amoebae*, *testaceans*, *thecamoebae* nebo *thecamoebians*) jsou polyfyletickou skupinou vodních nebo půdních prvoků, tedy jednobuněčných organizmů, jejichž cytoplazma je uzavřena v samostatné schránce (Ogden a Hedley 1980). Krytenky byly dříve řazeny mezi kořenonožce (Rhizopoda: *Testacea*). V současnosti by se však tento pojem již neměl používat, protože většina krytenek je řazena do dvou nově ustavených skupin: *Arcellinida* (Amoebozoa) a *Euglyphida* (Cercozoa). Krytenky obývají nejrůznější biotopy, z pohledu této disertační práce je nejdůležitější jejich přítomnost ve vlhké půdě vřesovišť a lesních ekosystémů. Na podobných stanovištích může být zastoupení krytenek v rámci půdní bioty značné. Například Gilbert a kol. (1998) uvádí, že krytenky představovaly 48% celkové mikrobiální biomasy (nepočítaje biomasu půdní hub) na rašeliništi dominovaném rašeliníkem (rod *Sphagnum*). V jiné studii zaměřené na výzkum struktury mikrobiálního společenstva v habitatu dominovaném druhy *Sphagnum fallax* a *Carex rostrata* Gilbert a kol. (1998b) zjistili, že prvoci tvořili přibližně 26% celkové mikrobiální biomasy (tentokrát houby nevyjímaje). Krytenky pak v rámci této skupiny přispěly téměř 14% z celkové mikrobiální biomasy. Oproti tomu půdní houby představovaly pouze 2% celkové mikrobiální biomasy. Z toho je zřejmé, že odumřelé krytenky (= chitinózní schránky + jejich obsah) představují, alespoň v rámci podobných, na živiny chudých ekosystémů, považení hodný zdroj (*pool*) živin. Ovšem zdroj živin organických, které jsou přímo nepřístupné primárním producentům, rostlinám.

Přítomnost půdních prvoků v rhizosféře signifikantně ovlivňuje růst rostlin (Bonkowski a kol. 2000) a předpokládá se, že tento vliv může být umocněn součinností s mykorhizními houbami (Bonkowski a kol. 2001). Mykorhizní houby jsou totiž, podobně jako půdní prvoci, považováni za hybatele (*drivers*) výměny živin v rhizosféře, kteří zcela zásadně ovlivňují hostitelské rostliny (Read a Perez-Moreno 2003, Read a kol. 2004). ErM houby, na které je tato DP zaměřená, tvoří ErM s vřesovcovitými rostlinami, které typicky dominují habitaty chudé na dostupný dusík (N), tedy např. rašeliniště a vřesoviště (Read 1996, Cairney a Meharg 2003). V takových podmínkách ErM houby zajišťují přísun N svým hostitelským rostlinám, přičemž jsou k tomu schopny využívat rozličných substrátů, např. chitinu (Kerley a Read 1995), houbového mycelia (Kerley a Read 1997) nebo rostlinné/mykorhizní nekromasy (Kerley a Read 1998). Posledně jmenovaná studie ukázala

že typická ErM houba *R. ericae* produkovala extracelulární proteinázy a chitinázy, když byla kultivována na nekromase mykorhizních kořenů, a byla schopna využívat chitin jako zdroj N, který posléze transportovala do hostitelské rostliny.

Interakce mezi půdní faunou a mykorhizními houbami zahrnují komplexní a dynamické procesy, které mohou ovlivnit všechny partnery, kteří se takových procesů účastní: klíčení spor, schopnost kolonizovat kořeny, růst mycelia a jeho sporulace může být ovlivněna na straně půdních hub (Fitter a Garbaye 1994), produkce biomasy na straně mykorhizních rostlin (Bonkowski a kol. 2001) a složení a struktura společenstev na straně půdních prvoků (Ingham a Massicotte 1994). Interakce mezi krytenkami a ostatní půdní biotou, zejména půdními houbami, nejsou příliš častým předmětem výzkumu. Z publikovaných prací však vyplývají zajímavé souvislosti. Gilbert a kol. (2003) zjistili, že spory a mycelium půdních hub představovaly 36% identifikovatelné potravy zástupců komplexu krytenek *Nebela tincta major-bohemica-collaris*. To naznačuje, že půdní houby (pravděpodobně i mykorhizní) představují podstatnou složku potravy těchto krytenek. Ingham a Massicote (1994) zjistili, že krytenky pravidelně osidlovaly mykorhizosféru EcM kořenů pěti konifer, a že jejich společenstva se kvalitativně i kvantitativně lišila v závislosti na druhu EcM houby a její hostitelské rostliny. To naznačuje, že složení EcM společestva může ovlivňovat složení společenstva krytenek, vyskytujících se v mykorhizosféře. Podstata takového vztahu zůstává bohužel neznámá. Ingham a Massicote (1994) hypotetizují, že mykorhizní houby by mohly ovlivnit společenstvo půdních prvoků prostřednictvím kontroly společenstva bakterií v mykorhizosféře, protože tyto jsou podstatnou složkou potravy prvoků.

Ačkoliv se mi nepodařilo nalézt práci, která by se přímo zabývala interakcí krytenek a mykorhizních hub, lze z dostupné literatury na příbuzná témata odvodit několik zajímavých souvislostí: **(i)** krytenky představují kvantitativně důležitou součást půdní bioty v živinově chudých habitatech (Gilbert a kol. 1998); **(ii)** takové habitaty jsou často dominovány vřesovcovitými rostlinami, tvořícími ErM a/nebo DSE-asociaci; **(iii)** v takových habitatech ErM houby (a pravděpodobně i DSE) zprostředkovávají rostlinám přístup k organickým živinám (Read a kol. 2004, Mandyam a Jumpponen 2005); **(iv)** mykorhizní houby mohou využívat "živočišný" dusík (Klironomos a Hart 2001); **(v)** půdní houby představují důležitou součást potravy krytenek (Gilbert a kol. 2003). Konečně, **(vi)** má předchozí nepublikovaná pozorování naznačují, že schránky pravděpodobně mrtvých krytenek jsou běžně asociovány s mimokořenovým myceliem, vyrůstajícím z ErM kořenů. Tyto skutečnosti dohromady tvoří velmi zajímavou mozaiku mykorhizosférních vztahů, zahrnující organizmy diametrálně odlišných skupin. Většina komponentů této hypotetické mozaiky je bohužel neprozkoumaná. V Článku 6 jsme se proto na některé z nich zaměřili.

Cílem Části III této disertační práce jmenovitě bylo:

1. **Prozkoumat asociaci krytenek a mycelia, vyrůstajícího z kořenů mykorhizních rododendronů**
2. **Prozkoumat složení společenstva krytenek, vyskytujících se v rhizopláně těchto rododendronů.**
3. **Prozkoumat, mohou-li vybrané ErM a DSE houby využívat schránky mrtvých krytenek jako zdroj živin pro svůj růst.**

   Tyto cíle jsou zpracovány v Článku 6 této disertační práce.

3. 2. ČLÁNEK 6

Testate Amoebae vs. Mycorrhizal Fungi: A Possible Novel Interaction in Mycorhizosphere of Ericaceous Plants?

(manuskript odeslán do časopisu *Microbial Ecology*)

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Testate Amoebae vs. Mycorrhizal Fungi: A Possible Novel Interaction in Mycorhizosphere of Ericaceous Plants?

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Abstract

Interactions between soil protozoans and mycorrhizal fungi, despite their sympatric distribution and proposed key role in nutrient cycling in the rhizosphere have been studied only to a limited extent. Studies focusing on the interaction between testate amoebae (TA) and mycorrhizal fungi are lacking. Here we report (i) field observations of TA, associated with ericaceous roots via mycelium of putative mycorrhizal fungi, (ii) results of preliminary screening of TA populations associated with the rhizoplane of three European *Rhododendrons*, and (iii) results of two *in vitro* experiments answering the question, whether TA shells may serve as a sole source of nutrient for ericoid mycorrhizal fungi (ErMF) and dark septate endophytes (DSE). Our field observations indicate that TA regularly associate with the mycorrhizosphere of ericaceous plants and that ErMF and/or DSE possibly exploit the TA shells as a nutrient source. Composition of TA communities associated with the rhizoplane did not differ among all three *Rhododendron* species. In the *in vitro* experiments, both ErMF *Rhizoscyphus ericae* and DSE *Phialocephala fortinii* regularly colonized TA shells, utilizing them as a sole source of nutrients. *P. fortinii* formed microsclerotia (hyphal structures typical for DSE-association in roots of higher plants) inside colonized TA shells. We hypothesize a multidimensional relationship between ErMF/DSE and TA. If corroborated, it would represent an interesting nutrient loop in mycorrhizosphere of ericaceous plants.

Introduction

Protozoans represent an important part of soil biota, playing a crucial role in decomposition of organic matter and thus in nutrient cycling (25, 4). Testate amoebae (TA; *Rhizopoda*, *Testacea*), also known as thecamoebae, are a polyphyletic group of aquatic or terrestrial unicellular organisms, whose cytoplasm is enclosed within a discrete shell/testa (19). TA inhabit various habitats, including heathlands and peat bogs, and are an important part of the community of soil protozoans. For example, Gilbert et al. (8)

found that TA represented 48% of the total microbial biomass in a *Sphagnum* peatland. Thus, dead TA constitute a considerable pool of nutrients in such nutrient-impoverished habitats, however in an organic form, they are directly unavailable to plants.

The presence of soil protozoans in the rhizosphere significantly affects plant growth (2) and this effect can be mediated by interactions with mycorrhizal fungi (3). Similarly to protozoans, mycorrhizal fungi are also accepted as drivers of nutrient exchange in the rhizosphere, with fundamental effects on plants fitness (22, 23). Among them, ericoid mycorrhizal fungi (ErMF) regularly associate with roots of *Ericaceae* to form ericoid mycorrhiza (ErM). Ericaceous plants typically inhabit soils poor in available nitrogen (N) and often dominate vegetation in heathlands and peat bogs (21, 6). Under these conditions, ErMF provide N supply to their hosts (24), being able to utilize such complex substrates as chitin (14), fungal mycelium (15) or plant/mycorrhizal necromass (16). The later study (16) showed that the typical ErMF *Rhizoscyphus ericae* (Read) Zhuang & Korf produced extracellular protease and chitinase when grown on mycorrhizal root biomass and used fungal chitin as a source of N, transporting it into host plant tissues.

Interactions between soil microfauna and mycorrhizal fungi comprise dynamic and complex processes that may influence all partners entering the interaction: spore germination, root colonization, growth of fungal mycelium or sporulation can be affected on the fungal side (7), production of biomass on the plant side (3) and composition of the community structure (11) on the protozoan side. Investigations of interactions between TA and soil fungi are rare, and reveal interesting consequences. Gilbert et al. (9) found that spores and mycelium of soil fungi represented 36% of the identified prey of the members of the TA complex *Nebela tincta major-bohemica-collaris*. This suggests that soil fungi, possibly including mycorrhizal, represent an important part of feed for this species. Ingham and Massicotte (11) found that TA regularly inhabited the mycorrhizosphere of ectomycorrhizal (EcM) roots of five conifers and that their communities quantitatively and qualitatively differed depending on host plants and EcM fungi colonizing their roots. This suggests that the composition of an EcM community might determine the composition of a TA community in the mycorrhizosphere. However, principles of this relationship are unknown. Ingham and Massicotte (11) suggested that mycorrhizal fungi could influence the protozoan community by controlling/altering the bacterial community in the rhizosphere.

Even though reports focused directly on interactions between TA and mycorrhizal fungi are lacking, several interesting facts can be extracted from the literature focused on related topics: **(i)** TA represent a quantitatively important part of soil biota in nutrient-poor peatland habitats (8); **(ii)** such habitats are often dominated by ericaceous plants, which regularly form ErM and/or DSE-association (21, 13, 6); **(iii)** in such habitats ErMF, and possibly also DSE, help plants to access organic nutrients, namely N (23, 18); **(iv)** mycorrhizal fungi can access animal N (17); **(v)** soil fungi represent an important part of TAs' feed (9). In addition, our previous observations indicate that **(vi)** shells of

probably dead TA are regularly associated with mycelium emerging from ericoid mycorrhizal roots (M. Vohník, unpublished data). These facts together indicate a possible multidimensional relationship between mycorrhizal fungi and TA, which might influence also mycorrhizal plants. However, almost all pieces of this hypothetic mosaic are unwrought.

Here we report our observations of the association between shells of TA and fungal mycelium emerging from *Rhododendron* mycorrhizal roots. In a preliminary screening, we describe the composition of the TA communities found in the rhizoplane of three autochthonous European *Rhododendron* species. In two *in vitro* experiments, we try to answer the question whether sterile TA shells could serve as propagule carriers and/or a sole source of nutrient for ErMF *R. ericae* and DSE *Phialocephala fortinii* Wang & Wilcox.

Materials and Methods

Screening of the association between TA shells and Rhododendron roots

Three European *Rhododendron* species (Table 1) were screened for the presence of TA shells in the rhizoplane, with special emphasis on the shells associated with the fungal mycelium emerging from their roots. For each species, four to seven individuals were screened. From each individual, three root samples (each containing approx. 15 cm of roots) were taken from the upper soil layer (depth 5 – 15 cm), inserted in plastic bags and stored in a fridge until screened. Additionally, roots of four rooted stem cuttings (size approx. 10 cm) of *Rhododendron* cv. Azurro, cultivated in a growth chamber in a non-sterile peat-based substrate were screened in the same manner except the TA species spectrum was not determined. All root samples were also screened for the presence of ErM and DSE-association.

Roots with adhering rhizospheric soil were gently washed under running tap water on a sieve (Ø 1 mm) to remove excessive substrate. Washed roots with adhering rhizoplanic substrate were divided into three parts. The first two parts were subjected to direct observation of associated TA shells using light/SEM microscope. Prior to observation, the first part of the roots was treated according to the methods commonly used for screening mycorrhizal colonization (5), i.e., autoclaved in 10% KOH for 20 min at 121 ºC, rinsed in 3% HCl, washed with running tap water and autoclaved in 0.05% trypan blue in lactoglycerol for 20 min at 121 ºC and left overnight at room temperature (= "common treatment"). The second part of the roots was directly immersed in a solution of 0.05% trypan blue in lactoglycerol and left overnight at room temperature (= "alternative treatment"). Roots were de-stained by immersing in de-ionized water and screened for the presence of associated shells of TA and mycorrhizal structures using binoculars. Because there were no TA shells associated with the roots in the common treatment, we further focused only on the roots from the alternative treatment. Here, mycorrhizal roots associated with TA shells via fungal mycelium were cut into 1-cm pieces and mounted onto non-permanent slides for light microscopy or were directly microscoped using SEM. Slides were microscoped at high magnification (400x or 1000x) using Olympus BX60 microscope

equipped with DIC. SEM photographs were taken in the ESEM™ mode at low temperatures (-6 ºC to –3 ºC) using an FEI Quanta 200 microscope.

Community structure of TA associated with the rhizoplane of three Rhododendron species

The third part of the roots was placed into 250-ml flasks with lactoglycerol (lactic acid : glycerol : de-ionized water = 1 : 1 : 3) and stored in the fridge for one month. After this period the rhizoplanic material, which separated from the roots of *R. hirsutum*, *R. kotschyi* and *R. luteum* due to gravitation, was collected from the bottom of the flasks with plastic pipettes and was screened for composition of the TA community. In each sample, at least 100 TA shells were collected and determined to the genus level according to Ogden and Hedley (19). Similarity of the TA spectra between different individuals of the three *Rhododendron* species was compared using cluster analysis (tree clustering, Ward's method) in Statistica™ 5.1 (StatSoft, USA).

Experiment 1 – colonization of TA shells by P. fortinii and R. ericae

Shells of TA belonging to *Cyclopyxis* and *Trygonopyxis* genera were extracted with fine forceps using binoculars from a water suspension of the substrate, collected from the rhizosphere of *Vaccinium myrtillus* L. in Modrava, Šumava NP, Czech Republic in September 2005. These two TA genera were used because of their abundance in the suspension and shell size suitable for manipulation. Grains of serially washed quartz sand (Provodínské písky Inc., CZ; fraction <1 mm) were used as a negative control and were treated in the same manner as the TA shells.

Extracted shells/sand grains were transferred onto moistened PRAGOPOR 6 nitrocellulose membranes (Ø 1 cm, pore size 0.4 µm; Pragochema Ltd., CZ) placed in glass Petri dishes, five shells/grains per membrane, and autoclaved for 20 min at 121 °C. Autoclaved membranes with adhering shells/grains were aseptically placed into plastic Petri dishes with water agar (WA). Small pieces of mycelium (approx. 1 mm^3) obtained from actively growing cultures of *P. fortinii* and *R. ericae* were inoculated approx. 3 mm from each membrane. *P. fortinii* was the strain "F" from Vohník et al. (26), which is deposited in the Culture Collection of Fungi (Department of Botany, Faculty of Science, Charles University in Prague, CZ) under the accession number CCF 3586. It corresponds to the *P. fortinii* cryptic species CSP7 (= *P. fortinii* s.s.) sensu Grünig et al. (10). *R. ericae* was the isolate UAMH 6735 (GenBank AJ319078) from Pearson and Read (20). There were three variants (*P. fortinii*-inoculated, *R. ericae*-inoculated and non-inoculated), each containing in total 60 autoclaved TA shells onto 12 membranes in four Petri dishes (three membranes in one dish, each membrane with five TA shells) and 60 autoclaved sand grains organized in the same manner as the shells. The dishes were sealed and incubated at room temperature in the dark for two months. After this period, the shells/grains were extracted with forceps and divided into three parts for each variant, each part containing 20 shells/grains on four membranes. The first part was screened for colonization with *P.*

fortinii or *R. ericae* using light and/or scanning electron microscopy. The sand grains were subjected to SEM only.

Experiment 2 – TA shells as a sole source of nutrients for P. fortinii and R. ericae

The second part of the shells/grains was aseptically transferred onto new autoclaved moistened nitrocellulose membranes, placed on water agar in plastic Petri dishes (Ø 9 cm), sealed and incubated at room temperature in the dark for two months. The third part of the shells/grains was transferred onto new autoclaved moistened nitrocellulose membranes, placed on serially washed quartz sand (the same provenience as the grains) in glass Petri dishes (Ø 5 cm), sealed and incubated at room temperature in the dark for two months. The mycelium emerging from the shells/grains was observed and documented periodically each week. After two months, the shells were screened using light and scanning electron microscopy. The grains were subjected to SEM only.

Results

Screening of the association between TA shells and Rhododendron roots

We found the association between TA shells and roots via the mycelium of putative mycorrhizal fungi in all samples of all three *Rhododendron* species and *Rhododendron* cv. Azurro in the alternative treatment. We estimate that there was at least one TA shell associated with the mycorrhizal root via the fungal mycelium per 5cm of the total root length. We found no associated TA shells in the samples from the common treatment.

In some cases, TA shells appeared to be only loosely attached to the root surface via fungal mycelium (Fig. 1), in other cases however, the association between TA shells and the root appeared to be very tight (Fig. 2). TA shells were often embedded in the fungal mycelium, often being partially decomposed (Fig. 3), which substantially hampered their identification as the TA shells. Some of the objects with the TA shells' appearance were filled with darkly pigmented thick-walled cells (Fig. 4), which resembled the colonization pattern of the DSE *P. fortinii* (see text below). All screened ericaceous plants were both ErM and DSE-associated (Table 1).

Community structure of TA associated with the rhizoplane of three Rhododendron species

In total, we found 13 genera of TA to be associated with the rhizoplane of the three *Rhododendron* species (alphabetically): *Arcella*, *Assulina*, *Centropyxis*, *Corythion*, *Cyclopyxis*, *Diplochlamys*, *Euglypha*, *Heleopera*, *Nebela*, *Pseudodifflugia*, *Tracheleuglypha*, *Trigonopyxis* and *Trinema* (Tab. 2). Shells of *Pseudodifflugia* were found only in the *R. hirsutum* samples, all other TA genera were found in the rhizoplane of all three rhododendrons. The most frequent genera (>10% of the community) were *Diplochlamys* and *Centropyxis*. In average, we were unable to determine 5.6% of the shells from the community of each *Rhododendron* species.

We found no apparent difference in structure of TA communities associated with the rhizoplane between the three *Rhododendron* species. Clustering of the TA communities of the individual samples revealed no general pattern with respect to the three *Rhododendron* species (Graph 1).

Experiment 1 – colonization of TA shells by P. fortinii and R. ericae

Both *Cyclopyxis* and *Trigonopyxis* shells were colonized by the mycelium of both ErMF *R. ericae* and DSE *P. fortinii*. Commonly, *R. ericae* mycelium almost completely covered the surface of the colonized shells (Fig. 5). *P. fortinii* was slower in colonization and its mycelium usually did not cover the whole surface of the shells (Fig. 6). *P. fortinii* hyphae often entered the shell via its aperture (Fig. 6). The intracellular colonization of such shells consisted of short, thick, darkly colored and thick cell-walled hyphae, which usually occupied the whole lumen of the shell (Fig. 7). This was connected with notable color change – the shells intracellularly colonized by *P. fortinii* were dark brown to black, which contrasted with the yellowish to light brownish color of the shells, which were colonized only superficially. Such intracellular colonization resembled microsclerotia, formed by DSE in the roots of higher plants (Fig. 11). The surface of the sand grains was only poorly colonized by either single hyphae or a very loose weft of the mycelium of *P. fortinii* or *R. ericae*.

Experiment 2 – TA shells as a sole source of nutrients for P. fortinii and R. ericae

After transferring the *P. fortinii*- and *R. ericae*- pre-colonized shells/grains onto new membranes and during their two-month cultivation on WA, both fungi were able to utilize the TA shells as a sole source of nutrients. This was indicated by vigorous growth of their hyphae, which radiated from the pre-colonized shells and lack of such growth from the pre-colonized sand grains. Vigorous growth was notable especially for the hyphae of *P. fortinii*, which expanded from the dark-colored pre-colonized shells in all directions, covering the whole surface of the shells and completely disorganizing their shape with progressing time (Fig. 8). Also the mycelium of *R. ericae* completely covered the surface of the pre-colonized shells and partially disorganized their shape with progressing time (Fig. 9).

When the membranes with the pre-colonized shells/grains were transferred into the dishes with serially washed quartz sand, a similar situation occurred: the pre-colonized shells gave rise to new abundant *P. fortinii* or *R. ericae* mycelium (Fig. 10), which was not the case of the pre-colonized sand grains. In the case of *P. fortinii*, the dark brown to black colored shells, which were intracellularly colonized as described above, gave rise to more abundant mycelium than the yellow- to light-brown-colored shells, which lacked significant intracellular colonization.

Discussion

The main impulse for this study originated from regular observations of associated TA shells while screening extraradical mycelium (ERM) of ErMF, DSE and other fungi inhabiting roots of ericaceous plants. Existence of the association between TA and ericaceous mycorrhizal roots might have significant impact on the understanding of nutrient cycling in the rhizosphere of ericaceous plants, which commonly dominate nutrient-poor habitats (22), particularly when considering that TA may represent up to 48% of the total microbial biomass in such habitats (8). Therefore, it was worthwhile to further investigate this interesting phenomenon.

The first step of the "alternative approach" used for this screening differed from the methodology commonly used for screening endomycorrhizal colonization (5). Instead of serial washings of the roots and their treatment with KOH or H2O2 in the "common approach", which destroy or remove most ERM and also root-associated protozoa, we immersed roots with adhering rhizospheric soil directly into trypan blue, the dye that for its ability to stain chitin is used to visualize fungal hyphae. Trypan blue stained also associated chitinous TA shells in our samples. We concluded that the "common approach" impaired observation of the association between TA shells and mycorrhizal roots and that the "alternative approach" would have to be employed to evaluate the association. This conclusion was confirmed in this study, because we found TA shells associated with mycorrhizal roots only in the alternative treatment, which did not include numerous washing steps.

Regularity of the association between TA shells and ericaceous mycorrhizal roots indicates that it is not an exceptional curiosity. Subsequently, a question arose why this association had not been reported earlier. We see two probable reasons. Firstly, the "common treatment" of roots prior to screening of their endomycorrhizal colonization prevents observation of fragile TA shells and their more fragile association with the fungal mycelium. Secondly, colonization by both *P. fortinii* and *R. ericae* notably changed anatomy and morphology of the colonized TA shells (Figs. 7 - 9). To date, TA species are determined mostly using the morphology of their shells (e.g. 19). Likely, TA shells colonized by soil fungi, especially in later stages of their decomposition, would not be recognized as TA at all (cf. Figs 3, 8 and 9).

As the next step, we determined the composition of TA communities living in the rhizoplane of ErM/DSE-associated ericaceous roots. This was done for two reasons: (i) we needed to select relevant TA candidates for *in vitro* experiments and (ii) we wanted to find out whether the TA communities differ between different hosts from different localities. Our working hypothesis was that if any closer relationship between TA and mycorrhizal roots exists, it is likely to be species-specific, as indicated by results of Ingham and Massicotte (11). Then, assuming that the composition of ErMF and DSE is similar in the roots of European rhododendrons, TA communities should not significantly differ in their composition in rhizoplanes of different *Rhododendron* species.

The TA spectrum revealed in our preliminary screening (Tab. 2) differed from this found by Ingham and Massicotte (11) around EcM roots of five conifer species, which was generally dominated by species of *Nebela*, *Valkanovia* and *Trinema*. The significance of the comparison of the TA spectra from ericaceous and coniferous mycorrhizosphere together with reasons for their similarity/difference is open to debate. On the other hand, studies focusing on ericaceous mycorrhizosphere are lacking and our preliminary screening pioneers this area of research.

There was no general pattern in the TA distribution among the three *Rhododendron* species, which indicates that the TA composition was variable between different individuals of the same species and generally, the variability was similar for all three *Rhododendron* species. Hypothetically, this can be either directly or indirectly due to similar spectrum of mycorrhizal fungi colonizing roots of European rhododendrons. However, it is needed to screen this spectrum to corroborate our hypothesis. It is also needed to compare the composition of TA communities between plants of different mycorrhizal status from the same environment prior to any general conclusion about possible reciprocal preferences between TA and mycorrhizal fungi. To our knowledge, such a comparison is to date lacking.

The association of TA shells (which likely represent a considerable pool of organic nutrients in soil) with ericoid mycorrhizal roots (which occur mostly in N-limited habitats; 6) via the mycelium of putative mycorrhizal fungi (which help ericaceous plants to access organic nutrients; 21) indicates that ErMF and probably also DSE may exploit the shells as a nutrient source. The results of our *in vitro* experiments confirm this possibility, because both *P. fortinii* and *R. ericae* were able to utilize TA shells as a sole source of nutrients. It can be argued that WA or the membranes served as an additional source of nutrients for the mycelium expanding from the shells. However, it was proved that the nitrocellulose membranes used in the experiments were resistant to microbial degradation (1) and the results from WA were repeated on serially washed quartz sand. Moreover, the differences between the growth of the mycelium from the pre-inoculated shells and the sand grains indicate that both fungi did utilize the shells as a sole source of nutrients.

To our knowledge, our study is the first observation of dead soil protozoans being exploited by mycorrhizal fungi under field conditions, and its verification *in vitro*. Our observations and results indicate the existence of an interesting nutrient loop in the rhizosphere of ericaceous plants. TA are known to feed on soil fungi (9) and it can be expected that also mycorrhizal fungi are part of their diet. Hypothetically, withered TA could be decomposed by the same mycorrhizal fungi that they fed on, and through their mycelium they could serve as a nutrient source for mycorrhizal plants. Several reports exist about the ability of ErMF to access organic N (in the form of chitin or plant, fungal and mycorrhizal necromass) and transport it to their host plants under laboratory conditions (14, 15, 16). However, this study showed that DSE and ErMF could directly access nutrients contained in withered protozoans.

In a laboratory study Klironomos and Hart (17) found that *Pinus strobus* ectomycorrhizal with *Laccaria bicolor* was able to derive up to 25% of its N from either dead or live soil-dwelling arthropods via its fungal partner. This revealed, according to the authors, "a nitrogen cycle of far greater flexibility and efficiency than was previously assumed, where the fungal partner uses animal-origin nitrogen to ´barter´ for the carbon from the host tree" (17). However, it remained unclear whether the phenomenon observed by the authors was widespread or functional under natural conditions. In our study, we report a very similar phenomenon, which is probably both widespread and functional in the rhizosphere of ericaceous plants under natural conditions. For direct evidence of its functioning and to reveal a nutrient flow between TA, mycorrhizal fungi and their host plants, labeled TA should be employed in a microcosm study with tracing of their elements in plant tissues.

In addition, the relationship between TA and ErMF/DSE might have more dimensions. For example, DSE fungi may survive unfavorable conditions in the form of microsclerotia embedded inside TA shells. Intracellular microsclerotia formed by DSE in roots of higher plants (Fig. 11) consist of short, thick, irregular, either dark or hyaline hyphae and are supposed to act as a nutrient storage and/or propagules, released after the root's disintegration (12). In our *in vitro* experiments, DSE *P. fortinii* formed similar structures inside *Centropyxis* and *Trigonopyxis* shells (Fig. 7). Thus, TA shells may serve as an efficient protecting envelope for these fungal structures. Moreover, fungal hyphae are often associated with the surface of TA shells (Fig. 12). This probably includes also hyphae of mycorrhizal fungi, because we isolated and amplified the DNA of *R. ericae* from TA shells collected from the peat-based substrate (M. Vohník, unpublished data). Thus, slowly motile TA might serve as propagule carriers for soil fungi. These are some additional pieces of the interesting "TA vs. mycorrhizal fungi" mosaic proposed in the introduction of this paper. We believe that our ongoing work will further elucidate its ecological significance.

Acknowledgments

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References

1. Baláž M, Vosátka M (2001) A novel inserted membrane technique for studies of mycorrhizal extraradical mycelium. Mycorrhiza 11: 291-296
2. Bonkowski M, Cheng W, Griffiths BS, Alphei J, Scheu S (2000) Microbial-faunal interactions in the rhizosphere and effects on plant growth. Eur J Soil Biol 36: 135-147
3. Bonkowski M, Jentschke G, Scheu S (2001) Contrasting effects of microbial partners in the rhizosphere: interactions between Norway Spruce seedlings (*Picea abies* Karst.), mycorrhiza (*Paxillus involutus* (Batsch) Fr.) and naked amoebae (protozoa). Appl Soil Ecol 18: 193-204

4. Bonkowski M (2004) Protozoa and plant growth: the microbial loop in soil revisited. New Phytol 162: 617-631
5. Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with mycorrhizas in forestry and agriculture. ACIAR Monograph 32, Canberra, 374 pp
6. Cairney JWG, Meharg AA (2003) Ericoid mycorrhiza: a partnership that exploits harsh edaphic conditions. Eur J Soil Sci 54: 735-740
7. Fitter AH, Garbaye J (1994) Interactions between mycorrhizal fungi and other soil organisms. Plant Soil 159: 123-132
8. Gilbert D, Amblard C, Bourdier G, Francez J.-A. (1998) The microbial loop at the surface of a peatland: structure, function, and impact of nutrient input. Microb Ecol 35: 83-93
9. Gilbert D, Mitchell EAD, Amblard C, Bourdier G, Francez A-J (2003) Population dynamics and food preferences of the testate amoeba *Nebela tincta major-bohemica-collaris* complex (Protozoa) in a Sphagnum peatland. Acta Protozool 42: 99-104
10. Grünig CR, McDonald BA, Sieber TN, Rogers SO, Holdenrieder O (2004) Evidence for subdivision of the root-endophyte *Phialocephala fortinii* into cryptic species and recombination within species. Fungal Genet Biol 41: 676-687
11. Ingham ER, Massicotte HB (1994) Protozoan communities around conifer roots colonized by ectomycorrhizal fungi. Mycorrhiza 5: 53-61
12. Jumpponen A, Trappe JM (1998) Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. New Phytol 140: 295-310
13. Jumpponen A (2001) Dark septate endophytes – are they mycorrhizal? Mycorrhiza 11: 207-211
14. Kerley SJ, Read DJ (1995) The biology of mycorrhiza in the Ericaceae. XVIII. Chitin degradation by *Hymenoscyphus ericae* and transfer of chitin-nitrogen to the host plant. New Phytol 131: 369-375
15. Kerley SJ, Read DJ (1997) The biology of mycorrhiza in the Ericaceae. XIX. Fungal mycelium as a nitrogen source for the ericoid mycorrhizal fungus *Hymenoscyphus ericae* and its host plant. New Phytol 136: 691-701
16. Kerley SJ, Read DJ (1998) The biology of mycorrhiza in the Ericaceae. XX. Plant and mycorrhizal necromass as nitrogenous substrates for ericoid mycorrhizal fungus *Hymenoscyphus ericae* and its host. New Phytol 139: 353-360
17. Klironomos JN, Hart MM (2001) Animal nitrogen swap for plant carbon. Nature 410: 651-652
18. Mandyam K, Jumpponen A (2005) Seeking the elusive function of the root-colonising dark septate endophytic fungi. Stud Mycol 53: 173-189
19. Ogden CG, Hedley RH (1980) An atlas of freshwater testate amoebae. Oxford University Press, Oxford. 222 pp
20. Pearson V, Read DJ (1973) The biology of mycorrhiza in the Ericaceae. I. The isolation of the endophyte and synthesis of mycorrhiza in aseptic culture. New Phytol 72: 371-379
21. Read DJ (1996) The structure and function of the ericoid mycorrhizal root. Ann Bot 77: 365-374
22. Read DJ, Perez-Moreno J (2003) Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? New Phytol 157: 475-492
23. Read DJ, Leake JR, Perez-Moreno J (2004) Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. Can J Bot 82: 1243-1263
24. Smith SE, Read DJ (1997) Mycorrhizal Symbiosis. Second Edition. Academic Press. London. 323-345 pp
25. Vargas R (1990) Avances en microbiologia de suelos: los protozoarios y su importancia en la mineralizacion del nitrogeno. Agronomía Costarricense 14(1): 121-134
26. Vohník M, Albrechtová J, Vosátka M (2005) The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro. Symbiosis 40: 87-96

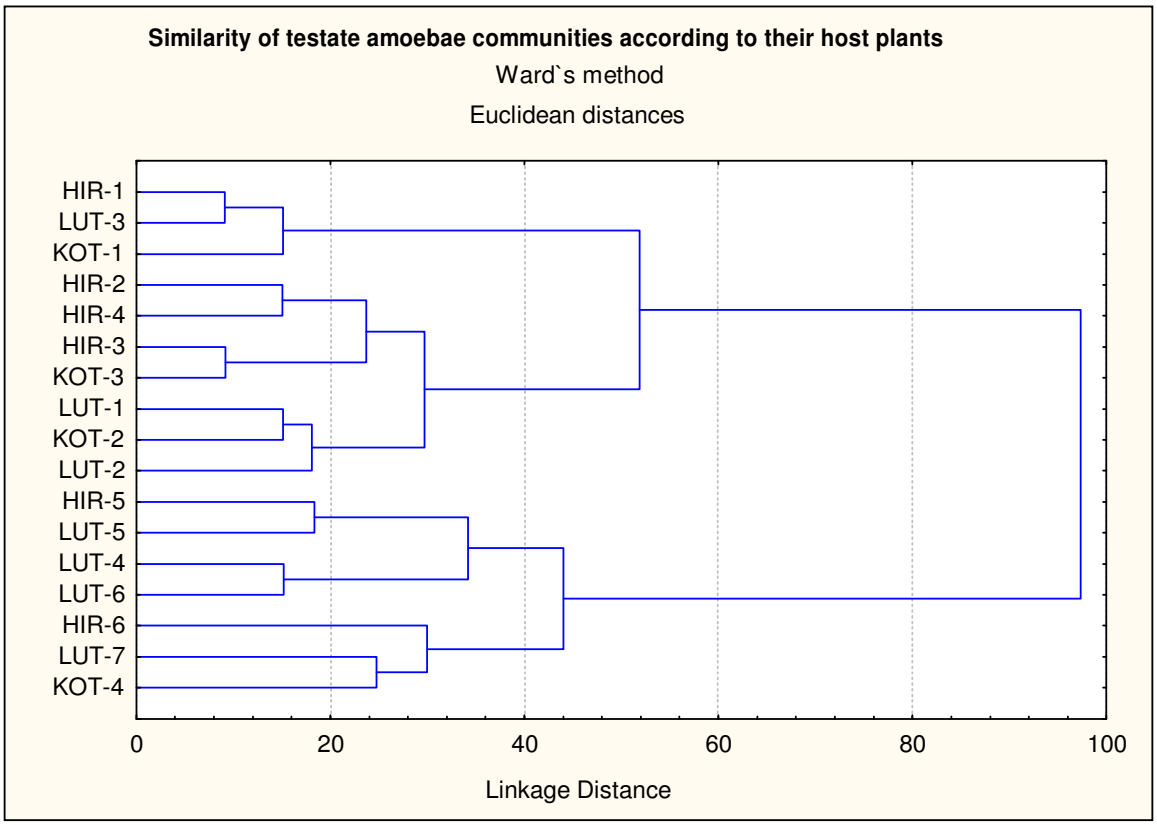
TABLE 1 Species, origin and date of collection of ericaceous host plants screened for presence of testate amoebae associated with the mycelium of putative mycorrhizal fungi. Additionally, information about the mycorrhizal status of the screened species is provided. **ErM** = ericoid mycorrhiza, **DSE** = association with Dark Septate Endophytes.

Host species	Origin	Date	Mycorrhiza
Rhododendron hirsutum	Slovenia, Velika Planina	June 2005	ErM + DSE
Rhododendron kotschyi	Romania, Carpathian Mts.	September 2005	ErM + DSE
Rhododendron luteum	Slovenia, near Boštanj	September 2005	ErM + DSE

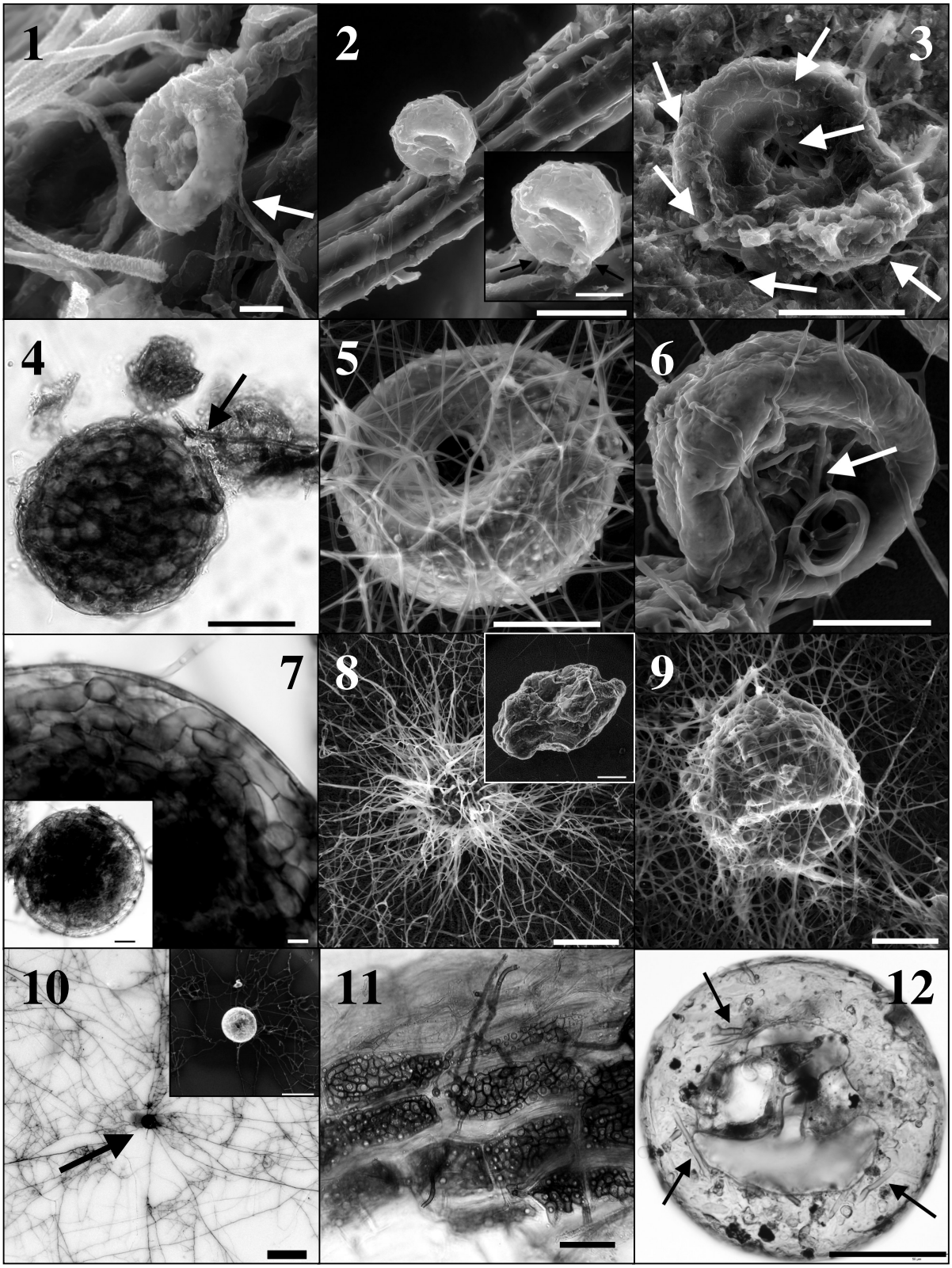
TABLE 2 Genus-level compositions of the communities of testate amoebae (TA) from the rhizoplanic material of three *Rhododendron* species. Frequency of TA in samples decreases from left to right. **HIR** = *R. hirsutum*; **LUT** = *R. luteum*; **KOT** = *R. kotschyi*; **ARC** = *Arcella*; **ASS** = *Assulina*; **CEN** = *Centropyxis*; **COR** = *Corythion*; **CYC** = *Cyclopyxis*; **DIP** = *Diplochlamys*; **EUG** = *Euglypha*; **HEL** = *Heleopera*; **NEB** = *Nebela*; **PSE** = *Pseudodifflugia*; **TRA** = *Tracheleuglypha*; **TRI** = *Trigonopyxis*; **TRN** = *Trinema*; **UDT** = undetermined. n = number of individuals screened per *Rhododendron* species.

Plant species /TA species	Frequency of TA shells (% ± SD) associated with the rhizoplane of three European *Rhododendron* species													
	DIP	CEN	CYC	EUG	TRN	ASS	COR	ARC	NEB	TRA	TRI	HEL	PSE	UDT
HIR (n=6)	21.6 ± 7.8	15.8 ± 6.4	11.6 ± 7.6	9.0 ± 3.0	7.7 ± 7.1	10.4 ± 11.0	4.7 ± 3.2	5.6 ± 3.6	1.3 ± 2.0	2.9 ± 5.5	2.0 ± 1.4	0.4 ± 1.0	1.0 ± 2.6	6.0 ± 4.9
LUT (7)	14.5 ± 7.6	12.5 ± 6.7	9.0 ± 3.1	13.4 ± 6.7	10.8 ± 7.6	6.7 ± 5.1	2.6 ± 3.3	2.4 ± 3.1	8.2 ± 4.7	10.0 ± 9.4	4.8 ± 4.4	0.5 ± 1.3	0.0	4.6 ± 4.2
KOT (4)	16.5 ± 9.4	11.4 ± 2.8	8.7 ± 7.6	10.0 ± 7.2	11.4 ± 10.8	11.0 ± 4.1	8.3%± 4.7	4.7 ± 4.9	9.0 ± 9.8	0.3 ± 0.7	2.2 ± 3.0	0.3 ± 0.6	0.0	6.2 ± 4.7

GRAPH 1 Similarity between the communities of testate amoebae from the rhizoplane of different *Rhododendron* individuals. HIR = *R. hirsutum*; **KOT** = *R. kotschyi*; **LUT** = *R. luteum*; numbers after abbreviations indicate different individuals of the same *Rhododendron* species. For details about the statistical method see Materials and Methods. For details about sampled *Rhododendron* species see Table 1. For details about the testate amoebae community composition for individual *Rhododendron* species see Table 2.



FIGURES Figure 1: A TA shell loosely associated with the mycorrhizal root of *Rhododendron* cv. Azurro via fungal hyphae emerging from the root (white arrow). SEM; bar = 10µm. **Figure 2:** A TA shell tightly associated with the mycorrhizal root of *Rhododendron* cv. Azurro via fungal hyphae. SEM; bar = 50µm. The detail shows the hyphae connecting the shell with the mycorrhizal root (black arrows). SEM; bar = 25µm. **Figure 3:** Partially decomposed TA shell, surrounded by fungal hyphae (white arrows). SEM; bar = 50µm. **Figure 4:** A TA shell from the root surface of *Rhododendron* cv. Azurro. The shell is associated with dark septate hypha (black arrow) and filled with septate multicellular object, which shows similarity to microsclerotia formed by DSE fungi either in TA shells (cf. Fig. 7) or rhizodermal cells of higher plants (cf. Fig. 11). DIC; bar = 50µm. **Figure 5:** A TA shell colonized by the mycelium of *R. ericae*, incubated on WA for 6 weeks. SEM; bar = 50µm. **Figure 6:** A TA shell colonized by the mycelium of *P. fortinii*, incubated on WA for 6 weeks. Contrary to *R. ericae*, there is only loose net of hyphae covering the shell's surface and the *P. fortinii* hyphae enter the shell's lumen via its aperture (white arrow). SEM; bar = 50µm. **Figure 7:** A detail of a TA shell intracellularly colonized by the mycelium of *P. fortinii*. The colonization pattern, i.e. dark, short and thick cells forming dense coiled hyphae, resembles microsclerotia formed by DSE fungi in the rhizodermal cells of higher plants (cf. Fig. 11). SEM; bar = 1µm. The detail shows general appearance of the colonized shell. DIC; bar = 10µm. **Figure 8:** A TA shell as a propagule carrier and a nutrient source for the *P. fortinii* mycelium. The pre-inoculated shell was cultivated on a nitrocellulose membrane placed on WA for two months. The mycelium emerges in all directions from the shell's lumen and completely disintegrates its structure and shape. SEM; bar = 100µm. The detail shows a sand grain, which served as a negative control. Nearly no mycelium developed from the pre-inoculated grain. SEM; bar = 100µm. **Figure 9:** A TA shell as a propagule carrier and a nutrient source for the *R. ericae* mycelium. The pre-inoculated shell was cultivated on a nitrocellulose membrane placed on WA for two months. The shell is completely covered by the *R. ericae* mycelium, which partly disintegrates its structure. SEM; bar = 50µm. **Figure 10:** A TA shell as a propagule carrier and a nutrient source for the *P. fortinii* mycelium. The pre-inoculated shell (black arrow) was cultivated on a nitrocellulose membrane placed on moistened, serially washed quartz sand for two months. The mycelium radiates from the shell across the membrane. Binoculars; bar = 500µm. The detail shows the same situation using SEM. Bar = 100µm. **Figure 11:** Microsclerotia formed by *P. fortinii* in the rhizodermal cells of *Vaccinium myrtillus* in an aseptic culture. DIC; bar = 25µm. **Figure 12:** A TA shell collected from the rhizosphere of *V. myrtillus* from the field. Note dark septate hyphae attached to its surface (black arrows). DIC; bar = 50µm.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3. 3. Část III: Diskuse

Hlavním podnětem vzniku Článku 6 a tedy i Části III této DP byla opakující se pozorování schránek pravděpodobně mrtvých krytenek, asociovaných s myceliem mykorhizních kořenů různých vřesovcovitých rostlin. Tyto rostliny byly zkoumány zejména v souvislosti s Článkem 1 této DP. Diskuse Článku 6 nastiňuje, jakým způsobem může "mykorhizní" metodika při zkoumání rhizosféry ovlivnit, resp. zúžit náhled pozorovatele na děje, které v ní probíhají.

Část III si kladla několik cílů (viz Úvod) a byla postavena na hlavní pracovní hypotéze: mykorhizní houby (ErM a DSE) dokáží využívat schránek mrtvých krytenek jako zdroje živin pro svůj růst. Tato hypotéza byla ve dvou in vitro experimentech potvrzena (viz výsledky a diskuse Článku 6). Zdá se tedy, že odumřelé krytenky mohou představovat kvalitativně, ale i kvantitativně zajímavý "živinový koktejl" jak pro mykorhizní houby, tak zprostředkovaně pro jejich hostitelské rostliny. Ekofyziologický význam této domněnky však stále čeká na své ozřejmení.

Dále jsem předpokládal, že existuje-li nějaký obousměrný vztah mezi mykorhizními houbami a krytenkami, měl by se projevit ve složení jejich společenstev. Nevím, do jaké míry lze předpokládat, že mykorhizní houby využívají jako zdroj živin preferenčně určitý typ krytenek, výsledky mých pozorování ale naznačují, že kolonizovány jsou především větší schránky s větším množstvím detritu, nalepeným na schránky ve formě xenozómů (např. rody *Centropyxis* a *Trigonopygis*) než schránky menší, s hladkým povrchem. Nevím také, do jaké míry lze předpokládat, že mykorhizní houby nějakým způsobem pozitivně zvýhodňují či přímo selektují určité typy krytenek v mykorhizosféře. Lze uvažovat, že mechanizmem takové selekce by mohla být schopnost některých krytenek využívat právě mycelium mykorhizních hub jako svojí potravu, jak je naznačeno např. v práci Gilbert a kol. (2003). Pokud výše uvedené hypotézy skutečně platí, je možné hypotetizovat dále: krytenky se v mykorhizosféře vřesovcovitých živí (otázkou zůstává, do jaké míry) myceliem ErM a/nebo DSE hub. Zároveň si na své schránky "lepí" mycelium těchto hub ve formě xenozómů. Často jsem pozoroval, že schránky krytenek na sobě mají přilepeny houbové hyfy; je však nutné zjistit, jsou-li takové krytenky ještě živé. Tím se ještě za svého života krytenky "preinokulují". Poté, co odumřou, jsou jejich preinokulované schránky využity jako zdroj živin mykorhizními houbami, potažmo jejich hostitelskými rostlinami. Efekt preinokulace by tak dal časový náskok mykorhizním houbám před ostatními destruenty. Optimálně zásobené mykorhizní rostliny mohou investovat dostatečné množství energie do extraradikálního mycelia, které je potravou mykofágních krytenek. Hypotetický cyklus se tím uzavírá.

Výsledky Článku 6 ukazují, že spektra krytenek, žijících v rhizoplâně tří druhů rododendronů, si byla alespoň do určité míry podobná, resp. nebyla navzájem rozdílná. Nijak nepřeceňují význam tohoto zjištění. Je nutné si uvědomit, že složení společenstev krytenek může být ovlivněno pestrou škálou faktorů. Předně jsem zjednodušeně předpokládal, že kořeny zkoumaných rododendronů jsou kolonizovány přibližně stejným spektrem mykorhizních hub. Článek I však ukazuje, že minimálně proporce ErM a DSE hub se u všech tří rododendronů liší jak mezidruhově, tak vnitrodruhově. To může vysvětlovat variabilitu společenstev krytenek v rámci

jednotlivých druhů rododendronů. Také jsem předpokládal, že chemizmus rhizoplány zkoumaných rododendronů je podobný. Ostatně, vřesovcovité rostliny obývají nápadně si podobné habitaty a totožná je i anatomie, morfologie, mykotrofie a fyziologie jejich vlasových kořenů (např. Read 1996, Cairney a Meharg 2003). Takto podobné podmínky by měly selektovat podobná spektra krytenek, vyskytujících se v rhizopláně (ale nejspíše i v mykorhizosféře) vřesovcovitých, na mykorhizní houby nehledě.

Z Článku III nicméně nepopiratelně vyplývá, že krytenky se přímo v rhizopláně vřesovcovitých rostlin vyskytují. Je z něj také zřejmé, o jaké spektrum krytenek se jedná. Obojí je kupodivu zcela originální, doposud nezkoumaná (nebo alespoň nepublikovaná) informace. Dále, houby asociované s kořeny mykorhizních vřesovcovitých rostlin mají nepopiratelně schopnost kolonizovat schránky mrtvých krytenek v přirozených podmínkách stanovišť, na kterých se vřesovcovité rostliny vyskytují. A v neposlední řadě, ErM a DSE houby mají schopnost takové schránky využívat jako zdroj živin, alespoň v in vitro podmínkách.

V rámci kontextu celé disertační práce je vhodné upozornit na způsob, jakým byly kolonizovány schránky krytenek DSE *P. fortinii* (Obr. 7, Článek 6). Hyfové struktury, které *P. fortinii* uvnitř schránek tvořila, zcela nepochybně připomínají vnitrobuněčná mikrosklerocia, tvořená DSE v kořenech vyšších rostlin (Obr. 11, Článek 6). Zdá se tedy, že tvorba mikrosklerocií DSE hubami nemusí být podmíněna nějakým aktivním impulsem ze strany "hostitele" (v tomto případě schránky mrtvého prvoka). Je otázkou, do jaké míry tato paralela platí i pro hostitele rostlinné. Doposud nebylo doloženo, že by mikrosklerocia byla směrem k rostlině fyziologicky aktivní. Je naopak nasnadě, že se jedná spíše o klidové/zásobní struktury DSE hub, kolonizujících kořen. Pak zůstává otázkou, proč jsou právě takové útvary, které již z podstaty rostlinu spíše zatěžují (minimálně inaktivují většinu obsahu kolonizované buňky), určovacím znakem DSE-asociace, která je prohlašována za mykorhizní, tedy rostlině mutualisticky prospěšnou (viz diskuse Článku 1).

Jak jsem již uvedl v úvodu této kapitoly, je problematika v ní obsažená z experimentálního hlediska velmi náročná. Pominu-li její mezioborovost, kladoucí na zúčastněné jedince vysoké nároky, zůstává stále celá řada nepříznivých faktorů. Zdá se, že vodní krytenky lze za určitých podmínek klonálně pěstovat, půdní krytenky však již při nepatrné změně prostředí encystují, jejich metabolismus je snížen na minimum. Nelze je získat v axenickém stavu a jejich molekulární taxonomie je doslova v plenkách. V České Republice existuje velmi omezené spektrum protozoologů, schopných (a ochotných) je podle schránek určit. Schránky krytenek, zejména v pokročilém stádiu rozkladu, nelze určit vůbec – často je i samotná skutečnost, že se jedná o krytenku, značně nejistá. Jejich miniaturní křehké schránky lze pouze s obtížemi pozorovat jednotlivě pomocí světelném mikroskopu, lze s nimi pouze obtížně manipulovat. Prakticky nelze získat takové množství jednotlivých krytenek, které by bylo z hlediska příjmu živin podstatné byť pro malý semenáček vřesovcovité rostliny. Skoro se zdá, že krytenky "Bůh snad proto učinil tak malé, aby se s nimi člověk vůbec nezaobíral". Nevím, platí-li tato parafráze výroku C. Linného,

doufám však, že nikoliv. Své doufání dokládám hrubými obrysy práce, kterou je záhodné vykonat pro lepší objasnění nastíněné problematiky. Dle mého názoru je třeba porovnat spektrum krytenek, vyskytujících se v stejném biotopu, avšak v rhizosféře rostlin s různou mykortrofií. Je třeba zjistit, jaké množství odumřelých krytenek je skutečně kolonizováno mykorhizními houbami a jaké množství takto získaných živin je dále transportváno do hostitelské rostliny. Je třeba ozřejmit, jaké jsou potravní preference krytenek, vyskytujících se v mykorhizosféře vřesovcovitých rostlin, a jestli mezi mykorhizními houbami a krytenkami existuje nějaká zpětná vazba, pravděpodobně založená právě na potravních preferencích. Velmi zajímavé by bylo zjištění, do jaké míry platí hypotéza o "preinokulaci" schránek, uvedená výše. Doufám, že se mi v blízké budoucnosti alespoň některé z těchto námětů podaří rozkrýt.

Shrnutí

Tato DP se zabývá vybranými interakcemi, probíhajícími v rhizosféře erikoidně mykorhizních rostlin. Zkoumané vztahy zahrnují: **(i)** interakce mezi erikoidně mykorhizními (ErM) houbami a tmavými přepážkovanými endofytickými (DSE) houbami; **(ii)** interakce mezi vřesovcovitými rostlinami a ErM houbami, ektomykorhizními (EcM) houbami a houbami s dosud nevyjasněným mykorhizním statutem; **(iii)** interakce mezi ErM a DSE houbami a půdními krytenkami. Hlavní poznatky, dosažené v rámci DP, jsou následující:

- **ErM a DSE-asociace jsou současně přítomny v kořenech všech zkoumaných rododendronů napříč evropským kontinentem, ačkoliv jejich proporce se liší zejména v závislosti na zeměpisné šířce. Kolonizace DSE houbami je negativně korelována s kolonizací ErM houbami. DSE houby tvoří v kořenech rododendronů morfologické struktury, velmi podobné strukturám tvořeným ErM houbami. To podstatně ztěžuje morfologické rozlišení ErM a DSE-asociace.**
- **V in vitro podmínkách má DSE-asociace na růst vřesovcovité rostliny neutrální až negativní vliv, zatímco ErM má vliv neutrální až pozitivní. Tento vliv je korelován s proporcemi DSE a ErM kolonizace v kořenech rostliny. ErM houby mají alespoň v in vitro podmínkách schopnost zmírňovat negativní vliv DSE hub.**
- **V ex vitro podmínkách mají ErM houby na růst a příjem živin vřesovcovitou rostlinou pozitivní vliv, který může být ovlivněn přítomností DSE houby. Efekt DSE-asociace je proměnlivý od pozitivního po neutrální až negativní, zejména v závislosti na zkoumaném izolátu DSE houby. Pozitivní vliv DSE houby nemusí být úměrný jejímu kolonizačnímu potenciálu.**
- ***Meliniomyces variabilis*, houba náležící do tzv. *Rhizoscyphus ericae* – agregátu, má schopnost tvořit struktury morfologicky shodné s ErM. Tato houba také dokáže intracelulárně kolonizovat typicky EcM hostitelské rostliny (*Picea abies*, *Pinus sylvestris*), aniž by se toto negativně odrazilo na jejich růstu. *M. variabilis* také může kolonizovat plodnice EcM houby *Hydnotrya tulasnei*, což svědčí o rozmanitosti životního stylu této houby.**
- **Vřesovcovité rostliny, vyskytující se často v podrostu typicky EcM rostlin, mohou být ovlivňovány i EcM houbami, i když tyto s nimi mykorhizu netvoří. Příkladem je *Cenococcum geophilum*, podporující růst a rozvoj kořenů u těchto rostlin. Mechanizmem je pravděpodobně tvorba rostlinných hormonů (IAA) některými kmeny EcM hub.**
- **Kořeny vřesovcovitých rostlin mohou být vnitrobuněčně kolonizovány i houbami, které nejsou běžně považovány za ErM, přičemž morfologie takové kolonizace připomíná ErM. Příkladem je půdní saprotrofní houba *Geomyces pannorum*, jejíž vliv na kolonizovanou rostlinu je v in vitro podmínkách negativní.**
- **Schránky pravděpodobně mrtvých půdních krytenek jsou pravidelně asociovány s mykorhizními kořeny vřesovcovitých rostlin. ErM a DSE houby jsou schopny tyto schránky kolonizovat a využívat jako zdroj živin pro svůj růst. Typická DSE houba *P. fortinii* tvoří v prázdných schránkách typická mikrosklerocia, tedy útvary charakteristické pro DSE-asociaci v kořenech vyšších rostlin.**

Summary

This doctoral dissertation focuses on selected interactions, which take place in the rhizosphere of ericoid mycorrhizal (ErM) plants. These include **(i)** interactions between ErM fungi and dark septate endophytic (DSE) fungi; **(ii)** interactions among ericaceous plants and ErM and EcM fungi, and fungi with yet unresolved mycorrhizal status; **(iii)** interactions among ErM and DSE fungi and soil testate amoebae. Main findings achieved in the frame of the doctoral dissertation are:

- **Ericoid mycorrhiza and DSE-association simultaneously occur in roots of all screened European *Rhododendron* species across the continent. However, their proportions differ depending mainly on latitude. DSE-colonization is negatively correlated with ErM colonization in roots of all screened rhododendrons. DSE fungi form intracellular structures, which morphologically resemble ericoid mycorrhiza.**
- **Colonization with selected DSE strains has neutral to negative influence on the growth of ericaceous plants in vitro, whereas the effect of the typical ErM fungus *Rhizoscyphus ericae* is neutral to positive. Effects of both types of fungi on the growth of host plants are correlated with the level of ErM and DSE colonization. ErM fungi can alter the negative effect of DSE fungi when present in the same root system in vitro.**
- **ErM fungus *Oidiodendron maius* increases the growth and nutrient uptake by ericaceous plants, which can be strain-specifically altered by the presence of a DSE fungus. The effect of DSE-association is variable and strain specific, ranging from positive to neutral to negative. Positive effect of the DSE fungus does not need to be connected with its colonization potential.**
- ***Meliniomyces variabilis*, a fungus with affinities to the *Rhizoscyphus ericae* – aggregate, forms intracellular structures resembling ErM in ericaceous roots. In addition, *M. variabilis* intracellularly colonizes ectomycorrhizal host plants (*Picea abies*, *Pinus sylvestris*) without causing a negative effect on their growth. *M. variabilis* can also colonize sporocarps of an EcM fungus *Hydnotrya tulasnei*, which illustrates its variable life-style.**
- **EcM fungi can indirectly affect ericaceous plants, often growing under the canopy formed by EcM plants, even without forming mycorrhizal association. For example, *Cenococcum geophilum* can support the growth and formation of roots and hence also overall growth of ericaceous plants, likely by producing phytohormones (IAA) in their rhizosphere.**
- **Roots of ericaceous plants can be intracellularly colonized by fungi, which are to date not regarded as their mykorrhizal associates. *Geomyces pannorum*, a soil-borne saprotrophic fungus, forms structures resembling ErM in roots of its ericaceous hosts, but apparently depresses their growth in vitro.**
- **Shells of probably withered soil testate amoebae are regularly associated with mykorrhizal roots of ericaceous plants. ErM and DSE fungi can colonize the shells and use them as a sole source of nutrients for their growth. Within the shells, the typical DSE fungus *P. fortinii* forms microsclerotia, which are a diagnostic character for DSE-association, formed in roots of most of higher plants.**

Citovaná literatura

Abuarghub SM, Read DJ. 1988a. The biology of mycorrhiza in the Ericaceae. XI. The distribution of nitrogen in soil of typical upland Callunetum with special reference to the "free" amino acids. *New Phytologist* 108: 425- 431.

Abuarghub, SM, Read DJ. 1988b. The biology of mycorrhiza in the Ericaceae. XII. Quantitative analysis of individual „free" amino acids in relation to time and depth in the soil profile. *New Phytologist* 108: 433-441.

Addy HD, Hambleton S, Currah RS. 2000. Distribution and molecular characterization of the root endophyte *Phialocephala fortinii* along an environmental gradient in the boreal forest of Alberta. *Mycological Research* 104: 1213-1221.

Addy HD, Piercey MM, Currah RS. 2005. Microfungal endophytes in roots. *Canadian Journal of Botany* 83: 1-13.

Ahlich K, Sieber TN. 1996. The profusion of dark septate endophytic fungi in non-ectomycorrhizal fine roots of forest trees and shrubs. *New Phytologist* 132: 259-270.

Allen TR, Millar T, Berch SM, Berbee ML. 2003. Culturing and direct DNA extraction find different fungi from the same ericoid mycorrhizal roots. *New Phytologist* 160: 255-272.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389-3402.

Bajwa R, Read DJ. 1985. The biology of mycorrhiza in the Ericaceae: IX. Peptides as nitrogen sources for mycorrhizal and non-mycorrhizal plants. *New Phytologist* 101: 459-467.

Bajwa, R., Abuarghub, S., Read, D. J. 1985. The biology of mycorrhiza in the Ericaceae. X. The utilization of proteins and the production of proteolytic enzymes by mycorrhizal plants. *New Phytologist* 101: 469-486.

Baláž M, Vosátka M. 2001. A novel inserted membrane technique for studies of mycorrhizal extraradical mycelium. *Mycorrhiza* 11: 291-296.

Barrow JR, Aaltonen RE. 2001. Evaluation of the internal colonization of *Atriplex canescens* (Pursh) Nutt. roots by dark septate fungi and the influence of host physiological activity. *Mycorrhiza* 11: 199-205.

Barrow JR. 2003. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in arid southwestern USA rangelands. *Mycorrhiza* 13: 239-247.

Berch SM, Allen TR, Berbee ML. 2002. Molecular detection, community structure and phylogeny of ericoid mycorrhizal fungi. *Plant and Soil* 244: 55-66.

Bergero R, Perotto S, Girlanda M, Vidano G, Luppi AM. 2000. Ericoid mycorrhizal fungi are common root associates of a Mediterranean ectomycorrhizal plant (*Quercus ilex*). *Molecular Ecology* 9: 1639-1649.

Bonfante-Fasolo P. 1980. Occurrence of a basidiomycete in living cells of mycorrhizal hair roots of *Calluna vulgaris*. *Transactions of British Mycological Society* 75: 320-325.

Bonkowski M, Cheng W, Griffiths BS, Alphei J, Scheu S. 2000. Microbial-faunal interactions in the rhizosphere and effects on plant growth. *European Journal of Soil Biology* 36: 135-147.

Bonkowski M, Jentschke G, Scheu S. 2001. Contrasting effects of microbial partners in the rhizosphere: interactions between Norway Spruce seedlings (*Picea abies* Karst.), mycorrhiza (*Paxillus involutus* (Batsch) Fr.) and naked amoebae (protozoa). *Applied Soil Ecology* 18: 193-204.

Bonkowski M. 2004. Protozoa and plant growth: the microbial loop in soil revisited. *New Phytologist* 162: 617-631.

Bougoure DS, Cairney JWG. 2005a. Fungi associated with hair roots of *Rhododendron lochiae* (Ericaceae) in an Australian tropical cloud forest revealed by culturing and culture-independent molecular methods. *Environmental Microbiology* 7(11): 1743-1754.

Bougoure DS, Cairney JWG. 2005b. Assemblages of ericoid mycorrhizal and other root-associated fungi from *Epacris pulchella* (Ericaceae) as determined by culturing and direct DNA extraction from roots. *Environmental Microbiology* 7(6): 819-827.

Bradley R, Burt AJ, Read DJ. 1982. The biology of mycorrhiza in the Ericaceae. VIII. The role of mycorrhizal infection in heavy metal resistance. *New Phytologist* 91: 197-209.

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N. 1996. *Working with mycorrhizas in forestry and agriculture*. ACIAR Monograph 32, Canberra. 374 pp.

Brundrett MC. 2002. Coevolution of roots and mycorrhizas of land plants. *New Phytologist* 154: 275-304.

Cairney JW. 2000. Evolution of mycorrhiza systems. *Naturwissenschaften* 87: 467-475.

Cairney JW, Ashford AE. 2002. Biology of mycorrhizal associations of epacrids (*Ericaceae*). *New Phytologist* 154: 305-326.

Cairney JWG, Meharg AA. 2003. Ericoid mycorrhiza: a partnership that expoits harsh edaphic conditions. *European Journal of Soil Science* 54: 735-740.

Cázares E, Trappe JM, Jumpponen A. 2005. Mycorrhiza-plant colonization patterns on a subalpine glacier forefront as a model system of primary succession. *Mycorrhiza* 15: 405-416.

Chaurasia B, Pandey A, Palni LMS. 2005. Distribution, colonization and diversity of arbuscular mycorrhizal fungi associated with central Himalayan rhododendrons. *Forest Ecology and Management* 207: 315-324.

Couture M, Fortin JA, Dalpé Y. 1983. *Oidiodendron griseum* Robak: An endophyte of ericoid mycorrhiza in *Vaccinium* spp. *New Phytologist* 95: 375-380.
Cudlín P. 1991. Epifluorescent microscopy for identification of ectomycorrhiza. *Methods in Microbiology* 23: 365-368.
Currah RS, Tsuneda A, Murakami S. 1993a. Conidiogenesis in *Oidiodendron periconioides* and ultrastructure of ericoid mycorrhizas formed with *Rhododendron brachycarpum. Canadian Journal of Botany* 71: 1481-1485.
Currah RS, Tsuneda A, Murakami S. 1993b. Morphology and ecology of *Phialocephala fortinii* in roots of *Rhododendron brachycarpum. Canadian Journal of Botany* 71: 1639-1644.
Dalpé Y. 1986. Axenic synthesis of ericoid mycorrhiza in *Vaccinium angustifolium* Ait. by *Oidiodendron* species. *New Phytologist* 103: 391-396.
Dalpé Y. 1989. Ericoid mycorrhizal fungi in the Myxotrichaceae and Gymnoascaceae. *New Phytologist* 113: 523-527.
Dalpé Y. 1991. Statut endomycorhizien du genre Oidiodendron. *Canadian Journal of Botany* 69: 1712-1714.
Dighton J, Coleman DC. 1992. Phosphorus relations of roots and mycorrhizas of *Rhododendron maximum* L. in the southern Appalachians, North Carolina. *Mycorrhiza* 1: 175-184.
Domsch KH, Gams W, Anderson, T. 1980. Compendium of Soil Fungi. Academic Press, New York.
Dostálková A. 1981. *Rododendrony (Rhododendrons).* Academia, Prague. 155 pp.
Douglas GC, Heslin MC, Reid C. 1989. Isolation of *Oidiodendron maius* from *Rhododendron* and ultrastructural characterization of synthesized mycorrhizas. *Canadian Journal of Botany* 67: 2206-2212.
Duclos JL, Fortin JA. 1983. Effect of glucose and active charcoal on *in vitro* synthesis of ericoid mycorrhiza with *Vaccinium* ssp. *New Phytologist* 94: 95-102.
Duddridge JA, Read DJ. 1982. Ultrastructural analysis of the development of mycorrhizas in *Rhododendron ponticum. Canadian Journal of Botany* 60: 2345-2356.
Eccher T, Noé N. 2002. Influence of ericoid endomycorrhizae inoculated in vitro on rooting and early growth of micropropagated plants of *Vaccinium corymbosum. Acta Horticulturae* 574: 373-378.
Ehrenberger, F., Gorbach, S. 1973. *Methoden der organischen Elementar- und Spurenanalyse*. Verlag Chemie, Weinheim.
Fitter AH, Garbaye J. 1994. Interactions between mycorrhizal fungi and other soil organisms. *Plant and Soil* 159: 123-132.
Francis R, Read DJ. 1994. The contribution of mycorrhizal fungi to the determination of plant community structure. *Plant and Soil* 159: 11-25.
Frank B. 1885. On the root-symbiosis-based nutrition of certain trees via underground fungi (v němčině). *Berichte der Deutschen Botanischen Gesellschaft* 3: 128-145.
Gay G. 1990. Effect of the ectomycorrhizal fungus *Hebeloma hiemale* on adventitious root formation in derooted *Pinus halepensis* shoot hypocotyls. *Canadian Journal of Botany* 68: 1265-1270.
Gianni C, Caretta G, Romano C. 2003. Skin infection due to *Geomyces pannorum* var. *pannorum*. *Mycoses* 46: 430-432.
Gilbert D, Amblard C, Bourdier G, Francez AJ. 1998. The microbial loop at the surface of a peatland: structure, function, and impact of nutrient input. *Microbial Ecology* 35: 83-93.
Gilbert D, Amblard C, Bourdier G, Francez AJ. 1998b. Short-term effect of nitrogen enrichment on the microbial communities of a peatland. *Hydrobiologia* 373/374: 111-119.
Gilbert D, Mitchell EAD, Amblard C, Bourdier G, Francez AJ. 2003. Population dynamics and food preferences of the testate amoeba *Nebela tincta major-bohemica-collaris* complex (Protozoa) in a Sphagnum peatland. *Acta Protozoologica* 42: 99-104.
Grünig CR, Sieber TN, Holdenrieder O. 2001. Characterisation of dark septate endophytic fungi (DSE) using inter-simple-sequence-repeat-anchored polymerase chain reaction (ISSR-PCR) amplification. *Mycological Research* 105: 24-32.
Grünig CR, McDonald BA, Sieber TN, Rogers SO, Holdenrieder O. 2004. Evidence for subdivision of the root-endophyte *Phialocephala fortinii* into cryptic species and recombination within species. *Fungal Genetics and Biology* 41: 676-687.
Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp* Ser 41:95-98.
Hambleton S, Currah RS. 1997. Fungal endophytes from the roots of alpine and boreal Ericaceae. *Canadian Journal Botany* 75: 1570-1581.
Hambleton S, Egger KN, Currah RS. 1998. The genus *Oidiodendron*: species delimitation and phylogenetic relationship based on nuclear ribosomal DNA analysis. *Mycologia* 90: 854-869.
Hambleton S, Sigler L. 2005. *Meliniomyces*, a new anamorph genus for root-associated fungi with phylogenetic affinities to *Rhizoscyphus ericae* (= *Hymenoscyphus ericae*), *Leotiomycetes*. *Studies in Mycology* 53: 1-27.
Hartley SE, Amos L. 1999. Competitive interactions between *Nardus stricta* L. and *Calluna vulgaris* (L.) Hull: the effect of fertilizer and defoliation on above- and below-ground performance. *Journal of Ecology* 87: 330-340.

Haselwandter K, Read DJ. 1980. Fungal associations of roots of dominant and sub-dominant plants in high-alpine vegetation systems with special reference to mycorrhiza. *Oecologia* 45: 57-62.
Hashimoto Y, Hyakumachi, M. 2001. Effects of isolates of ectomycorrhizal fungi and endophytic *Mycelium radicis atrovirens* that were dominant in soil from disturbed sites on growth of *Betula platyphylla* var. *japonica* seedlings. *Ecological Research* 16: 117-125.
Horton TR, Cázares E, Bruns TD. 1998. Ectomycorrhizal, vesicular-arbuscular and dark septate fungal colonization of bishop pine (*Pinus muricata*) seedlings in the first 5 months of growth after wildfire. *Mycorrhiza* 8: 11-18.
Hutton BJ, Dixon KW, Sivasithamparam K. 1994. Ericoid endophytes of Western Australian heaths (Epacridaceae). *New Phytologist* 127: 557-566.
Ingham ER, Massicotte HB. 1994. Protozoan communities around conifer roots colonized by ectomycorrhizal fungi. *Mycorrhiza* 5: 53-61.
Jansa J, Vosátka M. 2000. In vitro and post vitro inoculation of micropropagated Rhododendrons with ericoid mycorrhizal fungi. *Applied Soil Ecology* 15: 125-136.
Johansson M. 2000. The influence of ammonium nitrate on the root growth and ericoid mycorrhizal colonization of *Calluna vulgaris* from Danish heathlands. *Oecologia* 123: 418-424.
Johansson M. 2001. Fungal associations of Danish *Calluna vulgaris* roots with special reference to ericoid mycorrhiza. *Plant and Soil* 231: 225-232.
Johnson NC, Graham JH, Smith FA. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytologist* 135: 575-585.
Jones MD, Smith SE. 2004. Exploring functional definitions of mycorrhizas – are mycorrhizas always mutualisms? *Canadian Journal of Botany* 82: 1089-1109.
Jumpponen A, Trappe JM. 1998(a). Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. *New Phytologist* 140: 295-310.
Jumpponen A, Trappe JM. 1998(b). Performance of *Pinus concorta* inoculated with two strains of root endophytic fungus, *Phialocephala fortinii*: effects of synthesis system and glucose concentration. *Canadian Journal of Botany* 76: 1205-1213.
Jumpponen A, Mattson KG, Trappe JM. 1998. Mycorrhizal functioning of *Phialocephala fortinii* with *Pinus contorta* on glacier forefront soil: interactions with soil nitrogen and organic matter. *Mycorrhiza* 7: 261-265.
Jumpponen A. 2001. Dark septate endophytes – are they mycorrhizal? Mycorrhiza 11: 207-211.
Kaldorf M, Renker C, Fladung M, Buscot F. 2004. Characterisation and spatial distribution of ectomycorrhizas colonizing aspen clones released in an experimental field. *Mycorrhiza* 14: 295-306.
Kerley SJ, Read DJ. 1995. The biology of mycorrhiza in the Ericaceae. XVIII. Chitin degradation by Hymenoscyphus ericae and transfer of chitin-nitrogen to the host plant. New Phytologist 131: 369-375.
Kerley SJ, Read DJ. 1997. The biology of mycorrhiza in the Ericaceae. XIX. Fungal mycelium as a nitrogen source for the ericoid mycorrhizal fungus *Hymenoscyphus ericae* and its host plant. *New Phytologist* 136: 691-701.
Kerley SJ, Read DJ. 1998. The biology of mycorrhiza in the Ericaceae. XX. Plant and mycorrhizal necromass as nitrogenous substrates for ericoid mycorrhizal fungus *Hymenoscyphus ericae* and its host. *New Phytologist* 139: 353-360.
Klironomos JN, Hart MM. 2001. Animal nitrogen swap for plant carbon. *Nature* 410: 651-652.
Kolařík M, Kubátová A, Pažoutová S, Šrůtka P. 2004. Morphological and molecular characterization of *Geosmithia putterillii*, *G. pallida* comb. nov. and *G. flava* sp. nov., associated with subcorticolous insects. *Mycological Research* 108: 1053-1069.
Lacourt I, Girlanda M, Perotto S, Del Pero M, Zuccon D, Luppi AM. 2001. Nuclear ribosomal sequence analysis of *Oidiodendron*: towards a redefinition of ecologically relevant species. *New Phytologist* 149: 565-576.
Leake JR, Read DJ. 1989. The biology of mycorrhiza in the Ericaceae: XIII. Some characteristics of the extracellular proteinase activity of the ericoid endophyte *Hymenoscyphus ericae. New Phytologist* 112: 69-76.
Leake JR, Read DJ. 1989b. The biology of mycorrhiza in the Ericaceae. XV. The effect of mycorrhizal infection on calcium uptake by *Calluna vulgaris* (L.) Hull. *New Phytologist* 113: 535-544.
Leake JR, Shaw G, Read DJ. 1990. The biology of mycorrhiza in the Ericaceae. XVI. Mycorrhiza and iron uptake in *Calluna vulgaris* (L.) Hull in the presence of two calcium salts. *New Phytologist* 114: 651-657.
Mandyam K, Jumpponen A. 2005. Seeking the elusive function of the root-colonising dark septate endophytic fungi. *Studies in Mycology* 53: 173-189.
Martino E, Turnau K, Girlanda M, Bonfante P, Perotto S. 2000. Ericoid mycorrhizal fungi from heavy metal polluted soils: their identification and growth in the presence of zinc ions. *Mycological Research* 104: 338-344.
McLean, C. B., Lawrie, A. C. 1996. Patterns of root colonization in epacridaceous plants collected from different sites. *Annals of Botany* **77**: 405-411.

Mejías JA, Arroyo J, Ojeda F. 2002. Reproductive ecology of *Rhododendron ponticum* (Ericaceae) in relict Mediterranean populations. *Botanical Journal of the Linnean Society* 140: 297-311.
Melin E. 1922. On the mycorrhizas of *Pinus sylvestris* L. and *Picea abies* Karst. *Journal of Ecology* 9: 254-257.
Midgley DJ, Chambers SM, Cairney WG. 2004. Distribution of ericoid mycorrhizal endophytes and root-associated fungi in neighbouring Ericaceae plants in the field. *Plant and Soil* 259: 137-151.
Mitchell DT, Read DJ. 1981. Utilization of inorganic and organic phosphates by the mycorrhizal endophytes of *Vaccinium macrocarpon* and *Rhododendron ponticum*. *Transactions of the British Mycological Society* 76: 255-260.
Montecchi A, Sarafini M. 2000. Funghi ipogei d´ Europa. Associazione Micologica Bresadola, Trento, 714 pp.
Moore-Pankhurst S, Englander L. 1981. A method for the synthesis of mycorrhizal association between *Pezizella ericae* and *Rhododendron maximum* seedlings growing in a defined medium. *Mycologia* 73: 994-997.
Moyersoen B, Becker P, Alexander IJ. 2001. Are ectomycorrhizas more abundant than arbuscular mycorrhizas in tropical heath forests? *New Phytologist* 150: 591-599.
Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.
Newsham KK. 1999. *Phialophora graminicola*, a dark septate fungus, is a beneficial associate of the grass *Vulpia ciliata* ssp. *ambigua*. *New Phytologist* 144: 517-524.
Newton AC, Haigh JM. 1998. Diversity of ectomycorrhizal fungi in Britain: a test of the species-area relationship, and the role of host specificity. *New Phytologist* 138: 619-627.
Niemi K, Häggman H. 2002. *Pisolithus tinctorius* promotes germination and forms mycorrhizal structures in Scots pine somatic embryos in vitro. *Mycorrhiza* 12: 263-267.
Niemi K, Vuorinen T, Ernstsen A, Häggman H. 2002. Ectomycorrhizal fungi and exogenous auxins influence root and mycorrhiza formation of Scots pine hypocotyl cuttings in vitro. *Tree Physiology* 22: 1231-1239.
Nylund JE. 1988. The regulation of ectomycorrhiza formation – carbohydrate and hormone theories revised. *Scandinavian Journal of Forestry Research* 3: 465-470.
Ogden CG, Hedley RH. 1980. An atlas of freshwater testate amoebae. Oxford University Press, Oxford. 222 pp.
Ohki T, Masuya H, Yonezawa M, Usuki F, Narisawa K, Hashiba T. 2002. Colonization process of the root endophytic fungus *Heteroconium chaetospira* in roots of Chinese cabbage. *Mycoscience* 43: 191-194.
Pearson V, Read DJ. 1973. The biology of mycorrhiza in the *Ericaceae*. I. The isolation of the endophyte and synthesis of mycorrhiza in aseptic culture. *New Phytologist* 72: 371-379.
Pearson V, Read DJ. 1973b. The biology of mycorrhiza in the Ericaceae. II. The transport of carbon and phosphorus by the endophyte and the mycorrhiza. *New Phytologist* 72: 1325-1331.
Perotto S, Girlanda M, Martino E. 2002. Ericoid mycorrhizal fungi: some new perspectives on old acquaintances. *Plant and Soil* 244: 41-53.
Peterson RL, Massicotte, HB, Melville, LH. 2004. *Mycorrhizas: Anatomy and Cell Biology*. NRC Research Press, Ottawa.173 pp.
Piercey MM, Thormann MN, Currah RS. 2002. Saprobic characteristics of three fungal taxa from ericalean roots and their association with the roots of *Rhododendron groenlandicum* and *Picea mariana* in culture. *Mycorrhiza* 12: 175-180.
Rains KC, Nadkarni NM, Bledsoe CS. 2003. Epiphytic and terrestrial mycorrhizas in a lower montane Costa Rican cloud forest. *Mycorrhiza* 13: 257-264.
Read DJ, Haselwandter K. 1981. Observations on the mycorrhizal status of some alpine plant communities. *New Phytologist* 88: 341-352.
Read DJ. 1996. The structure and function of the ericoid mycorrhizal root. *Annals of Botany* 77: 365-374.
Read DJ. 1998. Plants on the web. *Nature* 396: 22.
Read DJ, Perez-Moreno J. 2003. Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytologist* 157: 475-492.
Read DJ, Leake JR, Perez-Moreno J. 2004. Mycorrhizal fungi as drivers of ecosystem processes in heathland and boreal forest biomes. *Canadian Journal of Botany* 82: 1243-1263.
Redecker D, Kodner R, Graham LE. 2000. Glomalean fungi from the Ordovician. *Science* 289: 1920-1921.
Redecker D. 2002. Molecular identification and phylogeny of arbuscular mycorrhizal fungi. *Plant and Soil* 244: 67-73.
Rice AV, Currah RS. 2001. Physiological and morphological variation in *Oidiodendron maius*. *Mycotaxon* 79: 383-396.
Richard C, Fortin J-A, Fortin A. 1971. Protective effect of an ectomycorrhizal fungus against the root pathogen *Mycelium radicis atrovirens*. *Canadian Journal of Forest Research* 1: 246-251.
Richard C, Fortin J-A. 1975. Protective role of *Suillus granulatus* against *Mycelium radicis atrovirens* on seedlings of *Pinus retinosa*. *Canadian Journal of Forest Research* 5: 452-456.

Rudawska ML, Kieliszewska-Rokicka B. 1997. Mycorrhizal formation by *Paxillus involutus* strains in relation to their IAA-synthesizing activity. *New Phytologist* 137: 509-517.
Ruotsalainen AL, Väre H, Vestberg M. 2002. Seasonality of root fungal colonization in low-alpine herbs. *Mycorrhiza* 12: 29-36.
Ruotsalainen AL, Kytöviita MM. 2004. Mycorrhiza does not alter low temperature impact on *Gnaphalium norvegicum*. *Oecologia* 140: 226-233.
Schadt CW, Mullen RB, Schmidt SK. 2001. Isolation and phylogenetic identification of a dark-septate fungus associated with the alpine plant *Ranunculus adoneus*. *New Phytologist* 150: 747-755.
Schild DE, Kennedy A, Stuart MR. 1988. Isolation of symbiont and associated fungi from ectomycorrhizas of Sitka Spruce. *European Journal of Forest Pathology* 18: 51-61.
Setaro S, Weiß M, Oberwinkler F, Kottke I. 2005. Sebacinales form ectendomycorrhizas with *Cavendishia nobilis*, a member of the Andean clade of Ericaceae, in the mountain rain forest of southern Ecuador. *New Phytologist* 169: 355-365.
Seviour RJ, Willing RR, Chilvers GA. 1973. Basidiocarps associated with ericoid mycorrhizas. *New Phytol*ogist 72: 381-385.
Shaw G, Read DJ. 1989. The biology of mycorrhiza in the Ericaceae. XIV. Effects of iron and aluminium on the activity of acid phosphatase in the ericoid endophyte *Hymenoscyphus ericae* (Read) Korf and Kernan. *New Phytologist* 113: 529-533.
Shaw G, Leake JR, Baker AJM, Read DJ. 1990. The biology of mycorrhiza in the Ericaceae. XVII. The role of mycorrhizal infection in the regulation of iron uptake by ericaceous plants. *New Phytologist* 115: 251-258.
Simard SW, Perry DA, Jones MD, Myrold DD, Durall DM, Molina R. 1997. Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* 388: 579-582.
Smith, SE, Smith FA. 1990. Tansley Review No. 20: Structure and function of the interfaces in biotrophic symbioses as they relate to nutrient transport. *New Phytologist* 114: 1-38.
Smith JE, Molina R, Perry DA. 1995. Occurrence of ectomycorrhizas on ericaceous and coniferous seedlings grown in soils from the Oregon Coast Range. *New Phytologist* 129: 73-81.
Smith SE, Read DJ. 1997. *Mycorrhizal Symbiosis*. Second Edition. Academic Press. London. 605 pp.
Sokolovski SG, Meharg AA, Maathuis FJM. 2002. *Calluna vulgaris* root cells show increased capacity for amino acid uptake when colonized with the mycorrhizal fungus *Hymenoscyphus ericae*. *New Phytologist* 155: 525-530.
Stoyke G, Currah RS. 1991. Endophytic fungi from the mycorrhizae of alpine ericoid plants. *Canadian Journal of Botany* 69: 347-352.
Stoyke G, Egger KN, Currah RS. 1992. Characterization of sterile endophytic fungi from the mycorrhizae of subalpine plants. *Canadian Journal of Botany* 70: 2009-2016.
Stoyke G, Currah RS. 1993. Resynthesis in pure culture of a common subalpine fungus-root association using *Phialocephala fotinii* and *Menziesia ferruginea* (Ericaceae). *Arctic and Alpine Research* 25: 189-193.
Stribley DP, Read DJ. 1974. The biology of mycorrhiza in the Ericaceae. III. Movement of carbon-14 from host to fungus. *New Phytologist* 73: 731-741.
Stribley DP, Read DJ. 1974b. The biology of mycorrhiza in the Ericaceae. IV. The effect of mycorrhizal infection on uptake of 15N from labelled soil by *Vaccinium macrocarpon* Ait. *New Phytologist* 73: 1149-1155.
Stribley DP, Read DJ, Hunt R. 1975. The biology of mycorrhiza in the Ericaceae. V. The effect of mycorrhizal infection, soil type and partial soil-sterilization (by gamma-irradiation) on growth of cranberry (*Vaccinium macrocarpon* Ait.). *New Phytologist* 75: 119-130.
Stribley DP, Read DJ. 1976. The biology of mycorrhiza in the Ericaceae. VI. The effect of mycorrhizal infection and concentration of ammonium nitrogen on growth of cranberry (*Vaccinium macrocarpon* Ait.) in sand culture. *New Phytologist* 77: 63-72.
Stribley DP, Read DJ. 1980. The biology of mycorrhiza in the Ericaceae. VII. The relationship between mycorrhizal infection and the capacity to utilize simple and complex organic nitrogen sources. *New Phytologist* 86: 365-371.
Summerbell RC. 1987. The inhibitory effect of *Trichoderma* species and other soil microfungi on formation of mycorrhiza by *Laccaria bicolor* in vitro. *New Phytologist* 105: 437-448.
Summerbell RC. 1989. Microfungi associated with the mycorrhizal mantle and adjacent microhabitats within the rhizosphere of black spruce. *Canadian Journal of Botany* 67: 1085-1095.
Summerbell RC. 2005. Root endophyte and mycorrhizosphere fungi of black spruce, *Picea mariana*, in a boreal forest habitat: influence of site factors on fungal distributions. *Studies in Mycology* 53: 121-145.
Summerbell RC. 2005b. From Lamarckian fertilizers to fungal castles: recapturing the pre-1985 literature on endophytic and saprotrophic fungi associated with ectomycorrhizal root systems. *Studies in Mycology* 53: 191-256.
Tedersoo L, Hansen K, Perry BA, Kjøller R. 2006. Molecular and morphological diversity of pezizalean ectomycorrhiza. *New Phytologist* 170: 581-596.

Urcelay C. 2002. Co-occurrence of three fungal root symbionts in *Gaultheria poeppiggi* DC in Central Argentina. *Mycorrhiza* 12: 89-92.

Usuki F, Junichi AP, Kakishima M. 2003. Diversity of ericoid mycorrhizal fungi isolated from hair roots of *Rhododendron obtusum* var. *kaempferi* in a Japanese red pine forest. *Mycoscience* 44: 97-102.

Usuki F, Narisawa K. 2005. Formation of structures resembling ericoid mycorrhizas by the root endophytic fungus *Heteroconium chaetospira* within roots of *Rhododendron obsutum* var. *kaempferi*. *Mycorrhiza* 15: 61-64.

van der Heijden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR. 1998. Mycorrhizal fungal diversity determines plant biodiversity, ecosystem variability and productivity. *Nature* 396: 69.

Vargas R. 1990. Avances en microbiologia de suelos: los protozoarios y su importancia en la mineralizacion del nitrogeno. *Agronomía Costarricense* 14: 121-134.

Villarreal-Ruiz L, Anderson IC, Alexander IJ. 2004. Interaction between an isolate from *Hymenoscyphus ericae* aggregate and roots of *Pinus* and *Vaccinium*. *New Phytologist* 164: 183-192.

Vohník M, Lukančič S, Bahor E, Regvar M, Vosátka M, Vodnik D. 2003. Inoculation of *Rhododendron* cv. Belle-Heller with two strains of *Phialocephala fortinii* in two different substrates. *Folia Geobotanica* 38: 191-200.

Vohník M, Albrechtová J, Vosátka M. 2005. The inoculation with *Oidiodendron maius* and *Phialocephala fortinii* alters phosphorus and nitrogen uptake, foliar C:N ratio and root biomass distribution in *Rhododendron* cv. Azurro. *Symbiosis* 40: 87-96.

Vrålstad T, Fossheim T, Schumacher T. 2000. *Piceirhiza bicolorata* – the ectomycorrhizal expression of the *Hymenoscyphus ericae* aggregate? *New Phytologist* 145: 549-563.

Vrålstad T, Schumacher T, Taylor FS. 2002. Mycorrhizal synthesis between fungal strains of the *Hymenoscyphus ericae* aggregate and potential ectomycorrhizal and ericoid hosts. *New Phytologist* 153: 143-152.

Vrålstad T, Myhre E, Schumacher T. 2002(b). Molecular diversity and phylogenetic affinities of symbiotic root-associated ascomycetes of the Helotiales in burnt and metal polluted habitats. *New Phytologist* 155:131-148.

Vrålstad T. 2004. Are ericoid and ectomycorrhizal fungi part of a common guild? *New Phytologist* 164:7-10.

Walker JF, Miller OK, Lei T, Semones S, Nilsen E, Clinton BD. 1999. Suppression of ectomycorrhizae on canopy tree seedlings in *Rhododendron maximum* L. (Ericaceae) thickets in the southern Appalachians. *Mycorrhiza* 9: 49-56.

Weiß M, Selosse M-A, Rexer K-H, Urban A, Oberwinkler F. 2004. *Sebacinales*: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycological Research* 108: 1003-1010.

Wurzburger N, Bledsoe CS. 2001. Comparison of ericoid and ectomycorrhizal colonization and ectomycorrhizal morphotypes in mixed conifer and pygmy forests on the northern California coast. *Canadian Journal of Botany* 79: 1202-1210.

Xiao G, Berch SM. 1995. The ability of known ericoid mycorrhizal fungi to form mycorrhizae with *Gaultheria shallon*. *Mycologia* 87: 467-470.

Yu T, Nassuth A, Peterson RL. 2001. Characterization of the interaction between the dark septate fungus *Phialocephala fortinii* and *Asparagus officinalis* roots. *Canadian Journal of Microbiology* 47: 741-753.