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The role of DSE (Dark Septate Endophytes) in plant communities in forest ecosystem

Role DSE (Dark Septate Endophytes) v rostlinném společenstvu lesního ekosystému

MASTER THESIS

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Tereza Lukešová

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Abbreviations

AM – arbuscular mycorrhiza

AMF – arbuscular mycorrhizal fungi

CMN – common mycorrhizal network

EcM - ectomycorrhiza

EcMF – ectomycorrhizal

EeM - ectendomycorrhiza

EeMF – ectendomycorrhizal fungi

ErM – ericoid mycorrhiza

ErMF – ericoid mycorrhizal fungi

DSE – dark septate endophytes

OrM – ochideoid mycorrhiza

OrMF – orchideoid mycorrhiza

PAC - *Phialocephala fortinii – Acephala applanata* species complex

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Abstrakt

Všechny rostlinné druhy žijí v symbióze s endofytními houbami – mohou s nimi vytvářet mutualistické, komenzální či parazitické symbiózy. Symbióza skupiny kořenových endofytů nazývaných podle jejich tmavých přehrádkovaných hyf Dark Septate Endophytes (DSE) a rostlin je na rozdíl od mykorhizní symbiózy často opomíjená, i když její role pro rostlinná společenstva může být velmi významná. Navzdory tomu, že se DSE vyskytují ve všech terestrických ekosystémech a byli izolováni i z vodních ekosystémů, je jejich význam pro hostitelské rostliny stále nejasný. Výsledky dosud publikovaných studií jsou nejednoznačné – některé připisují DSE pozitivní vliv na růst hostitelské rostliny a jiné negativní. Důvodem je hlavně komplikovaná taxonomie a obtížné určení jednotlivých kryptických druhů, které jsou morfologicky nerozlišitelné. Díky zahraniční spolupráci se nám podařilo zkompletovat ucelenou sbírku DSE, především z komplexu kryptických druhů *Phialocephala fortinii* s.l. – *Acephala applanata* (PAC), která zahrnuje nejběžnější druhy vyskytující se v lesních společenstvech.

Cílem této diplomové práce bylo popsat chování jednotlivých druhů DSE v kořenech rostlin typických pro lesní ekosystémy střední Evropy a objasnit jejich fyziologický vliv na hostitele. Pro určení kolonizačního potenciálu zkoumaných DSE jsme využili *in vitro* resyntézní pokusy a pro sledování toku látek mezi houbou a rostlinou značení radioaktivními i neradioaktivními izotopy.

Všechny použité izoláty DSE kolonizovaly kořeny všech tří hostitelských rostlin (*Betula pendula*, *Picea abies* a *Vaccinium myrtillus*) v *in vitro* podmínkách. Žádný z námi použitých izolátů kryptických druhů nevytvářel mykorhizní struktury a jejich vliv na růst hostitelských rostlin byl velmi podobný. Blízký příbuzný kryptických druhů PAC, endofyt *Acephala macrosclerotiorum* byl schopen tvořit ektomykorrhizní struktury u *P. abies*. Ačkoliv byl endofyt *A. macrosclerotiorum* schopen chovat se jako slabý parazit, měl pozitivní vliv na biomasu *V. myrtillus* a *B. bendula* a u *P. abies* se nám podařilo pozorovat tok ^{33}P z endofyta do rostliny.

Ačkoliv jsou kryptické druhy PAC často izolovány z mykorhizních kořenů, naše výsledky ukazují, že nevytvářejí v kořenech *V. myrtillus* a *P. abies* mykorhizní struktury. Nechovají se tedy jako mykorhizní houby, ale pouze se společně s nimi vyskytují v kořenech. Význam této koexistence je stále nedostatečně prozkoumán. Podle výsledků dosažených v mé práci důvodem pro

variabilitu pozorovaných životních strategií DSE není rozdílnost v chování kryptických druhů – všechny měly negativní vliv na biomasu hostitelské rostliny. Ne všechny druhy DSE se ale chovaly jako slabí parazité, druh *A. macrosclerotiorum* vytvářel mykorhizní struktury a měl pozitivní vliv na růst rostlin. Čím byl tento prospěšný vliv DSE způsoben, zůstává nejasné, ale naše výsledky napovídají, že endofyt může mít přímý vliv na transport živin do rostliny.

Abstract

All plants live in symbiosis with fungal endophytes – they can form mutualistic, commensal or parasitic symbioses. Symbiosis of root endophytes, called for their darkly pigmented and septated hyphae *dark septate endophytes* (DSE), and plants has been often overlooked although its role for plant communities can be very important. Despite their ubiquitous presence in roots of terrestrial and also aquatic plants the influence of DSE on their host plants is still unresolved. Results of previous studies are inconsistent - some reported that DSE have positive effects on their host plant growth and some negative. The main reason for this inconsistency might be their complicated taxonomy and difficult identification of different cryptic species which are morphologically indistinguishable. We were able to complete a unique collection of the most common DSE species, mainly members of the *Phialocephala fortinii – Acephala applanata* species complex. The collection includes the most common DSE species isolated from roots of forest plant communities.

The goal of my thesis was to describe behaviour of DSE in roots of typical forest plants and elucidate their physiological influence on host plants. *In vitro* resynthesis experiments were used to observe root colonization patterns. Nutrient flow between the plant and the fungus was traced by radioactive and non-radioactive isotopes.

All tested isolates colonized roots of all three host plants (*Betula pendula*, *Picea abies* and *Vaccinium myrtillus*) in *in vitro* conditions. No mycorrhizal structures were formed by any of isolates of the *Phialocephala fortinii* s. l. cryptic species and their influence on growth of their hosts was very similar. A close relative of the cryptic species, *Acephala macrosclerotiorum*, was able to form ectomycorrhizal structures in roots of *P. abies*. Although the endophyte *A. macrosclerotiorum* acted as a weak parasite in *P. abies*, it had positive influence on biomass of *V. myrtillus* and *B. pendula* and ^{33}P flow was detected from the fungus to the host plant.

Although *P. fortinii* s. l. cryptic species have been often isolated from mycorrhizal roots our result show that they do not form mycorrhizal structures in roots of *P. abies* and *V. myrtillus.* They thus do not behave as mycorrhizal fungi but they live together with mycorrhizal fungi as their co-associates – the importance of this

coexistence is still unresolved. According to the findings of my work the reason for high variability of life strategies of DSE observed so far is not the difference in behaviour of the cryptic species. All species used in our experiments had negative influence on host plant biomass. Not all of the DSE species were acting as weak parasites – endophyte *A. macrosclerotiorum* formed mycorrhizal structures and had positive influence on host plant growth. What are the mechanisms of the positive influence of DSE remains unclear but our results suggest that endophytes can have direct influence on nutrient transport to the host plant.

Key words:

Plant – fungal assotiations, fungal root endophytes, dark septate endophytes, *Vaccinium myrtillus*, *Picea abies*, *Acephala macrosclerotiorum*

1. Introduction

Fungal root endophytes are colonizers of most plant species in the world, but despite their ubiquitous occurrence, their ecophysiological role for hosts is very unclear. One of the most common groups of root enophyted forms dark and septated hyphae and is, thus, called the Dark Septate Endophytes (DSE). They are commonly isolated from ectomycorrhizal (EcM) (Vohník *et al.*, 2013) and ericoid mycorrhizal (ErM) roots (Vohník & Albrechtová, 2011). My thesis is mainly focused on two forest species – *Picea abies* L. Karst as an EcM plant and *Vaccinium myrtillus* L. as ErM forming plant and their interactions with DSE. And *Betula pendula* L. was choosen as a common pioneering tree species in *P. abies* forests.

Thanks to international cooperation a unique collection of DSE species was colleceted in the Institute of Botany where my thesis was worked out, the isolates were identified and were available for the use in the studies performed under my Thesis project. Isolate of *A. macrosclerotium* was kindly provided by Babette Münzenberger Institute of Landscape Matter Dynamics, Leibniz-Centre for Agricultural Landscape Research (ZALF), Germany. Isolates of *Phialocephala fortinii* s.s. and *Phialocephala turiciensis* were identified by Christoph R. Grünig Institute of Integrative Biology (IBZ), Forest Pathology and Dendrology, ETH Zurich, Switzerland.

The main goal of my Master Thesis was to contribute and help understanding of this very important component of soil biota for temperate forest plant communities by testing the following hypotheses:

Hypothesis tested

1. The life strategy of DSE is not uniform; they can form parasitic, comensalic and mutualistic symbioses.
2. DSE do influence nutrient uptake of *P. abies, B. pendula* and *V. myrtillus.*

Aims of the present study

Aims of my thesis were to answer following questions:

1. How do selected members of DSE influence host plants typical for temperate forest ecosystem?

1.1 Are the selected DSE species able to colonize all three forest plant species (*P. abies, B. pendula* and *V. myrtillus)*?

1.2 What are colonization patterns of selected DSE in roots of *P. abies, B. pendula* and *V. myrtillus*?

1.3 How do different DSE influence growth of *P. abies, B. pendula* and *V. myrtillus*?

2. Is there a bidirectional flow of nutrients between selected DSE and host plants typical for temperate forest ecosystem?

2.1 Do selected DSE species enhance biomass of *P. abies, B. pendula* and *V. myrtillus?*

2.2 How do selected DSE species influence nutrient uptake of *P. abies?*

2. Literature review

2.1 (Hi)story of plant-fungus symbioses

Plant and fungi have coexisted for a very long time. Endophytic fungi may have been crucial for colonization of the land by plants (Pirozynski & Malloch, 1975) either as lichen-forming fungi in lichens or as root/rhizoid colonizing fungi. In lichens, the alga is protected by stroma of the fungal partner and is thus able to endure wide range of unfavourable conditions (drought, cold, heat, rocky substrates, intensive light and other). This feature may have given lichen organisms the ability to survive harsh conditions that may have prevailed on land during its colonization (Selosse & Le Tacon, 1998).

The most common plant-fungus symbiosis – arbuscular mycorrhiza (AM) – is a partnership between fungi belonging to the Glomeromycota and vascular plants. Structures strongly resembling typical AM structures, arbuscules, were observed already in Early Devonian fossils of the land plant *Aglaophyton major* (Kidston & Lang) D.S. Edwards (Remy *et al.*, 1994). Moreover, according to a phylogenetic analysis, AM-like fungi originated around 600 million years ago (Redecker *et al.*, 2000) what supports a hypothesis that fungi colonizing plant organs may have played a key role in colonizing land by plants. A recent study presented data that Mucoromycotina associating with liverworts, hornworts and ferns may predate Glomeromycota and thus be better candidates for facilitating terrestrialization during the Mid-Ordovician (475 million years ago)(Bidartondo *et al.*, 2011). The “mycorrhizal landing” hypothesis is additionally supported by another recent study showing that three genes required for mycorrhiza formation were present in a common ancestor of land plants and that they were vertically inherited during land plant evolution (Wang *et al.*, 2010).

AM is an ancestral type of mycorrhiza - the ability to form mutualistic symbiosis with glomeromycetous fungi has been inherited since the establishment of the symbiosis in the common ancestor of all land plants (Wang & Qiu, 2006). All recent plant species that do not form AM either lost the ablity to do so or started to form other mycorrhial symbiosis. The most common changes are to the non-mycorhizal status, to dual mycorrhiza (Ectomycorrhiza (EcM) and AM) or to EcM (Brundrett, 2002). The results of a Bayesian relaxed molecular clock analysis

performed with fungi, plants, and other eukaryotes show that the EcM symbiosis has evolved independently in eight clades of Agaricomycetes in associations with gymnosperms and at least in 6 clades of gymnosperms (Hibbett & Matheny, 2009).

Not only fungi living in mutualistic symbioses with early land plants were described up to date but also fungal endophytes. In fossils from Rhynie chert – an Early Devonian hot springs paleoecosystem, three fungal endophytes were observed in rhizoids of *Nothia aphylla* Lyon ex El-Saadawy & Lacy. One of them is thought to be a parasite but the ecophysiological role of the others is unclear. They do not form arbuscules or any other mycorrhizal structures but the plant response (such as secondary thickening of cell walls) suggests that the endophytes were colonizing living tissues (Krings *et al.*, 2007). Endophytes were also reported from permineralized calamite roots collected in Upper Pennsylvanian Grand-Croix cherts of France, where they heavily colonized cells of the outer cortex (Taylor *et al.*, 2012). Though, their ecophysiological role remains unclear.

2.2 Endophytes – life inside plants

The word *endophyte* is formed from two Greek words. The prefix *endo*- originates from Greek *endon* meaning *within*, *inside* or *internal* (literally *in the house*) and *phyton* referring to plant or a plant characteristic. It can be thus used to describe any organism inhabiting plant tissues – for example bacteria, fungi, algae and also insects (Schulz & Boyle, 2005).

Fungal endophytes can be defined as “organisms inhabiting plant organs that at some time in their life can colonize internal plant tissues without causing apparent harm to their host” (Petrini, 1991). Such definition identifies as endophytes fungi performing different life strategies such as mutualistic, parasitic, saprobic or even exploitive. It also refers only to the current state of the symbiosis, but endophytic fungi or plants are able to change their life strategy in time. They can, for example, react on changes of environmental conditions, or in the case of facultative saprobes await senescence of the plant tissue. Although the mycobionts can vary in many characteristics all the endophytic interactions have in common the provision of nutrients and a buffer from environmental stresses and microbial competition (Schulz & Boyle, 2005).

Fungal endophytes are usually divided into three distinct groups – mycorrhizal endophytes, clavicipitaceous endophytes and nonclavicipitaceous endophytes. Mycorrhizal fungi can be distinguished from endophytes for they form specialised interfaces to enhance nutrient transfer and their development is synchronised with the plant (Brundrett, 2004). Clavicipitaceous endophytes colonize mainly grass species forming systemic infection in their shoot and are able to synthesize alkaloids which may play role in repelling herbivores (Faeth, 2002). The last group is a highly diverse, polyphyletic assemblage of fungi with mostly unknown ecophysiological roles (Rodriguez *et al.*, 2009).

2.2.1 Mycorrhizal endophytes

The term “mycorrhiza” was first used in 1885 by a German scientist Albert Bernhard Frank in a context with bidirectional flow of nutrients in fungus-conifer associations (Frank, 1885). It is composed of two words – *mykós*, meaning *fungus* and *riza* corresponding to *root*. Plant-fungal interactions were described even earlier by Franz Kamienski in 1882 when he hypothesized that *Monotropa hypopitys* L. forms a mutualistic symbiosis with fungi that associate with the roots of neighbouring trees (republished by Berch *et al.*, 2005).

The exact definition of mycorrhizal symbiosis is since its first use problematic. In the beginning of mycorrhizal research it was described as mutualistic (Trappe, 1994) but later on not only positive responses of plant growth to mycorrhizal associations were observed (Johnson & Graham, 2013). Brundrett defines mycorrhiza as “a symbiotic association essential for one or both partners, between a fungus and a root of a living plant that is primarily responsible for nutrient transfer”. Furthermore he states that mycorrhizal fungus and mycorrhizal plant form an intimate relationship which results from their synchronised development (Brundrett, 2004). This definition allows to include also “exploitative mycorrhizas” in which the nutrient flow is unidirectional – from fungus to mycoheterotrophic plant lacking chlorophyll, that is fully dependent on this relationship with fungi (Leake, 1994).

Mycorrhizal associations are traditionally divided into three groups according to morphology of structures formed in host plants – 1. Endomycorrhizas (arbuscular mycorrhiza, ericoid mycorrhiza (ErM), orchideoid mycorrhiza (OrM)), 2.

Ectomycorrhiza (EcM), and 3. Ectendomycorrhizas (EeM) (arbutoid and monotropid mycorrizas) (Smith & Read, 2008) (see Fig. 1).

Arbuscular mycorrhiza is an ancestral and predominant type of mycorrhiza in land plants (Wang & Qiu, 2006). It is defined by formation of hyphal emergences inside of the plant cells separated from the cytoplasm by the cytoplasmatic membrane. Two different types of arbuscules can be observed in the root – the highly branched Arum-type or heavily curled coils of the Paris type (Smith & Read, 2008) together with intermediate structures (Dickson, 2004). The AMF are usually described as obligatory symbionts of their plant hosts (Smith & Read, 2008). Plants do not obligatory form AM symbiosis but it is a preferred life strategy of almost all plants in natural environments (Feddermann *et al.*, 2010). AMF belong to the Glomeromycota and are a monophyletic group representing one phylum (Schüßler *et al.*, 2001) (). Their hyphae are coenocytic – they lack septa and have many genetically different nuclei coexisting in common cytoplasm (Koch *et al.*, 2004) and are able to form anastomoses even between two distinct individuals, which may thus exchange their genetic information (Croll *et al.*, 2009). The effect of the infection by AMF on the plant growth is in most cases positive thanks to a better P uptake especially in soils of low or imbalanced nutrient status (Smith & Read, 2008).

The root colonized by EcMF is characteristic by presence of the Hartig net (labyrinth of hyphae in between cells), a hyphal mantle on the surface of the root and the extraradical mycelium (Smith & Read, 2008). According to the location of the Hartig net in the root EcM colonization can be divided into two categories – cortical (typical for Gymnosperms, for example members of Pinaceae) and paraepidermal (mainly seen in Angiosperms such as *Eucalyptus, Betula, Populus* or *Fagus*) (Brundrett, 2004). The architecture of the root undergoes several structural changes after fungal infection – it has higher branching densities and limited apical growth of root tips (Brundrett, 2002). In pine species dichotomous branching of short lateral roots can be seen as a result of plant growth regulators supplied by the EcMF (Kaska *et al.*, 1999). EcMF are members of Basidiomycota, Ascomycota and Zygomycota, taxa that contain both ectomycorrhizal fungi and saprotrophs (Tedersoo *et al.*, 2010). This mutualistic symbiosis has evolved repeatedly from saprotrophic precursors and it is not evolutionary stable – multiple

reversals to free-living conditions were noted (Hibbett *et al.*, 2000). EcMF mediate mineral uptake (especially N, P, K) and water to their host plants gaining photosynthetic sugars in exchange (Smith & Read, 2008).

Ericoid mycorrhizal fungi (ErMF) colonize members of Ericaceae, the colonization by intracellular fine hyphal coils occurs in fine hair roots especially in the external layer of cells (Cairney & Ashford, 2002). ErMF belong mostly to Ascomycota and Sebacinales although sheathed ericoid mycorhizal colonization formed by fungus belonging to Basidiomycota was also observed (Vohník *et al.*, 2012). Members of Ericaceae usually grow on nutrient poor organic substrates where N is a limiting source and ErMF possess strong saprotrophic enzymes and are thus able to mobilize N and P from recalcitrant substrates and transport the nutrients into their plant hosts (Smith & Read, 2008).

Orchids also host fungi in their roots, most of them belong to Basidiomycota and many of them to the genus Rhizoctonia (Rasmussen, 2002). Fungi form coiled structures inside of the host plant cortical cells called pelotons (Dearnaley, 2007). In OrM symbiosis only unidirectional nutrient flow from the plant to the fungus was anticipated but in 2006 a first mutualistic relationship was observed between the green-leaved terrestrial orchid *Goodyera repens* Br. and the fungus *Ceratobasidium cornigerum* (Bourdot) Rogers (Cameron *et al.*, 2006).

Monotropoid mycorrhizal colonization is similar to EcM – the Hartig net surrounding epidermal cells and a hyphal mantle on the surface of the root are formed. Moreover in epidermal cells penetrating hyphae induce the plant cell wall to invaginate and together they form a fungal peg with membrane protuberances, which seem to be the main sites of nutrient transport (Robertson & Robertson, 1982). Fungi forming monotropoid mycorrhizas typically form also EcM associations (Brundrett, 2002). The monotropoid plant hosts belong to Ericaceae and are all achlorophyllous and herbaceous. They are nourished through the common mycelium from the neighbouring autotrophs (Kamiensky 1982 re-pulication by Berch *et al.*, 2005, Taylor & Bruns, 1997).

Also arbutoid mycorrhiza appears in the roots of Ericaceae – in three genera Arbutus, Arctostaphylos and Pyrola (Brundrett, 2004). Mycobionts belong to Basidiomycota and usually form EcM with trees surrounding the arbutoid mycorrhizal plants (Vincenot *et al.*, 2008). Similarly to some orchids in tribe

Pyroleae the mixotrophic strategy in gaining C was proved by stable isotope analysis (Tedersoo *et al.*, 2007).

Ectendomycorrhizal colonization combines ecto- and edomycorrhizal features – the Hartig net and a hyphal mantle are formed extracellularly while the epidermal and cortical cells show intracellular infection (Peterson & Farquhar, 1994). This type of association is common in *Pinus* and *Larix* nurseries and is formed by fungi formerly described as E-strain fungi, now identified as a part of Ascomycota (Trevor *et al.*, 2001). Ectendomycorrhiza-like colonization was also observed in roots of *Helianthemum almeriense* Pau., a perennial growing in semi-arid ecosystems (Navarro-Ródenas *et al.*, 2012).

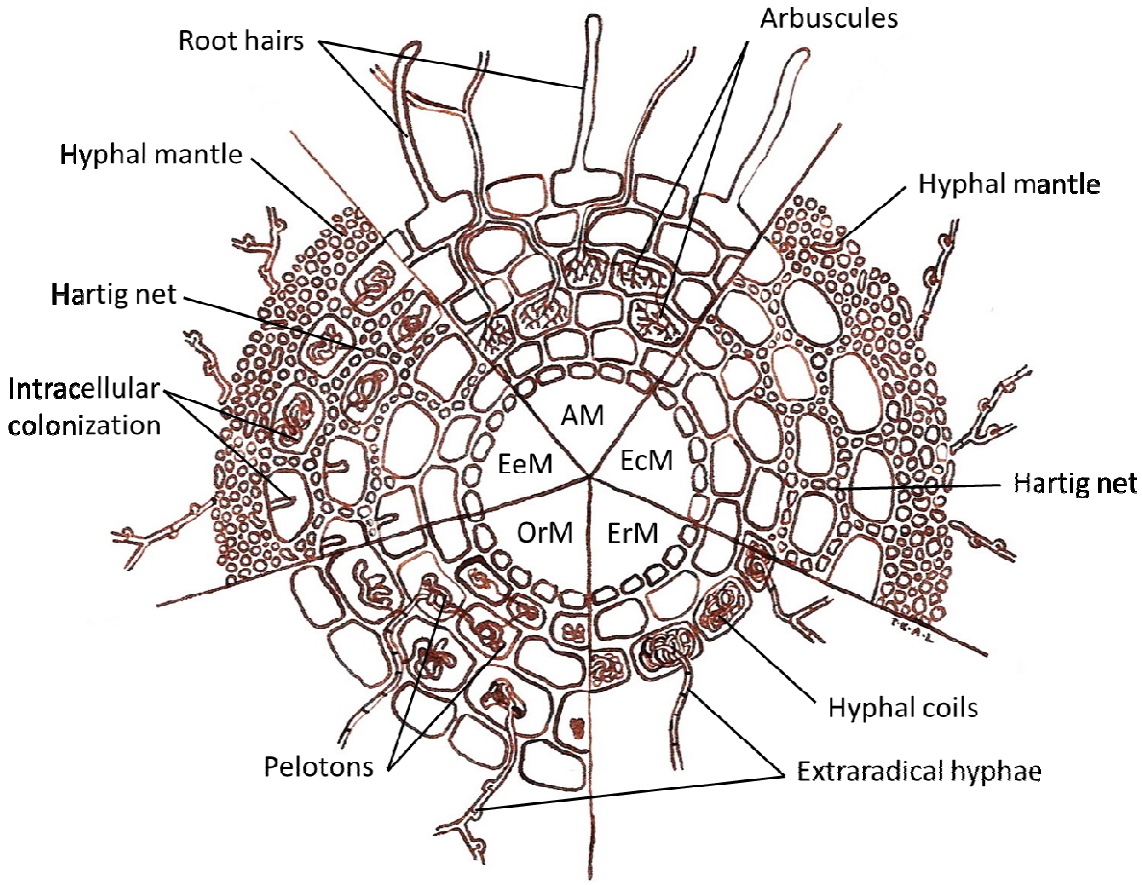

Figure 1 Diagram of colonizing patterns of different types of mycorrhizae – Arbuscular mycorrhiza (AM), Ectomycorrhiza (EcM), Ericoid mycorrhiza (ErM), Orchideoid mycorrhiza (OrM) and Ectendomycorrhiza (EeM).

2.2.2 Clavicipitaceous endophytes

This group of fungi contains fungal endophytes from the genera Epichloë and Neotyphodium which belong to the Ascomycota. They colonize cold-season grass hosts and are able to spend most of their life cycle in the host tissue (Craven, 2012). Species Neotiphodium non-destructively colonizes developing ovaries of the host plant and associates with seeds without causing any symptoms of infection. Thanks to this ability Neotipodium does not need to leave plant tissues during its whole life cycle (Saikkonen *et al.*, 2002). Clavicipitaceous fungi are referred as mutualists which are able to enhance plant performance by synthesizing alkaloids that aid protection of the host plant against herbivores (Clay & Schardl, 2002). One of the clavicipitaceous fungi – *Epichole typhina* (Pers. Ex Fr.) can produce sesquiterpens and chokols A-G that have fungitoxic properties and thus help to defend its host against leaf spot pathogen *Cladosporium phlei* (C.T. Greg.) G.A. de Vries (Kumar & Kaushik, 2013).

2.2.3 Non-clavicipitaceous endophytes

Non-clavicipitaceous fungal endophytes are a diverse group of fungi. They belong mostly to the Ascomycota but their life strategies, phylogeny, host preference and colonization pattern differ (Schulz & Boyle, 2005). They can be thus divided in at least three different functional groups. Rodriguez et al. (2009) distinguishes class 2, 3 and 4 of fungal non-clavicipitaceous endophytes.

Class 2 endophytes belong to Dicaria, mainly to the Ascomycota and few to the Basidiomycota. They extensively colonize shoots, roots and rhizomes of a broad range of hosts, but their *in planta* diversity is low. Transmission from plant to plan can be either vertical or horizontal. They are reported to act mainly as mutualists – they can increase plant root and shoot biomass (Barrow, 2003), enhance resistance to various abiotic stresses such as high temperature, salinity and drought (Redman *et al.*, 2002) and also various biotic stresses (Macia-Vicente, 2008).

Fungal endophytes that infect primarily or exclusively above ground tissues were classified as Class 3 endophytes. They can be found in photosynthetically active tissues but also in flowers (Kumar & Hyde, 2004) as well as in wood and bark (Tejesvi *et al.*, 2005). Their host range is broad and *in planta* biodiversity high

but the colonization rates are limited. Fungal species of Class 3 belong mostly to the Ascomycota, minority to the Basidiomycota. Their ecophysiological role is unresolved mainly due to the high diversity within host tissues (Arnold *et al.*, 2001). The plans infected by Class 3 endophytes typically do not show any changes in biomass production in *in vitro* conditions (Rodriguez *et al.*, 2009) but were reported to decrease leaf necrosis and leaf mortality caused by *Phytoshora sp.* (Arnold *et al.*, 2003). On the other hand they are also capable of decreasing plant growth (Schulz *et al.*, 1999).

This thesis is focused on Class 4 endophytes and they are thus described in detail in a special chapter 2.4.

2.3 Endophytic continuum and symbiotic lifestyle switching

Plant-endophyte interactions can vary from mutualistic to parasitic – this phenomenon is called the “endophytic continuum” (Schulz & Boyle, 2005). Fungi are thought to express only one life strategy – either they increase (mutualism), decrease (parasitism) or have no influence (commensalism) on host plant performance (Redman *et al.*, 2001). However, according to recent observations the life strategy does not seem to be stable, fungal isolates and/or species are able to switch it according to their host plant and environmental conditions (Rodriguez & Redman, 2008).

The fungal species *Colletotrichum magna* is considered to be a virulent pathogen in some host plant species. But when different cultivars of *Solanum lycopersicum* L. were inoculated, the fungus performed a wide range life strategies - mutualistic, commensal, and/or parasitic (Rodriguez & Redman, 2008). The life strategy can be affected by many factors as for example host genotype, imbalance in nutrient exchange or environmental factors (Aly *et al.*, 2011).

Another study on *Colletotrichum* species showed three different strategies – they either had a narrow host range and a single lifestyle or a wide host range and a single lifestyle or a wide host range and multiple lifestyles. For example, *Colletotrichum gloeosporioides* (Penz.) Sacc. colonized only one host and was acting as a parasite. *Colletotrichum* coccodes (Wallr.) S. Hughes was able to colonize various host plants and performed parasitic symbiosis. Lastly *Colletotrichum orbiculare* (Berk. & Mont.) Arx expressed different life strategies

(parasitism, commensalism, mutualism) with different host plants (Redman *et al.*, 2001). In the endophyte *Discula quercina* (West.) Arx. colonizing Mediterranean oaks a change from mutualistic/commensal lifestyle to parasitism was observed. This change of behaviour may be caused by an atypical change of conditions – prolonged dry season may cause the endophyte to act as a parasite and start colonizing the plant tissues epiphytically instead of intercellulary (Moricca & Ragazzi, 2008).

Barbara Schulz and Christine Boyle assume that the nature of plant-fungus symbiosis depends on balancing the fungal virulence and host plant defence (Fig. 2). Symptomless symbiosis can appear only when the antagonisms of both partners are put in balance (Schulz & Boyle, 2005).

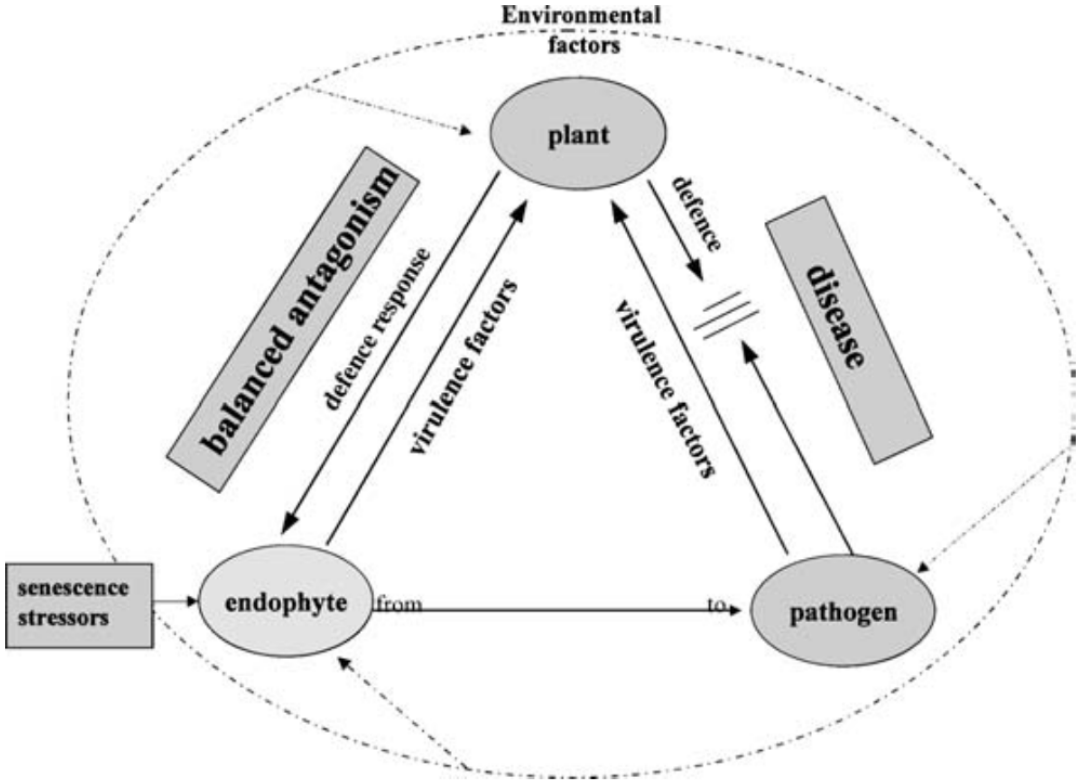

Figure 2 A diagram illustrating balanced antagonisms between plant defence response and fungal endophyte virulence factors. Balanced symbiosis can result in asymptomatic colonization. Source (Schulz & Boyle, 2005)

Change of life strategy by a mutation of a single DNA locus was observed. When a virulent isolate of *C. magna* was exposed to UV radiation a mutation occurred and the mutant was no longer able to cause disease symptoms in the host (Freeman & Rodriguez, 1993). Disruption of the fungus gene by restriction

enzyme-mediated integration (REMI) with selectable plasmid also resulted in non-pathogenic mutants. Authors hypothesise that both of the mutants lost the ability to switch between the lifestyles and thus remained restricted to mutualistic or commensal symbiosis (Rodriguez & Redman, 2008).

The ability to perform different life strategies brings up many questions. Does the evolution of fugal life strategies show any directionality? Do fungi tend to evolve from parasitism to mutualism? The EcM mutualistic behaviour seems to have evolved repeatedly in saprotrophic fungi and according to a phylogenetic analysis of EcM and free living fungi reversals to non-mutualistic life style were also not rare (Hibbett *et al.*, 2000). Recent mathematical model created to analyse evolutionary persistence of mutualistic symbioses shows that it is not regulated by positive density-dependence (meaning the mutualistic symbioses should be rare in nature due to their increased risk of extinction). Additionally, the trade-off between given and obtained benefits reduces opportunities for cheating, because if one of the partners requires more than it gives the other partner is driven to extinction (Johnson & Amarasekare, 2013). Fungi able of symbiotic life strategy switching can be undergoing an evolutionary transition or simply use this ecological flexibility to enhance their performance (Rodriguez & Redman, 2008).

2.4 Dark Septate Endophytes

Fungi with dark and septate mycelia were isolated form plant roots in the beginning of 20th century by Gallaud and Peyronel (Jumpponen & Trappe, 1998a). In 1922, Elias Melin described dark septate fungi isolated from EcM roots as “pseudomycorrhizal” because they were not able to form ECM structures in resynthesis experiments (Melin, 1922). The fungi were called *Mycelium radicis atrovirens* (MRA) literally translated as “dark root mycelium”; this name was used until 1991 when it was replaced by Dark septate endophytes (Stoyke & Currah, 1991).

2.4.1 DSE species

Dark septate endophytes are polyphyletic group of fungi; they can be defined by their life strategy and morphology as root endophytes with dark and septate mycelia. They belong to Class 4 of non-clavicipitaceous endophytes according to (Rodriguez *et al.*, 2009).

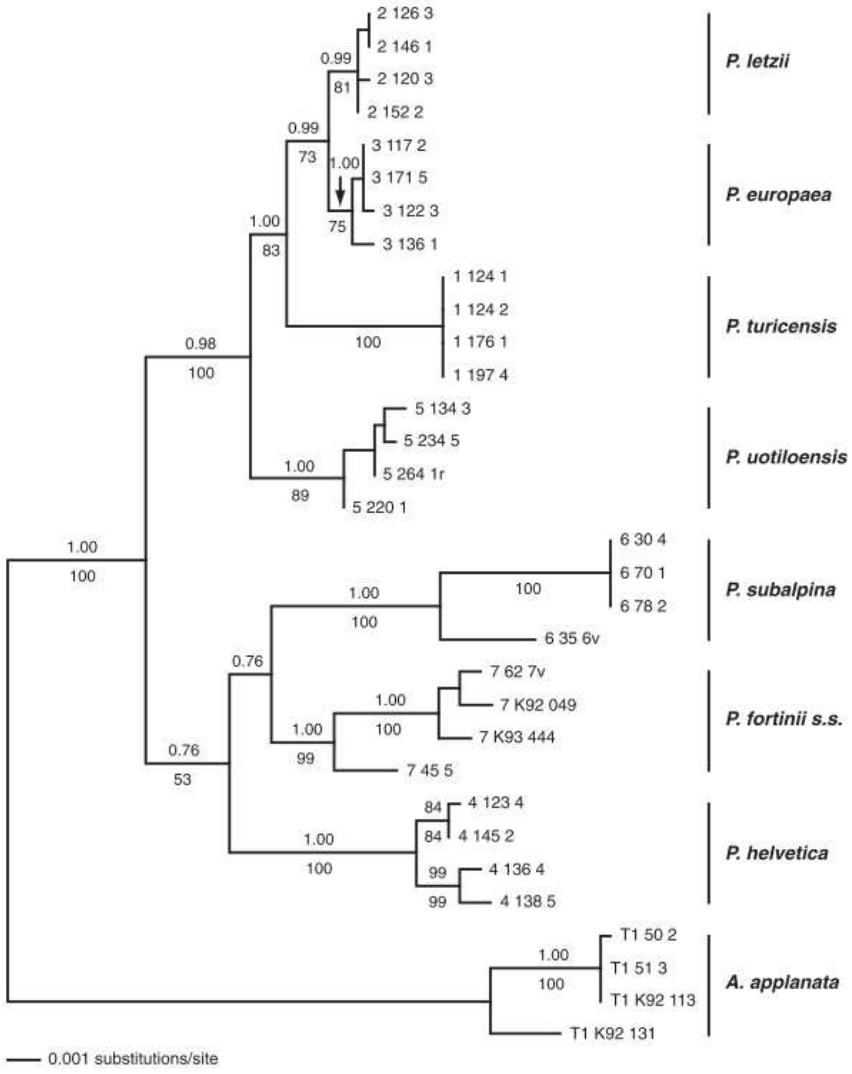

Figure 3 Phylogenetic tree for PAC species based on three mt loci Posterior probabilities of BI (above branches) and bootstrap values of ML analysis (below branches) are indicated. *Acephala applanata* was chosen as outgroup. Source: Duò *et al.*, 2012

The most studied group of DSE is fungi belonging to the *Phialocephala fortinii-Acephala applanata* species complex (PAC). This species complex comprises fungi that were formerly marked as species *Phialocephala fortinii* Wang & Wilcox (Wang *et al.*, 1985). In the year 1995 the species was, thanks to molecular analyses and morphology, divided into two species and a new genus Acephala with only one species *Acephala applanata* Grünig & Sieber was formed (Grünig & Sieber, 2005a). Three years later seven morphologicaly indistinguishable although reproductively isolated cryptic species (CSP) were found within *P. fortinii* s. l. (*Phialocephala europaea* Grünig & Sieber, *Phialocephala helvetica* Grünig & Sieber, *Phialocephala letzii* Grünig & Sieber,

Phialocephala subalpina Grünig & Sieber, *Phialocephala turiciensis* Grünig & Sieber, *Phialocephala uotolensis* Grünig & Sieber and P. fortinii s.s. for more information see Fig. 3) (Grünig *et al.*, 2008a). Up to date *P. fortinii* s. l. was shown to be composed of at least 21 reproductively isolated lineages (Duò *et al.*, 2012). All these species are now classified into PAC and belong to Helotiales (Ascomycota) (Grünig & Sieber, 2002, Duò *et al.*, 2012).

Not only members of PAC and related species can be included into DSE. For example *Cadophora finlandica* Wang & Wilcox, *Chloridium paucisporum* Wang & Wilcox, *Heteroconium chaetospira* Grove or *Leptodontidium orchidicola* Singler & Currah are endophytes possessing dark septate hyphae but they are in comparison studied much less than the species related to *P. fortinii*.

2.4.2 Geographical range

DSE fungi were isolated from wide range of ecosystems – from tropical (Rains *et al.*, 2003) to polar regions (Newsham *et al.*, 2009) and also in aquatic habitats (Kohout *et al.*, 2012) Most of the studies were conducted in the Northern Hemisphere – in Europe and North America. There is very little known about composition of DSE communities in plant roots in Australia and South America (Grünig *et al.*, 2008b). Acoording to recent study PAC have a broad range geographical distribution and they lack biogeographic structure (Queloz *et al.*, 2011).

2.4.3 Colonization pattern

Two morphological structures are typical for roots colonized by DSE – dark septate hyphae growing inter- and intracellulary in the root and microsclerotia. Invagination of the cytoplasmatic membrane around intracellulary growing hyphae has not yet been observed (Peterson *et al.*, 2008) but a fibrillar material probably of a plant origin was seen close to the fungal cell wall (Yonezawa *et al.*, 2004). Hyphae penetrating plant cell wall could form appressorium-like structures but the colonization usually does not trigger host defence reaction (Yonezawa *et al.*, 2004).

Hyphae show brownish or blackish colouring caused by large amounts of melanin in the fungal cell wall. Some DSE species are able to form also non melanised

hyphae that need to be visualised by special staining techniques (Barrow & Aaltonen, 2001).

Intracellular colonization is characteristic by formation of microsleclerotia – aggregations of irregularly lobed hyphae (Stoyke & Currah, 1991). They usually fill the entire volume of the plant cell by thick walled dark and highly septated hyphae. Their storage substances are similar to true sclerotia (Yu *et al.*, 2001), structures formed on the surface of the root composed of thick-walled, melanised hyphae that allow survival of the fungus during unfavourable conditions (Willetts, 1972). Some DSE also form sclerotia on host plant roots – for example *Acephala macrosclerotiorum* Münzenberger & Bubner (Münzenberger *et al.*, 2009). Both sclerotia and microsclerotia accumulate glycogen, proteins and polyphosphates (Yu *et al.*, 2001). Although the function of microsclerotia has not yet been resolved the composition of substances they contain indicates that they may serve as propagules (Grünig & McDonald, 2004).

Some DSE species are also able to form EcM structures in roots of their hosts. This behaviour was described by Babette Munzenberger for *A. macrosclerotiorum* and *Pinus sylvestris* L. (Münzenberger *et al.*, 2009).

2.4.4 Influence of the mycobiont on the plant

Although the DSE fungi colonize the majority of vascular plants their influence on the plant hosts is still unclear. The main focus of studying the effect of inoculation by DSE is on three areas – 1. nutrient transport, 2. increasing abiotic stress resistance and 3. protection against biotic stresses. The influence of DSE on plant nutrient uptake is usually studied in *in vitro* conditions, which greatly influence the behaviour of both the fungus and the plant. For example C:N ratio in the media showed high influence on the colonization rates of the endophyte *Heteroconium chaetospira* (Grove) M.B. Ellis in an *in vitro* experiment with Chinese cabbage. At the high level glucose condition (C:N ratio 40:1) the fungus did not enter the roots and grew as a saprophyte in the substrate. The fungal biomass in the host roots increased as a reaction to decrease in the C:N ratio (Usuki & Narisawa, 2007).

Results of resynthesis experiments are inconsistent – some show positive effect on plant growth (Newsham, 1999; Usuki & Narisawa, 2007; Wu *et al.*, 2010) other negative (Wilcox & Wang, 1987; Stoyke & Currah, 1993; Tellenbach, 2011). Recently a meta-analysis was performed on 18 research articles in which plants

were inoculated by DSE species on sterile substrates. Surprisingly only positive influence of inoculation was identified (Newsham, 2011). Due to the lack of specialized interfaces for nutrient transfer between most DSE and their host plants the cause of the positive influence is unresolved. Two main hypotheses were introduced, the synthesis of plant hormones and mineralization of the organic N-containing substrates (Newsham, 1999; Mandyam & Jumpponen, 2005; Addy, 2005).

DSE can influence plant performance also by enhancing abiotic stress resistance. Plants growing on geothermal soils in Lassen Volcanic and Yellowstone National Parks, USA showed much less mortality in soil of temperature 50 ºC when inoculated by the DSE *Curvularia sp*. They were then able to tolerate intermittent soil temperatures as high as 65 ºC for 10 days. Moreover both the fungus and the plant were not able to survive separately when exposed to temperatures higher than 38 ºC (Redman *et al.*, 2002). In 2007, a study was reported that the heat protection of the host plant does not work without another symbiotic partner – a mycovirus CThTV (Curvularia thermal tolerance virus) (Márquez *et al.*, 2007). DSE fungi are frequently isolated from roots of plants growing on naturally metalyferrous soils (Urban *et al.*, 2008; Zhang *et al.*, 2013). Authors of these studies hypothesise that DSE contribute to metal tolerance and nutrient acquisition on these sites.

Although inoculation by DSE may have negative effects on plant performance, their presence in roots may be tolerated for their indirect benefits to the host. Christoph Tellenbach and Thomas N. Sieber observed reduction of mortality of *P. abies* seedlings infected by two pathogenic oomycetes when inoculated by *Phialocephala subalpina* Grünig & T.N. Sieber (Tellenbach & Sieber, 2012). Recently, *Phialocephala europaea* Grünig & T.N. Sieber was reported to inhibit growth of a pathogenic oomycete in *in vitro* conditions. The chemical compounds were identified as sclerin, sclerolide, sclerotinin A and sclerotinin B (Tellenbach *et al.*, 2013). Reduction of infection by the pathogenic fungus *Verticilium longisporum* Krapapa Stark was observed in *Brassica campestris* L. colonized by an unknown DSE fungus (Narisawa *et al.*, 2004). The mechanisms of this protection are not yet clarified but the endophyte can produce toxins with antifungal activites (Tellenbach *et al.*, 2013), compete with the pathogen for

infection sites, nutrinents or space, or rise plant host resistance (Tellenbach & Sieber, 2012).

The effects of DSE on host plant performance are diverse. This diversity is caused by many factors – one of the most important is the factor of strain which is surprisingly stronger than the species factor. In a recent study the variation in virulence of PAC species was much higher within than among species in interaction with *P. abies* in *in vitro* conditions. Moreover the virulence was not correlated with phylogenetic relatedness of the isolates (Tellenbach, 2011). To similar conclusions came also Vanessa Reininger in her recent study but she observed also influence of strain-plant combination on plant growth and competition between PAC strains was alleviating adverse effects of some pathogenic strains (Reininger & Sieber, 2012a). Adverse effects of virulent PAC strains may be reduced not only by other PAC species but also by mycorrhizal fungi that may form physical and/or physiological barriers in the host root (Reininger & Sieber, 2012b). Coexistence of mycorrhizal fungi and DSE in natural habitats is ubiquitous but its effect on the host plant is still poorly studied.

2.5 Common mycorrhizal networks (CMN) and the role of fungal endophytes

Most endophytic fungi are not host specific and are able to simultaneously colonize many individuals of one or more plant species. And they are not fastidious in choosing their plant partners – they can live in symbiosis with small or large plant species (Heijden & Horton, 2009). Different plant individuals thus can be connected by mycelia and to form common mycorrhizal or mycelial networks (CMN) which can enable uni- or bidirectional nutrient flow between plant individuals (Selosse *et al.*, 2006). This phenomenon was described by (Simard *et al.*, 1997) on tree species *Betula papyrifera* Marsh. and *Pseudotsuga menziesii* (Mirb.) Franco by ^{13}C and ^{14}C isotope tracking. The trees shared seven EcM morphotypes which colonized over 90% of their root tips and indicated possible fungal network between the plants. In three consecutive years a bidirectional C flow was observed by the authors.

Specialised trophic interactions such as mycoheterotrophy and mixotrophy have evolved due to the existence of CMN. Mycoheterotrophic plants are not able to

photosynthesize or lack chlorophyll and use carbon transferred by fungi from other green plants. This behaviour can be observed in about 200 species of Ochideaceae (Leake, 1994). Some orchid species and also species from the genera *Pyrola*, *Orthilia* and *Chimaphila* combine photosynthesis and partial heterotrophy as carbon sources (Selosse & Roy, 2009, Bidartondo *et al.*, 2004) and are considered mixotrophic.

Another interesting feature enabled by CMN is the “nurse effect”. Mycelial networks enable connection between adult trees and seedlings (Nara, 2006). The growth rate and mortality of seedlings is often related to presence of mycorrhizal symbionts in their roots. These promote their performance not only by obtaining nutrients from the soil but also by providing nutrients from neighbouring adult individuals through CMN (Simard *et al.*, 1997, Warren & Brooks, 2008).

CMN can play also an importand role in supplying plant hosts by water. Plant roots mediate passive movement of water from wet to dry soil parts in process called hydraulic redistribution (Prieto *et al.*, 2012). Neighbouring plants can access redistributed water by two main ways – indirectly from soil or directly by through CMN (Egerton-Warburton *et al.*, 2007). Similarly to nutrient transport in nurse effect, CMN can facilitate flow of hydraulically redistributed water from large trees to seedlings in field condition. Transport of water from large threes to seedlings via EcMF was recorded using water enriched in D_2O. Authors hypothesize that the flow of water can mediate C or nutrient uptake, but more importantly it may help roots and root associated fungi survive seasonal or annual drought (Warren *et al.*, 2008).

3. Materials and methods

3.1 Experiment 1. In vitro resynthesis experiment with P. abies, V. myrtillus and 10 different DSE species

To describe the colonization potential of different DSE species and isolates in roots of ectomycorrhizal and ericoid mycorrhizal forest plants an aseptic *in vitro* experiment on *P. abies* and *V. myrtillus* was accomplished. Eleven different fungal species were used - 8 members of PAC (*A. applanata, P. europea, P. fortinii* s. s., *P. helvetica, P. letzii, P. subalpina, P. turiciensis, P. uotolensis*), the newly described DSE/ectomycorrhizal fungus *A. macrosclerotiorum, P. glacialis* and as a positive ericoid mycorrhizal control *R. ericae* (for more information see Table 1). From each fungal species two different isolates were used for inoculating both host plants. Due to a high number of isolates only 3 replicates, each containing 1 *P. abies* and 2 *V. myrtillus* seedlings, were prepared.

Species	Isolate	Origin	Isolated from	GenBank nr.
Acephala applanata	AAP-1	CR, Šumava	*Picea abies*	EF093158
Acephala applanata	AAP-2	CR, Šumava	*Picea abies*	-
Acephala macrosclerotiorum	AMA-1	Germany, Hubertusstock	*Pinus sylvestris*	EU882732
Acephala macrosclerotiorum	AMA-11	CR, Č. Švýcarsko	*Pinus sylvestris*	-
Phialocephala europea	PF-EU-1	Switzerland, Zürichberg	*Picea abies*	JN091538
Phialocephala europea	PF-EU-2	Switzerland, Zürichberg	*Picea abies*	JN091540
Phialocephala fortinii s.s.	PFO-F	CR, Jeseníky	*Vaccinium myrtillus*	EF446149
Phialocephala fortinii s.s.	PFO-9	CR, Příbram	*Pinus sylvestris*	-
Phialocephala helvetica	PF-HE-1	Switzerland, Zürichberg	*Picea abies*	JN091541
Phialocephala helvetica	PF-HE-2	Switzerland, Zürichberg	*Picea abies*	JN091543
Phialocephala letzii	PF-LE-1	Switzerland, Zürichberg	*Picea abies*	JN091534
Phialocephala letzii	PF-LE-2	Switzerland, Zürichberg	*Picea abies*	JN091536
Phialocephala subalpina	PF-SU-1	Switzerland, Bödmeren	*Vaccinium myrtillus*	JN091551
Phialocephala subalpina	PF-SU-2	Switzerland, Bödmeren	*Picea abies*	JN091553
Phialocephala turiciensis	PFO-2	CR, Šumava	*Picea abies*	EF093162
Phialocephala turiciensis	PFO-6	Czech republic, Šumava	*Picea abies*	EF093157
Phialocephala uotolensis	PF-UO-1	Switzerland, Uetliberg	*Picea abies*	JN091547
Phialocephala uotolensis	PF-UO-2	Switzerland, Uetliberg	*Picea abies*	JN091548
Phialocephala glacialis	PF-GL-1	Switzerland, Creux du Van	*Vaccinium myrtillus*	EU434843
Phialocephala glacialis	PF-GL-2	Switzerland, Creux du Van	*Picae abies*	EU434842

Table 1 Fungal isolates used in the study

3.1.1 Experimental design Plant and fungi cultivation

V. myrtillus and *P. abies* seeds were surface sterilized in 30% H_2O_2 for 12 and 21 minutes, respectively, and then rinsed twice in autoclaved deionised water. *P. abies* seeds were placed on MMN agar media ((Molina and Palmer, 1982), content: $(NH_4)_2HPO_4$ 0,25 g, KH_2PO_4 0,5 g, $MgSO_4.7H_2O$ 0.15 g, $CaCl2.2H_2O$ 0.05 g, NaCl 0.025 g, Fe EDTA 0,02g, glucose 10.0 g, malt extract 3g, thiamine 100 μg, agar 7,5 g, deionized water 1000 ml) and germinated in Petri dishes (9 cm in diameter) at room temperature in the dark for 2 weeks. *V. myrtillus* seeds germinated on water agar (10g agar, 1000 ml deionised water) at room temperature in the dark.

The isolates listed in Table 1 were grown on MMN media (as described above) for 2 weeks at room temperature in the dark.

Host plants and fungi were transferred to square Petri dishes (12 x 12 cm). The dishes were filled with MMN media without glucose and malt extract. Two thirds of the solidified media were then removed leaving approximately 40 ml of media in one part of the dish. The dish was then placed vertically with media block on the bottom part. One *P. abies* and two *V. myrtillus* seedlings were inserted into each Petri dish. The roots were placed on the agar media covered by an autoclaved cellulose foil together with 9 agar plugs well-colonized by newly formed hyphae (three plugs for each of the plants). To prevent desiccation of the roots a moistened autoclaved filter paper was placed on the agar block covering the roots and fungal plugs. Folded autoclaved filter paper was

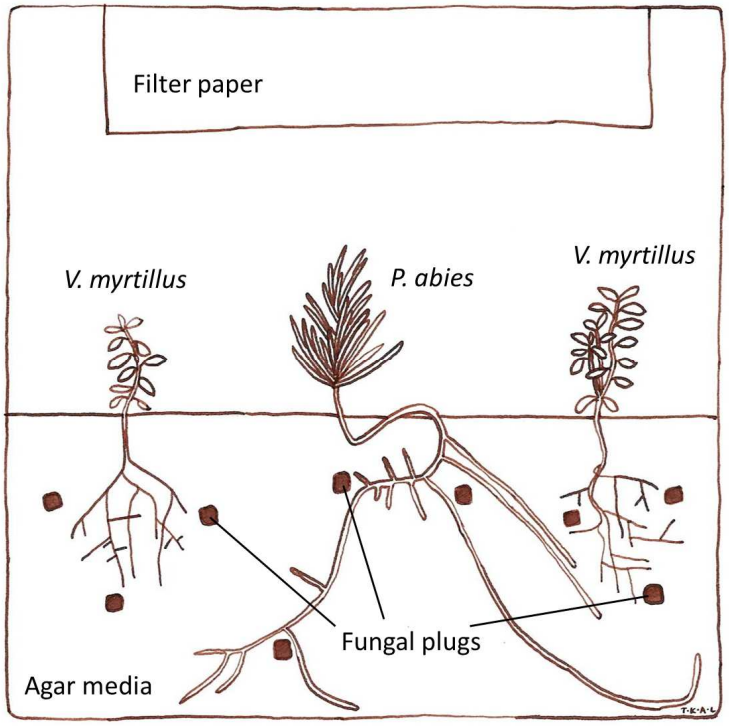

Figure 4 Experimental design of the Experiment 1. Plants and fungi were growing on agar media covered by filter paper in 9 x 9 cm Petri dishes. Front view.

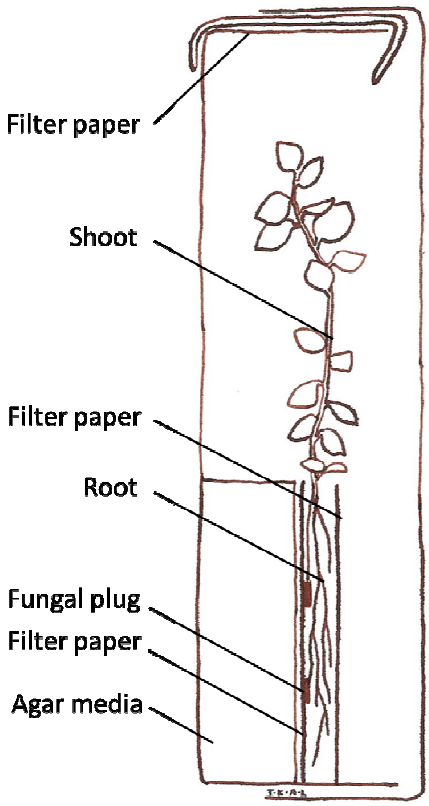

Figure 5 Experimental design of the Experiment 1. Roots and fungal plugs were placed in between of two layers of filter paper. Side view.

inserted in between the lid and the bottom of the dish to enable gas exchange (see Figures 4 and 5) and the dish was then sealed with an air permeable foil. To keep the roots shaded the bottom part of the dish was wrapped in aluminium foil. The Petri dishes were stored vertically in a growth chamber (16 hours of light at 20°C, one tungsten lamp and relative humidity 80%; Fitotron, SANYO)

3.1.2 Data collection

One of the microcosms per each treatment was harvested after 3 months, the other two month later (5 months since the start of the experiment). Roots were washed in tap water and prepared for microscopy.

V. myrtillus roots were placed into 10% KOH solution, autoclaved for 10 minutes and then rinsed in tap water and acidified by 3% HCl. To stain fungal structures the roots were put into a solution of trypan blue in lactoglycerol (1:1:3 v.v. lactic acid : glycerol : deionized water as 0.05 % solution) and then de-stained in lactoglycerol. An upright microscope with differential interference contrast at high magnifications (400× and 1000×) was used to and to observe structures formed inter- and intracellulary by the fungal hyphae.

P. abies roots were observed under a dissecting microscope, mycorrhizal and non-colonized root tips were counted and the total root length was measured. One or two colonized root tips per each strain were cross-sectioned using a razor blade. Cross-sections were then observed with the upright microscope at high magnifications (400x and 1000x).

3.2 Experiment 2. In vitro peat resynthesis experiment with V. myrtillus and 10 different DSE species on peat substrate

The goal of this experiment was to describe interactions between DSE and *V. myrtillus* in *in vitro* conditions. The same isolates as in Experiment 1 (see Table 1) were used. A new experimental design was chosen to prevent some adverse effect of *in vitro* conditions on the seedlings. Six replications were prepared for each treatment as well as for non-inoculated control.

3.2.1 Plant and fungi cultivation

V. myrtillus seeds (origin: Šumava mountains, collected in 2011) were sterilized and cultivated in the same manner as in Experiment 1 (see 3.1.1) in October 2011 on MMN medium. Fungal isoaltes were precultivated similarly to Experiment 1 (see 3.1.1)

3.2.2 Experimental design

One plug of agar media well colonized by newly formed hyphae was placed into 50 ml sterile autoclavable falcon tube on the surface of MMN media (see 3.1.1) without glucose and malt extract (see Fig. 6). The tubes were then placed in a dark cultivation box at room temperature. After 2 months approximately, 10 ml of thrice autoclaved peat (pH 3,9 before and 4,0 after autoclaving) was added and the tubes were stored in the dark for another 2 weeks to enable growth of the fungus into the peat substrate. Two-months-old sterile *V. myrtillus* seedling were then transferred into the system, tubes were closed by lids, sealed with an air

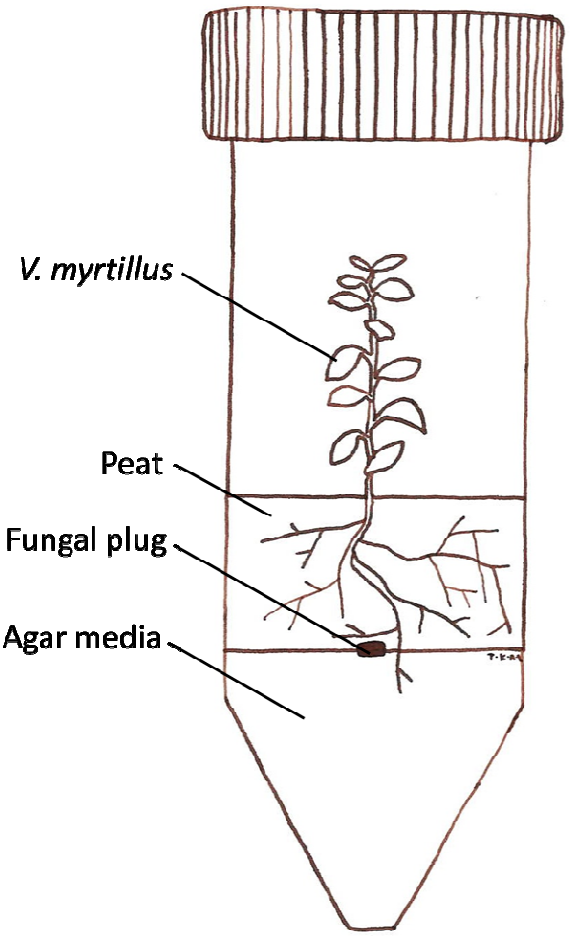

Figure 6 Experimental design of the Experiment 2. *V. myrtillus* seedling were grown in 50 ml falcon tubes in peat substrate.

permeable foil and stored in a growth chamber (16 hours of light at 20°C, one tungsten lamp and relative humidity 80%, Fitotron, SANYO).

3.2.3 Data collection

The seedlings were harvested after 3.5 months. The roots were cleaned in tap water, surface dried with towel paper, weighted and stained in Trypan blue (see 2.1.3). Roots were de-stained in lactoglycerol and then observed with an upright microscope with differential interference contrast at high magnifications (400× and 1000×) and percentage of colonized cells was measured (in each root system 500 cells was counted randomly). The shoots were dried (90 min at 65 º C) and weighted.

3.2.4 Data processing

Data were analysed using STATISTICA 12 (StatSoft Inc.). Normal distribution of the data was checked and logarithmical transformation was used when appropriate to treat the data with non-normal distribution. The differences between colonization rates, fresh root weight and dry shoot weight were evaluated by Breakdown and one-way ANOVA followed by Tukey´s HSD test. Means were compared at a significance level of $P < 0.05$.

3.3 Experiment 3. Pilot in vitro resynthesis experiment with P. abies and A. macrosclerotiorum labelled by ^{33}P to trace nutrient transfer from fungus to plant

Host plants were inoculated in the Czech Republic and then transported to the University of Aberdeen (Scotland, UK) to the lab of David Johnson to perform radioactive P labelling.

3.3.1 Plant and fungi cultivation

P. abies seeds (origin: Kušné hory, collected in 2003) were surface sterilized in 30% H_2O_2 for 21 minutes, rinsed twice in autoclaved tap water and seeded to water agar (7,5 g agar, 1000 ml water) in 9 cm Petri dishes and cultivated in the dark at room temperature in February 2012. After approximately two weeks the sealed dishes were transported to a growth chamber (16 hours of light at 20°C,

one tungsten lamp and relative humidity 80%) and cultivated for one month. Fungal isolate AMA-1 was selected for this experiment due to its ability to form EcM in *P. abeis* roots.

3.3.2 Inoculation of *P. abies* seedlings by fungal strains

For inoculation square Petri dishes (12 x 12 cm) filed to one third with MMN media without malt extract and glucose (similarly to Experiment 1, see Figure 3) were

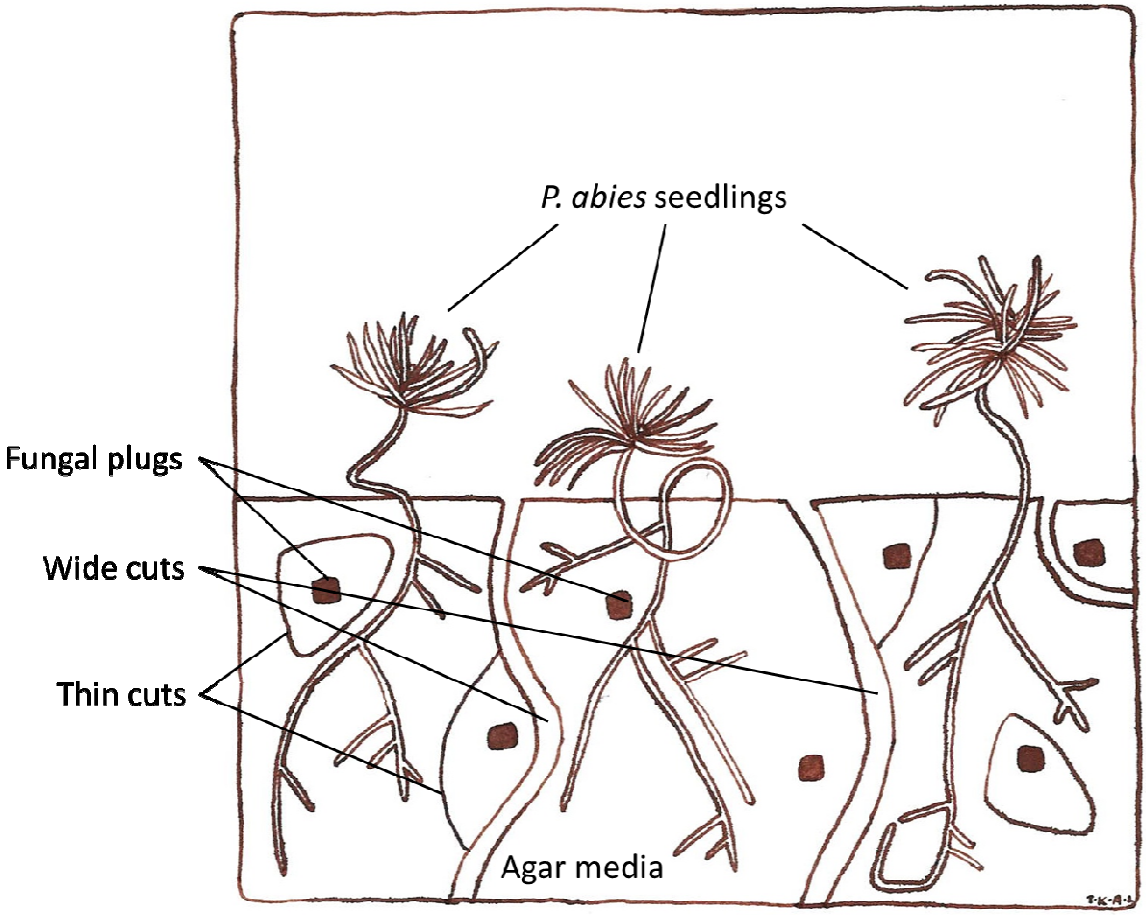

Figure 7 Experimental design of the Experiment 3. Three *P. abies* seedlings were grown on agar media in 9x9 cm Petri dish. For labelling the media was cut into three parts and in controls the fungal plugs and adjoining hyphae were separated by thin cut. Front view.

used. The roots of the seedlings were placed on the media together with 7 AMA - 11 inoculated agar plugs. To prevent growth of the roots into the media, sterilized filter paper was placed on the surface of the media. To prevent desiccation of the roots, another sheet of filter paper was placed to cover the roots. Three seedlings were placed in each of the dishes. In total 15 seedling per each treatment were prepared and cultivated for approximately 4 months in a growth chamber (16

hours of light at 20°C, one tungsten lamp and relative humidity 80%, Fitotron, SANYO) and then transported to the laboratory in Aberdeen (Scotland, UK).

3.3.3 Labelling design

Two Petri dishes with plants colonized by AMA-1 were selected for labelling by ^{33}P. The roots of the three plants were separated by approximately 2 mm wide cut in the agar media to prevent diffusion of the ^{33}P solution between the seedlings (see Fig. 7). To each Petri dish ^{33}P-phosphoric acid (specific activity 111TBq mmol-1, manufacturer: HARTMANN ANALYTIC GmBH, Germany) was added to 2 mycelial patches (20 µl of ^{33}P-phosphoric acid per patch) per plant. In control plants the mycelial patches were separated from the rest of the agar media by thin cut to interrupt the mycelial connections between the plant and the labelled patch (see Fig. 7).

3.3.4 Data collection

After 21, 26 and 45 hours changes in the location of the radioactive isotope were observed. Fujifilm imaginig plates were placed for 10 minutes on the surface of the agar media to enable it to accumulate the radiation. Imagining plates were then scanned using Fujifilm Image Analysis System (FLA 5100) and images automaticky analysed by AIDA software (Advance Imaging Data Analyser version 4.10.020). The digital autoradiograph collects light intensity at a scale of 50.0 µm.

3.4 Experiment 4. In vitro resynthesis experiment with P. abies and A. macrosclerotiorum labelled by ^{13}C and ^{15}N isotopes

The host plant and fungi cultivation was accomplished in the laboratory of the Institute of Botany, Academy of Sciences of the Czech Republic (IB ASCR) in Průhonice and stable isotope labelling was performed in the University of Aberdeen (Scotland, UK) in the lab of Dr. David Johnson.

3.4.1 Plant and fungi cultivation and inoculation

The *P. abies* plants and one isolate of *A. macrosclerotiorum* (AMA -1) were cultivated and inoculated in the same manner as in experiment 4 (3.4.1 and 3.4.2).

3.4.2 Labelling design

After 4 months from inoculation the seedlings were transferred to new microcosms in July 2012. Approximately 200 ml of autoclaved peat/vermiculite substrate (1: 5: 6, v.v. peat : vermiculite : water) was placed into Magenta dishes. To create fungal and root compartments 50 µm nylon mesh (4,5 cm x 10,5 cm) was inserted into the dishes and, thus, created 2 equally sized compartments (see Fig. 8). In one of the compartments, one colonized *P. abies* seedling was planted. In total 16 microcosms were prepared - 6 with seedlings inoculated by AMA-1 and 6 control plants. The systems were closed and placed in a growth chamber (16 hours at high light, at 20 ºC, Fitoron PG660, SANYO).

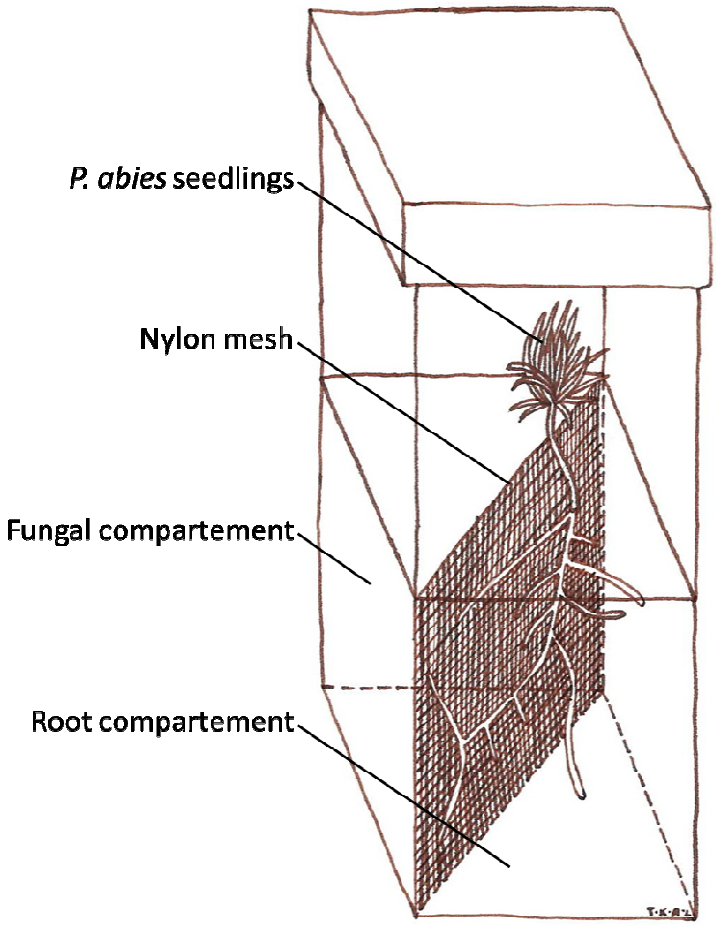

Figure 8 Experimental design of the Experiment 4. *P. abies* seedlings were grown in Magenta dishes devided into two compartements by nylon mesh.

After 2.5 months (end of September 2012) 2 needles per each plant were sampled to obtain time zero ^{13}C abundance. In three selected plants the majority of sclerotia was cut of the root tips and samples of approx. 2 µg of sclerotia was prepared. Three ml of 97% $(NH_4)(NO_2)$ were added in 6 doses into 6 different places in the fungal compartment of the microcosm not closer to the mesh than 1 cm. After 3 days the microcosms were opened and labelled by ^{13}C (>99 atom percent $^{13}CO_2$ at 350ppm CO_2 for 3 hours using a flow rate 0.2 to 1 l per minute). The $^{13}CO_2$ labelling was repeated after 15 hours.

3.4.3 Data collection

The plants were harvested 24 hours after the first $^{13}CO_2$ labelling and together with the substrate were stored in a freezer and then sent to IB ASCR.

Soil was dried (90 minutes in 70 º C) and milled. The shoots were dried (90 min 65 ºC) and weighted. The colonization rate was assessed in the roots using a dissecting microscope – numbers of colonized and non-colonized root tips were counted and root length was measured. Some of the well colonized root tips were cross-sectioned and observed using an upright microscope (Olympus BX 60 with differential interference contrast) at high magnifications (400× and 1000×) for presence of the Hartig net and a hyphal mantle. Microphotographs were taken by OLYMPUS DP70 camera. Root tips cross sections were observed under scaning electron microscope FEI Quanta 200 ESEM (Environmental Scanning Electron Microscopy) with GSED electron detector, sampes were cooled on 12.5 ºC, no preparation technique was used. In three selected plants the majority of sclerotia were cut of the root tips and fungal samples were thus prepared. The roots were then dried (90 min 65 ºC) and weighted. Substrate from root and fungal compartments and shoots were sent back to Aberdeen for further analyses.

Samples for mass spectrometry were dried (12 hours in 75 ºC), weighted and encased in aluminium foil and then the isotopic composition of needle samples, sclerotia samples, root compartement soil samples and fungal compartement soil samples were measured.

3.4.4 Data processing

Data were analysed using STATISTICA 12 (StatSoft Inc.). Normal distribution of the data was checked and logarithmical transformation was used when needed. The differences between colonization rates, fresh root weight and dry shoot weight were evaluated by Breakdown and one-way ANOVA followed by Tukey´s HSD test. Means were compared at a significance level of $P < 0.05$.

3.5 Experiment 5. In vitro experiment with B. pendula and DSE (A. macrosclerotiorum, P. fortini s.s., A. applanata)

In this experiment we wanted to determine whether *A. macrosclerotiorum* is able to associate with roots of a broad leaf tree - European birch (*B. pendula*) in *in vitro* conditions. For each treatment and negative control 6 replications were prepared.

3.5.1 Plant and fungi cultivation

Birch seeds were rid of the wings, surface sterilized in 30% H_2O_2 for 12 minutes and then rinsed twice in autoclaved tap water. Germination occurred in 9 cm Petri dishes filled with MMN media without glucose and malt extract at room temperature in the dark. After 3 weeks the seedlings were planted on a new media and placed in a growth chamber (16 hours of light at 20°C, one tungsten lamp and relative humidity 80%) for one month.

Four fungal isolates were selected for this experiment: AMA 1, AMA11, AAP 1 (see table 1) and the isolate PIN5 of *Paxillus involutus* (Batsch) Fr. (Origin: Varnsdorf, Czech republic, isolated from a fruit body) as positive ectomycorrhizal control.

3.5.2 Experimental design

Experimental system was similar to the Experiment 2 - fungal plugs were inserted into falcon tubes with 10 ml of MMN media without glucose and malt extract and cultivated for one month in the dark. Approximately 10 ml of twice autoclaved peat vermiculite substrate (1: 5 : 6, v.v. peat : vermiculite : water) was then added to the system and one birch seedling was planted into the substrate. The microcosms were then closed and placed in a growth chamber (16 hours of light at 20°C, one tungsten lamp and relative humidity 80%, Fitotron, SANYO).

3.5.3 Data collection

The seedlings were harvested after 6 months. The roots were cleaned in tap water, surface dried with towel paper, weighted and stained in Trypan blue (see 2.1.3). Roots were de-stained in lactoglycerol and then observed with an upright microscope with differential interference contrast at high magnifications (400× and 1000×) and presence of microsclerotia and hyphae was counted in 80 microscopic

fields per each root system under 400× magnification, similarly to Kohout *et al.*, 2012. To determine shoot dry weight the shoots were dried (90 min at 65 º C) and then weighted.

From each of the microcosms 4 samples of peat were aseptically taken and placed on MMN media in 9 cm Petri dishes. Dishes were stored for a month in dark at 20 °C and repeatedly checked for signs of fungal growth.

3.5.4 Data processing

Data were analysed using STATISTICA 12 (StatSoft Inc.). Normal distribution of the data was checked. The differences between colonization rates, fresh root weight and dry shoot weight were evaluated by Breakdown and one-way ANOVA followed by Tukey´s HSD test. Means were compared at a significance level of $P < 0.05$.

4. Results

4.1 Experiment 1. In vitro resynthesis experiment with P. abies, V. myrtillus and 10 different DSE species

In the roots of all inoculated plants fungal colonization was observed. Non-inoculated controls were successfully kept free of fungal infection and neither inter- nor intracellular colonization were detected. Structures observed in the roots are listed in Table 2.

Fungi		***P. abies***					***V. myrtillus***		
Species	Isolate	S	MS	InC	HM	HN	MS	C	EC
Acephala applanata	AAP1	-	+	+	-	-	+	-	-
Acephala applanata	AAP2	-	+	+	-	-	+	-	-
Acephala macrosclerotiorum	AMA1	+	-	-	+	+	+	+	-
Acephala macrosclerotiorum	AMA 11	+	-	-	+	+	+	+	-
Phialocephala europea	PF-EU-1	-	+	+	-	-	+	-	-
Phialocephala europea	PF-EU-2	-	+	+	-	-	+	-	-
Phialocephala fortinii s.s.	PFOF	-	+	+	-	-	+	-	-
Phialocephala fortinii s.s.	PFO9	-	+	+	-	-	+	-	-
Phialocephala helvetica	PF-HE-1	-	+	+	-	-	+	-	-
Phialocephala helvetica	PF-HE-2	-	+	+	-	-	+	-	-
Phialocephala letzii	PF-LE-1	-	+	+	-	-	+	-	-
Phialocephala letzii	PF-LE-2	-	+	+	-	-	+	-	-
Phialocephala subalpina	PF-SU-1	-	+	+	-	-	+	-	-
Phialocephala subalpina	PF-SU-2	-	+	+	-	-	+	-	-
Phialocephala turiciensis	PFO2	-	+	+	-	-	+	-	-
Phialocephala turiciensis	PFO6	-	+	+	-	-	+	-	-
Phialocephala uotolensis	PF-UO-1	-	+	+	-	-	+	-	-
Phialocephala uotolensis	PF-UO-2	-	+	+	-	-	+	-	-
Phialocephala glacialis	PF-GL-1	-	-	+	-	-	+	+	-
Phialocephala glacialis	PF-GL-2	-	+	+	-	-	+	+	-
Rhizoscyphus ericae	RER-1	-	-	-	-	-	-	+	+
Rhizoscyphus ericae	RER-6	-	-	-	-	-	-	+	+

Table 2 Fungal structures observed in the roots of *V. myrtillus* and *P. abies* colonized by 10 different DSE species, two isolates per each. (S – sclerotium, MS – microsclerotium, InC – intracellular colonization, HM – hyphal mantle, HN – Hartig net, C – coils, EC – ericoid mycorrhizal coils). + denotes presence of the structure, - denotes absence of the structure in the root sample.

Conditions in microcosms used were favorable for formation of ErM because *R. ericeae* was able to form intracellular hyphal coils typical for ErM in roots of *V. myrtillus*.

Almost all species belonging to PAC formed intracellular microsclerotia (Fig 9) in both plant hosts species. They consisted of melanised or hyaline hyphae (Fig 9). Only *A. macrosclerotiorum* colonized *P. abies* strictly intercellulary and formed Hartig net and hyphal mantle (Fig. 10). Additionally darkly pigmented sclerotia were formed on the surface of some roots. In *V. myrtillus* roots coils similar to ErM and microsclerotia were observed (see Fig. 9)

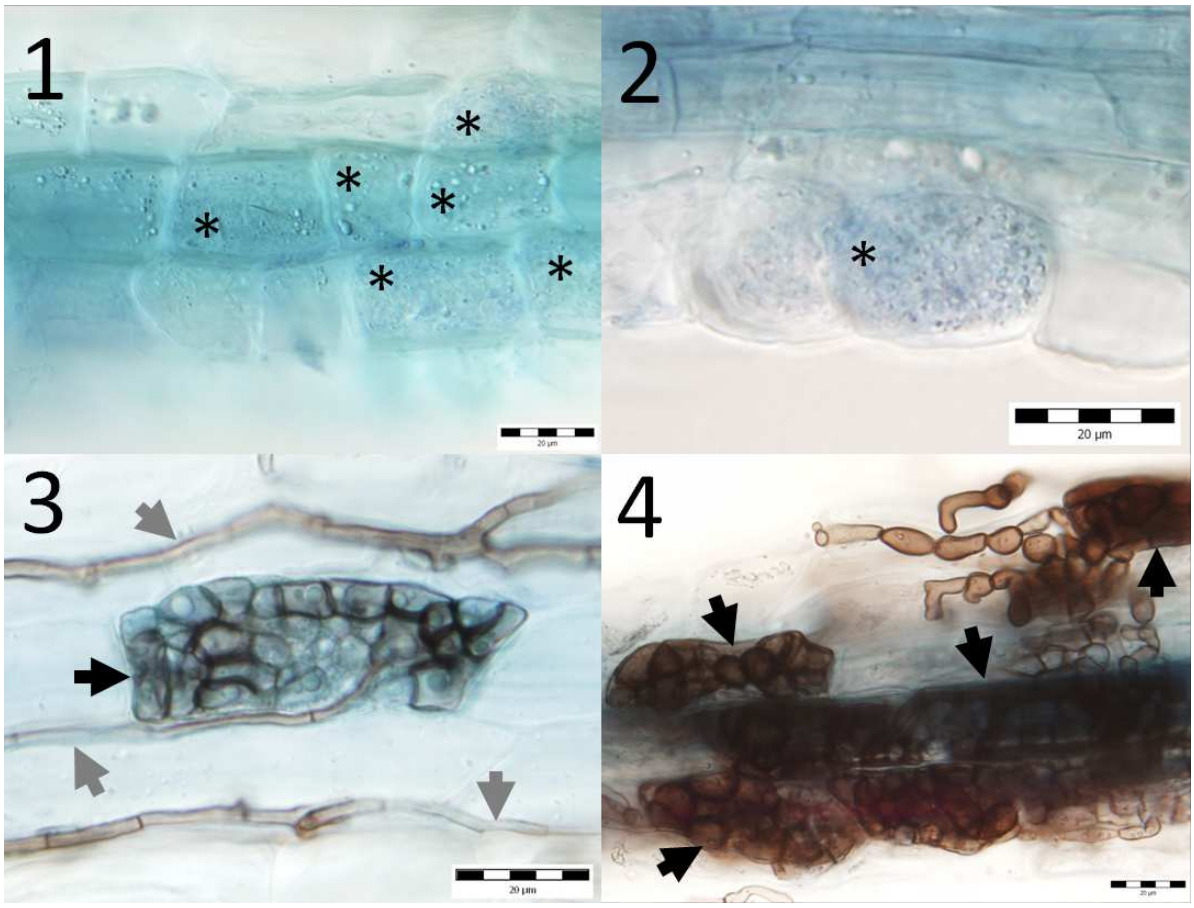

Figure 9 Structures formed in roots of *V. myrtillus* by *A. macrosclerotiorum* (1 and 2), by *P. helvetica* (3) and by *P. fortinii* (4) 1, 2 - Intracelullar hyphal loops (asterisks), 3 – microsclerotium (black arrow) and melanised hyphae (grey arrows), 4 – massive intercellular colonization by microsclerotia. All bars correspond to 20 µm, all roots were stained in trypan blue, observed by light microscopy, bright field.

Colonization resembling ErM was formed also by *P. glacialis* which however did not form EcM structures and colonized roots of *P. abies* intracellulary.

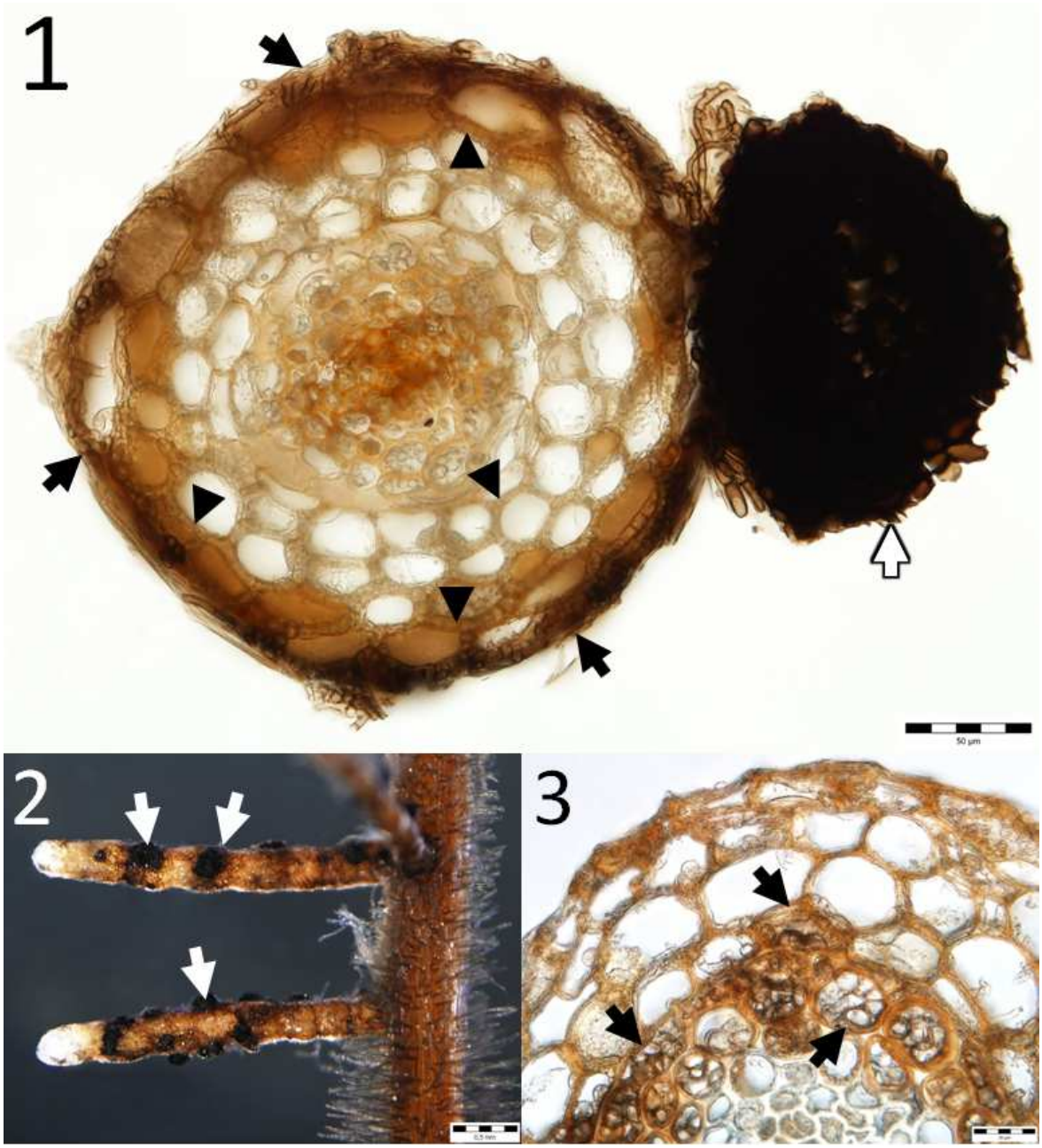

Figure 10 Structures formed in roots of *P. abies* by *A. macrosclerotiorum* (1, 2) and *P. Helvetica* (3) 1 – Root tip cross-section with sclerotium, hyphal mantle (black arrows) and Hartig net (black arrowheads) bar corresponds to 50 µm, 2 - Root tips with sclerotia (white arrowheads) bar corresponds to 0.5 mm, 3 – Intracellular colonization by microsclerotia (black arrows) bar corresponds to 20 µm, 1,3 observed by light microscopy: bright field, 2 – dissecting microscopy.

Differences in seedling survival were observed between the isolates but due to low number of replicates no statistical analysis of the data was possible.

4.2 Experiment 2. In vitro resynthesis experiment with V. myrtillus and 10 different DSE species on peat substrate

All of the inoculated plants had fungal structures developed in the roots. *V. myrtillus* seedlings were colonized in a similar manner as in the Experiment 1. ErM structures were formed only in the roots of plants inoculated with *R. ericeae*. Intracellular hyphal coils similar to ErM were formed by AAP-1, AAP-2, AMA-1, AMA-11, PH-GL-1 and PH-GL-2. Other isolates formed melanised or hyaline microsclerotia. In contrast to the previous results sclerotia were observed on root the surface of seedlings inoculated by AAP-1, AMA-1 and AMA-11 while both *A. macrosclerotiorum* isolates did not form microsclerotia. Control plants did not show any fungal infection.

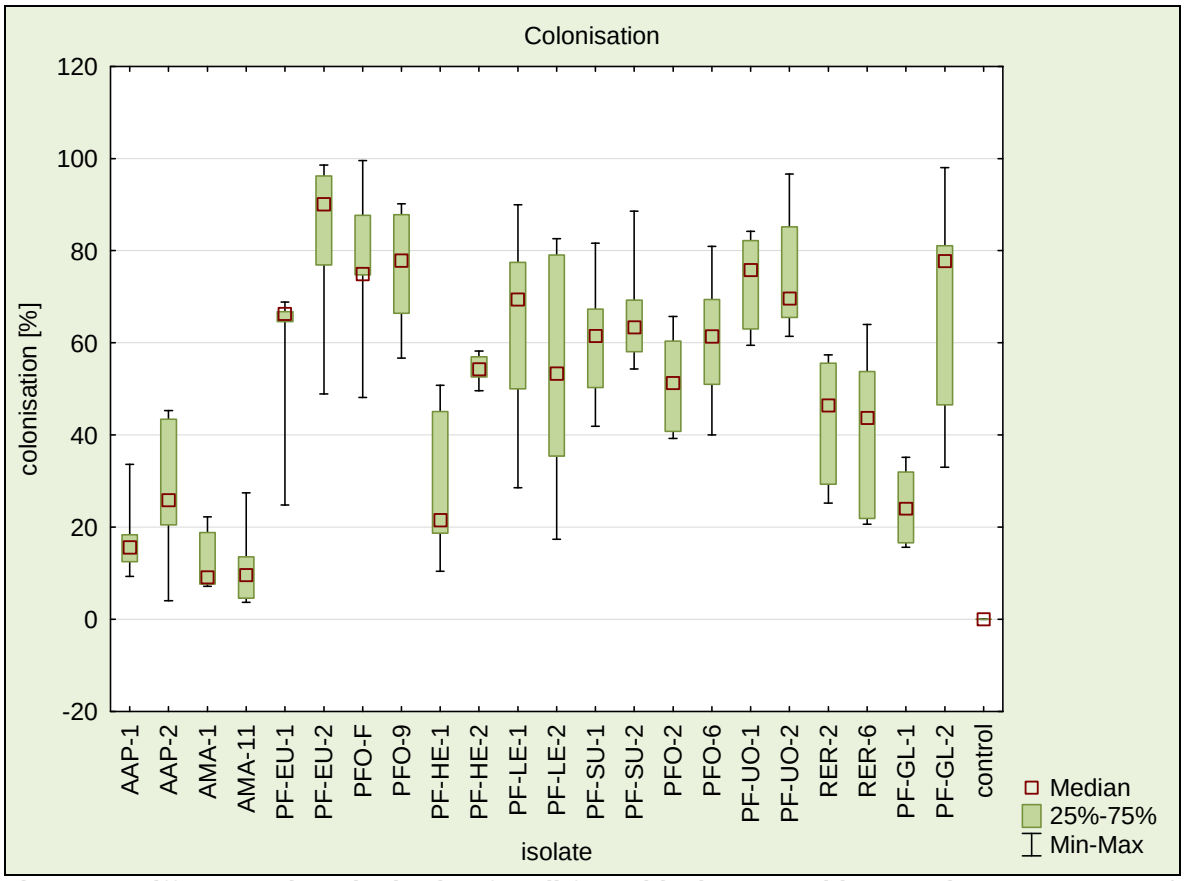

Figure 11 Differences in colonization for all fungal isolates used in Experiment 2 Compared at a significance of $P < 0.05$. The results of ANOVA significances are given in Table attachement 1.

As is to be seen on Fig. 11 the colonization varied between isolates. The isolates tended to form two groups – group 1 consisted of *A. applanata*, *A. macrosclerotiorum*, PF-HE-1, PF-GL-1 with low colonization rates (they were not statistically different from control) and Group 2 consisting of P*. europea*, *P. fortinii*, PF-HE-2, *P. letzii*, *P. subalpina*, *P. turiciensis*, *P. uotolensis* with higher percentage of colonized cells. Some isolates of the same species showed high dissimilarity (*P. helvetica, P. glacialis*) and were even significantly different from each other. Table with statistical significances attached (Attachement 1).

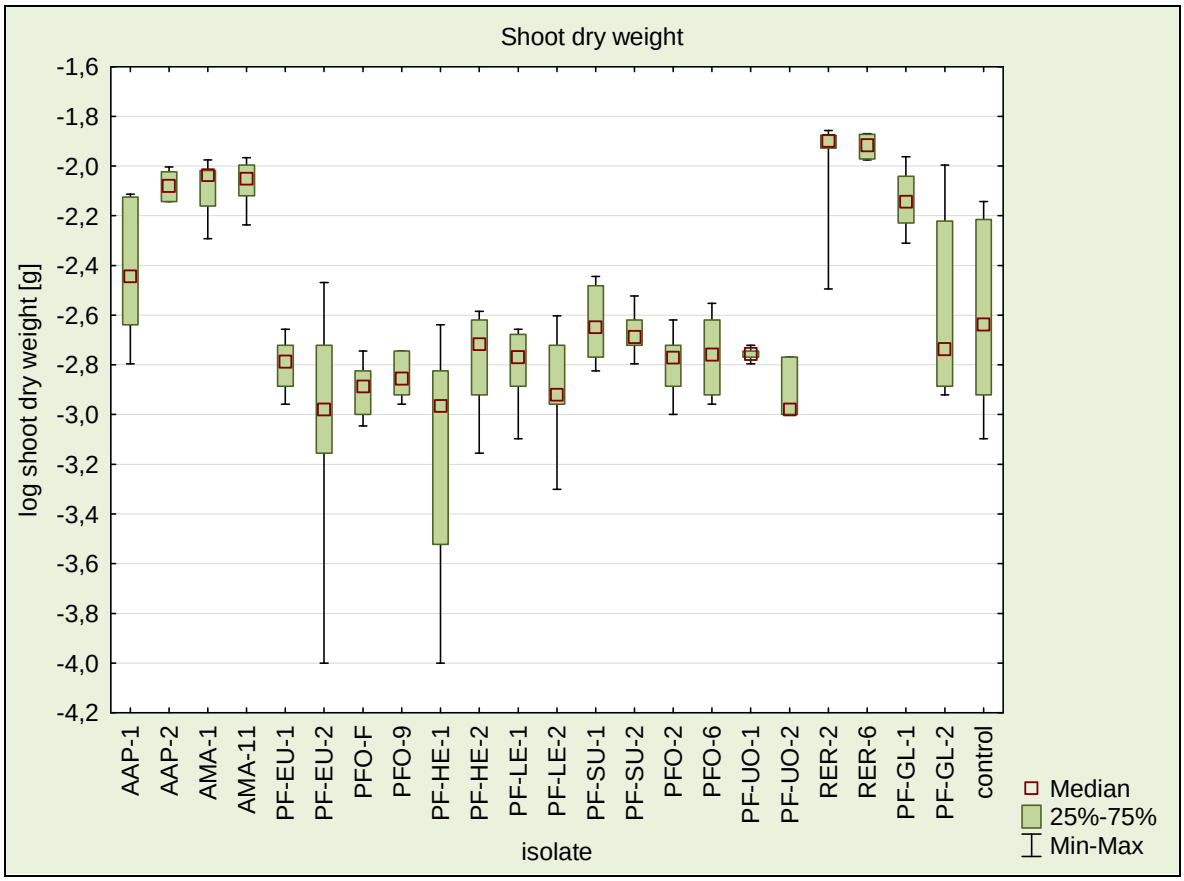

Figure 12 Differences in dry shoot weight for all fungal isolates used in Experiment 2 Compared at a significance of $P < 0.05$. The results of ANOVA significances are given in Attachement 2.

Influence of fungal isolate on dry shoot weight is displayed on Fig. 12. Similarly to the colonization rates a trend to form two groups is to be seen. Group one is formed by AAP-2, both isolates of *A. macrosclerotiorum* and the isolate PGL-1 and does not significantly differ from the positive mycorrnizal control (*R.*

ericeae). Group 2 consists of *P. europea*, *P. fortinii*, *P. hevetica, P. letzii*, *P. subalpina*, *P. turiciensis*, *P. uotolensis* and the isolate PF-GL-2. The isolate AAP-1 does not seem belong to either of the groups – it does differ from one of the positive control isolates but not from the other. Isolates AAP-1 and PF-GL-2 differed greatly from the other isolate of the same species. Table with statistical significances attached (Attachement 2).

Fresh root weight of the seedlings was also influenced by fungal isolate although inoculated plants did not differ from the uninoculated control (see Fig. 13). The tendency of forming two separate groups is noticeable for this parameter, too. The Group 1 consists of *A. applanata, A. macrosclerotiorum* and isolates PF-GL-1 and PF-SU-2 and is not statistically different from the positive mycorrhizal control. The Group 2 that differs from most of the isolates in the Group 1 and from the positive mycorrhizal control is formed by *P. europea*, *P. fortinii*, *P. hevetica, P. letzii*, PF-SU-1, *P. turiciensis* and *P. uotolensis.* The difference between isolates of one species is most visible in *P. subalpina and P. glacialis.* Table with statistical significances attached (Attachement 3).

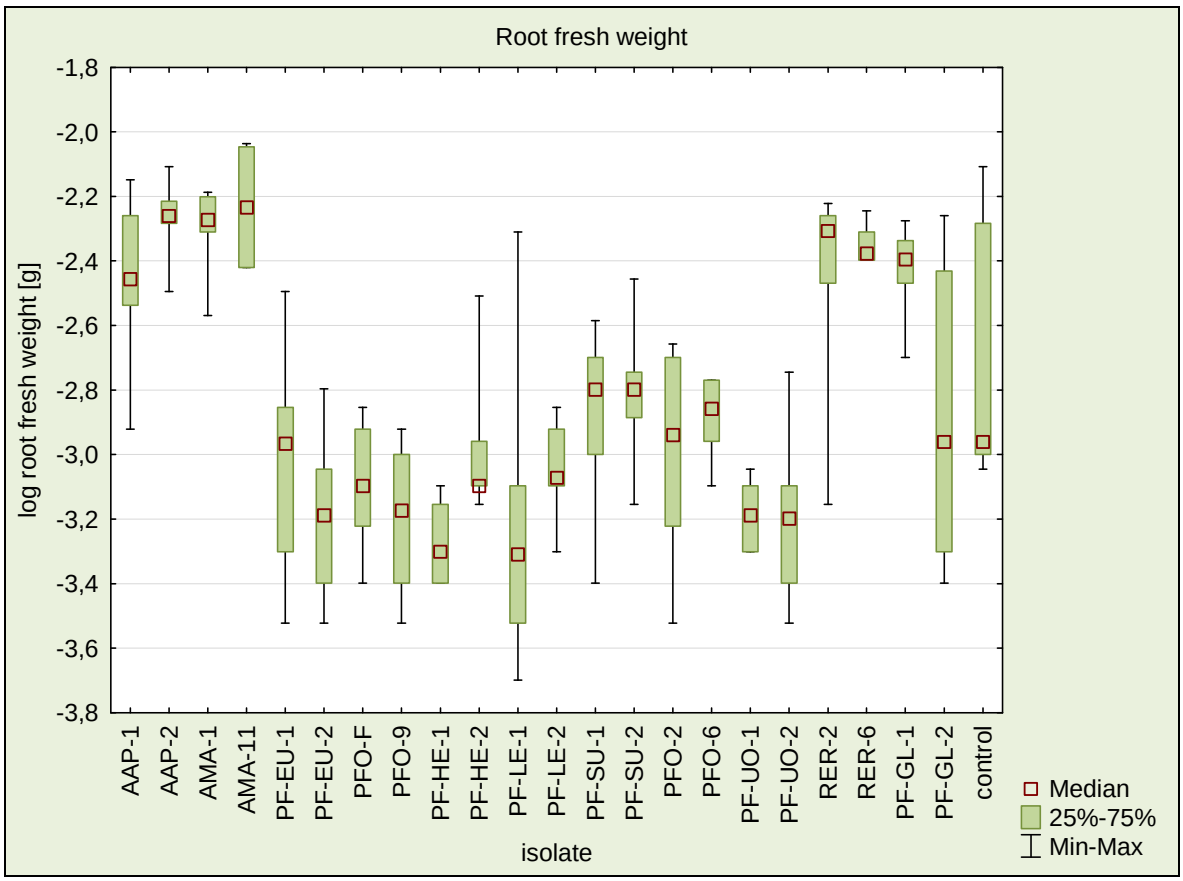

Figure 13 Differences in root fresh weight for all fungal isolates used in Experiment 2 Compared at a significance of $P < 0.05$. The results of ANOVA significances are given in Attachement 3.

4.3 Experiment 3. Pilot in vitro resynthesis experiment with P. abies and A. macrosclerotiorum labelled by ^{33}P to trace nutrient transfer from fungus to plant

Digital autoradiography does not provide information about anexact amount of radioactive ^{33}P transferred into the shoot but enables us to see its distribution in the experimental system.

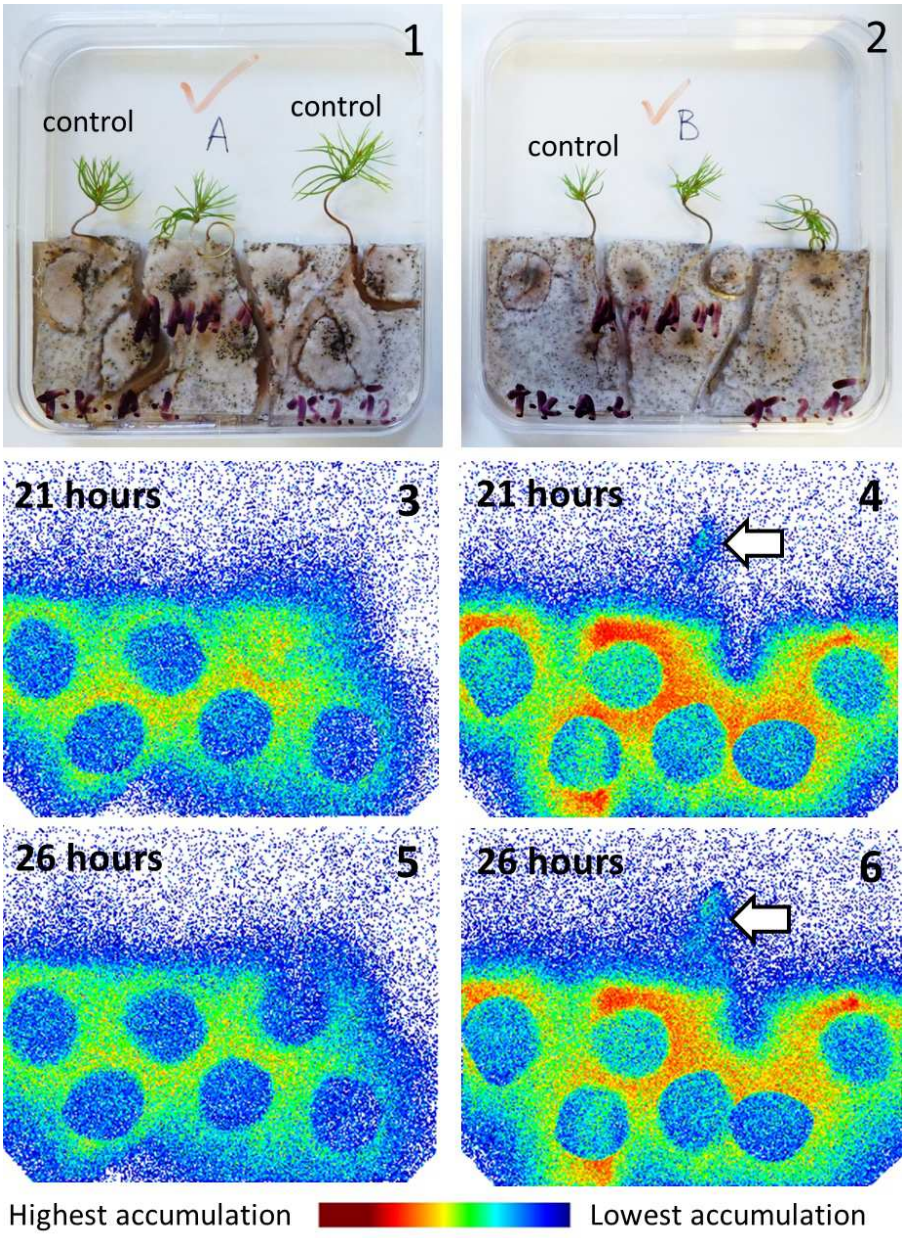

Figure 14 Transport of ^{33}P to shoot mediated by *A. macrosclerotiorum* 1,2 – Photographs of Petri dishes with 3 seedlings of *P. abies* in each with three control plants, 3,4 – digital autoradiography of the dishes 21 hours after applying ^{33}P 5,6 - digital autoradiography of the dishes 26 hours after applying ^{33}P, shoot of middle seedling with increased ^{33}P content marked by white arrow

In the pilot experiment one of the seedlings (dish 2, middle section seedling B) showed an apparent increase in the content of ^{33}P in the shoot 21 and 26 hours after applying solution with ^{33}P (see Fig. 14). Control plants did not show ^{33}P enrichment in shoot. All of the plants were colonized by *A. macrosclerotiorum* and typical structures for EcM were formed.

Although we were not able to measure the quantity of ^{33}P transported to the shoot of *P. abies,* the nutrient transport was detected in one of the labelled plants. Based on the results of the pilot Experiment 5 was made to test whether bidirectional transport of nutrients between *P. abies* and *A. macrosclerotiorum* occurs.

4.4 Experiment 4. In vitro resynthesis experiment with P. abies and A. macrosclerotiorum labelled by ^{13}C and ^{15}N isotopes

All plants inoculated by *A. macrosclerotiorum* were colonized. The root tips were covered by hyphal mantle with sclerotia (see Fig. 15 and 16) and Hartig net was formed intercellulary (see Fig. 15).

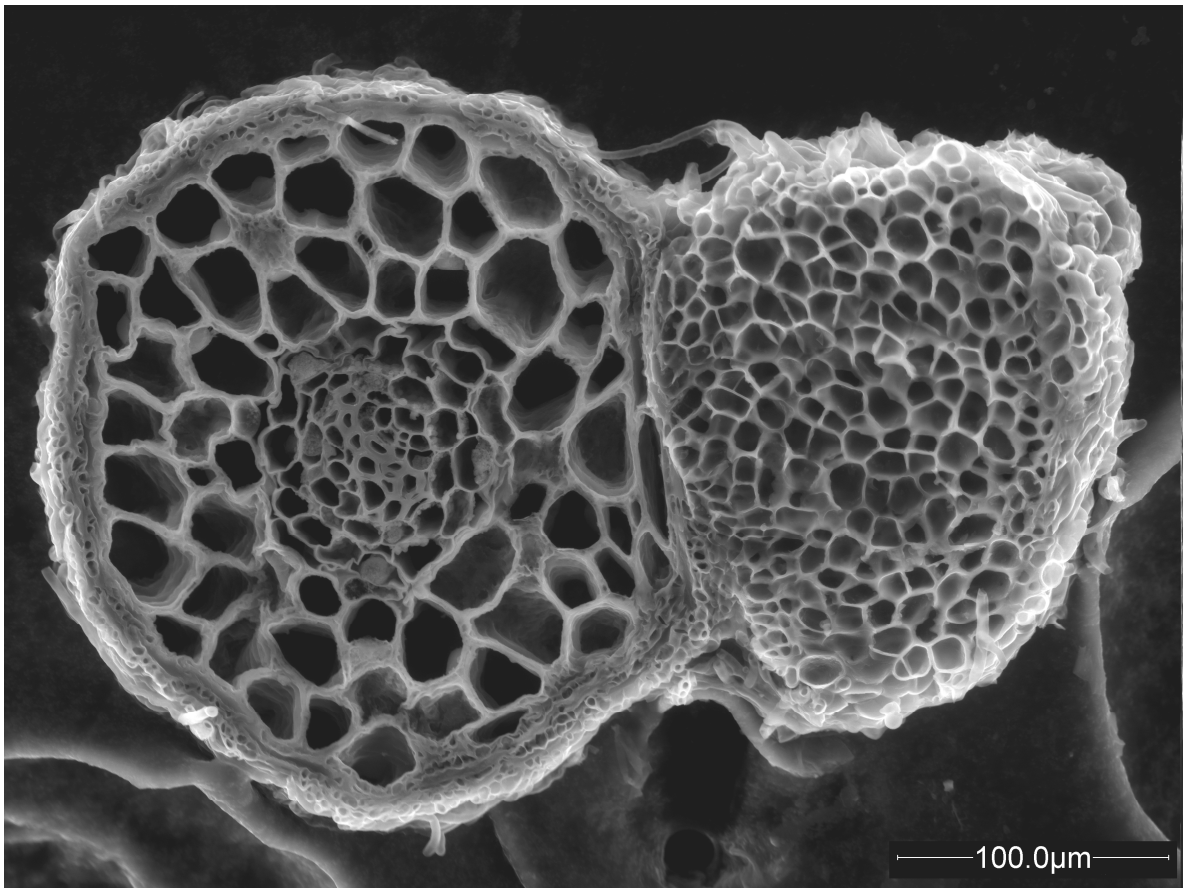

Figure 15 Cross-section of a root tip of *P. abies* colonized by *A. macrosclerotiorum.* Hyphal mantle is visible on the root surface and Hartig net between cortex cells. A sclerotium formed by melanised hyphae is attached on right side of the root. Scanning electron microscopy, bar corresponds to 100 µm.

A flow of ^{13}C from plant to fungal structures was observed. Sclerotia from labelled host plants showed significantly higher content of ^{13}C than sclerotia form non-labelled plants.

Percentage of ^{13}C in the fungal compartment was not significantly higher in the inoculated plants than in the control plants. Similar results were measured in the root compartment soil. A carbon flow from plant tissues to hyphae and soil in both compartments was not observed.

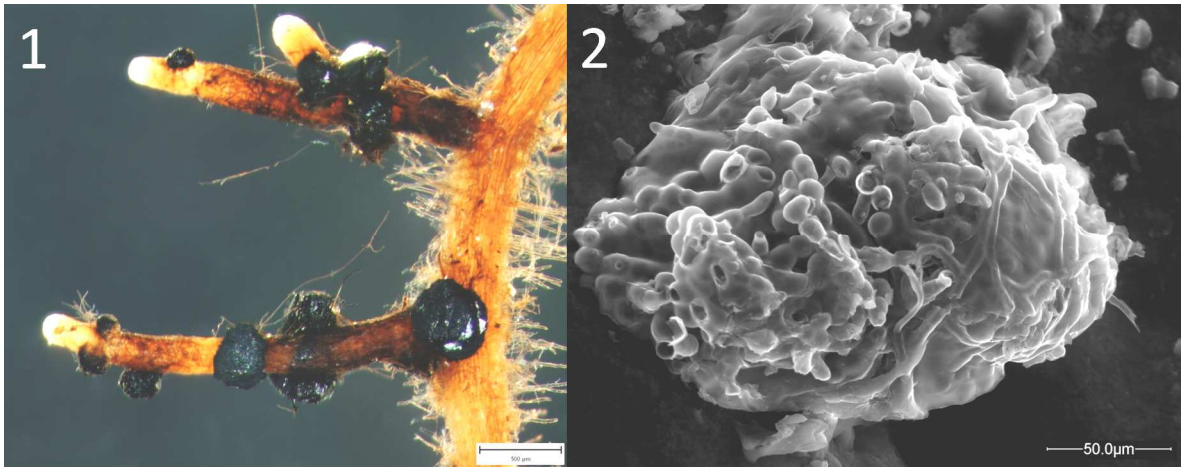

Figure 16 Structures formed on roots of *P. abies* by *A. macrosclerotiorum* 1 – Root tips with sclerotia. Dissecting microscopy, bar corresponds to 500 µm, 2 – Surface structure of sclerotia. Scanning electron microscopy, bar corresponds to 50 µm

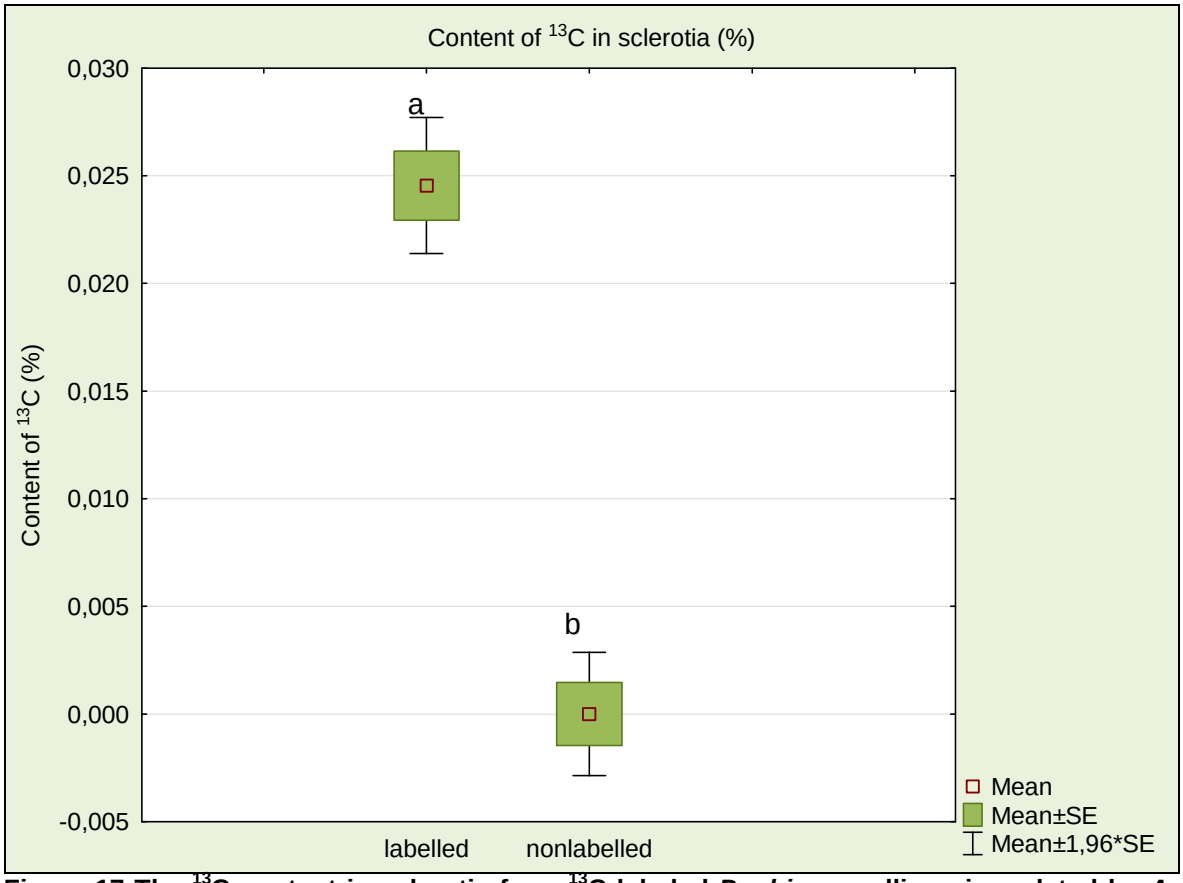

Figure 17 The ^{13}C content in sclerotia from ^{13}C labeled *P. abies* seedlings inoculated by *A. macrosclerotiorum* and in non-labelled plants. Different letters correspond to significant differences at $P < 0.001$

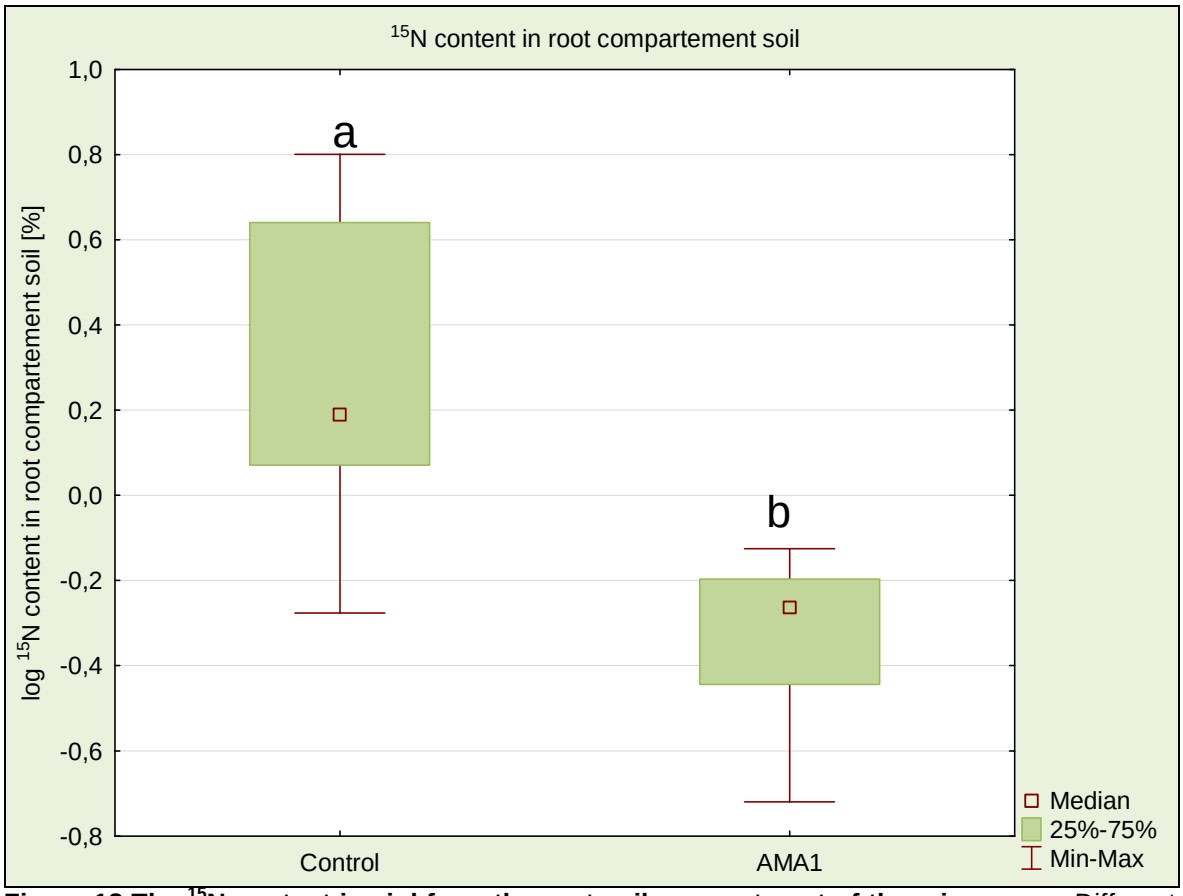

Figure 18 The ^{15}N content in siol from the root soil compartment of the microcosm. Different letters correspond to significant differences at $P < 0.05$

A significantly higher amount of ^{15}N was measured in the root compartment of the control uninoculated plants than in the inoculated plants (see Fig. 18). The percentage of ^{15}N in needles and shoot biomass did not significantly differ between control and inoculated plants, the values were lower for inoculated plants in both cases.

Total content of N in the shoot biomass was significantly higher in the control plants than in the inoculated plants (see Fig. 19)

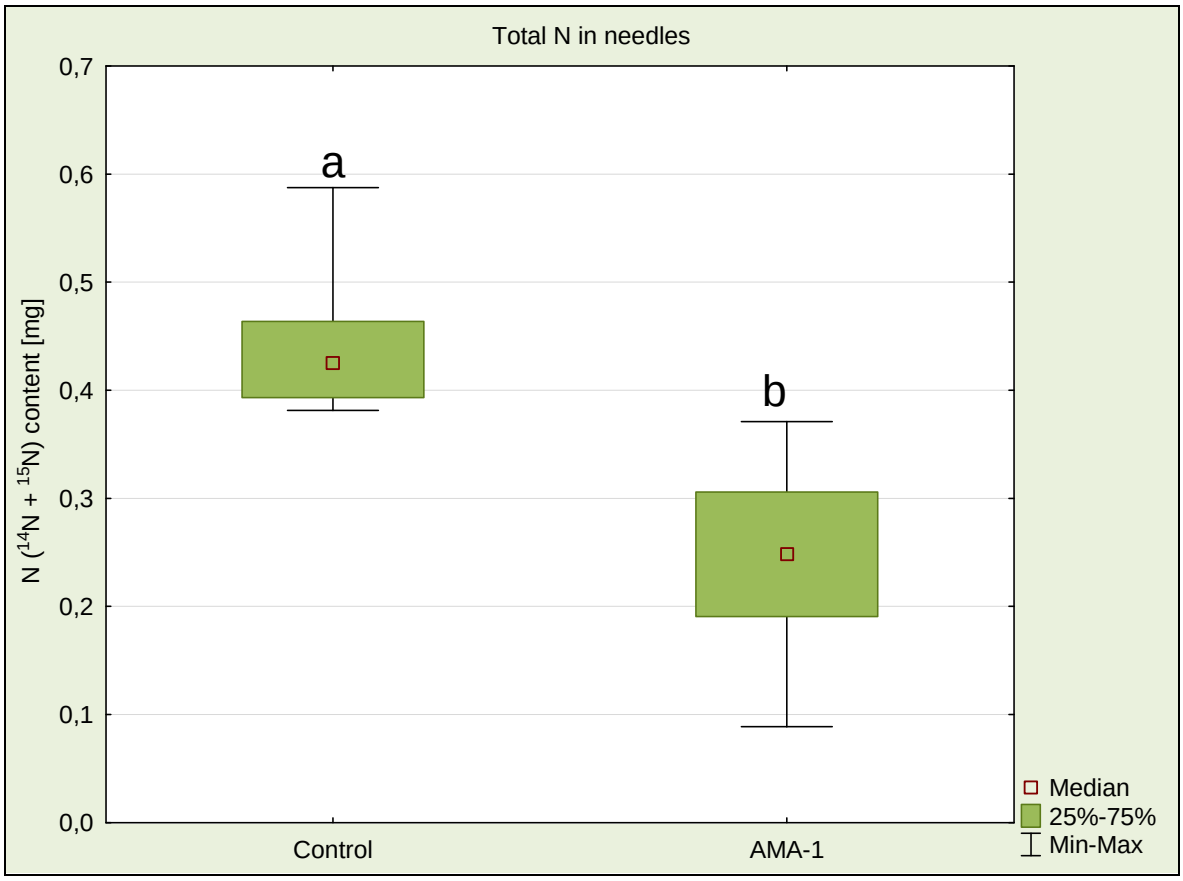

Figure 19 Total shoot N in control and AMA - 1 inoculats seedlings. Different letters correspond to significant differences at $P < 0.05$

4.5 Experiment 5. In vitro experiment with B. pendula and DSE A. macrosclerotiorum, P. fortini s. s. and A. applanata

All of the seedlings inoculated by fungi were colonized. The percentage of root sections colonized was significantly higher in AAP-1 and AMA-1 than in AMA-11 (see Fig. 20)

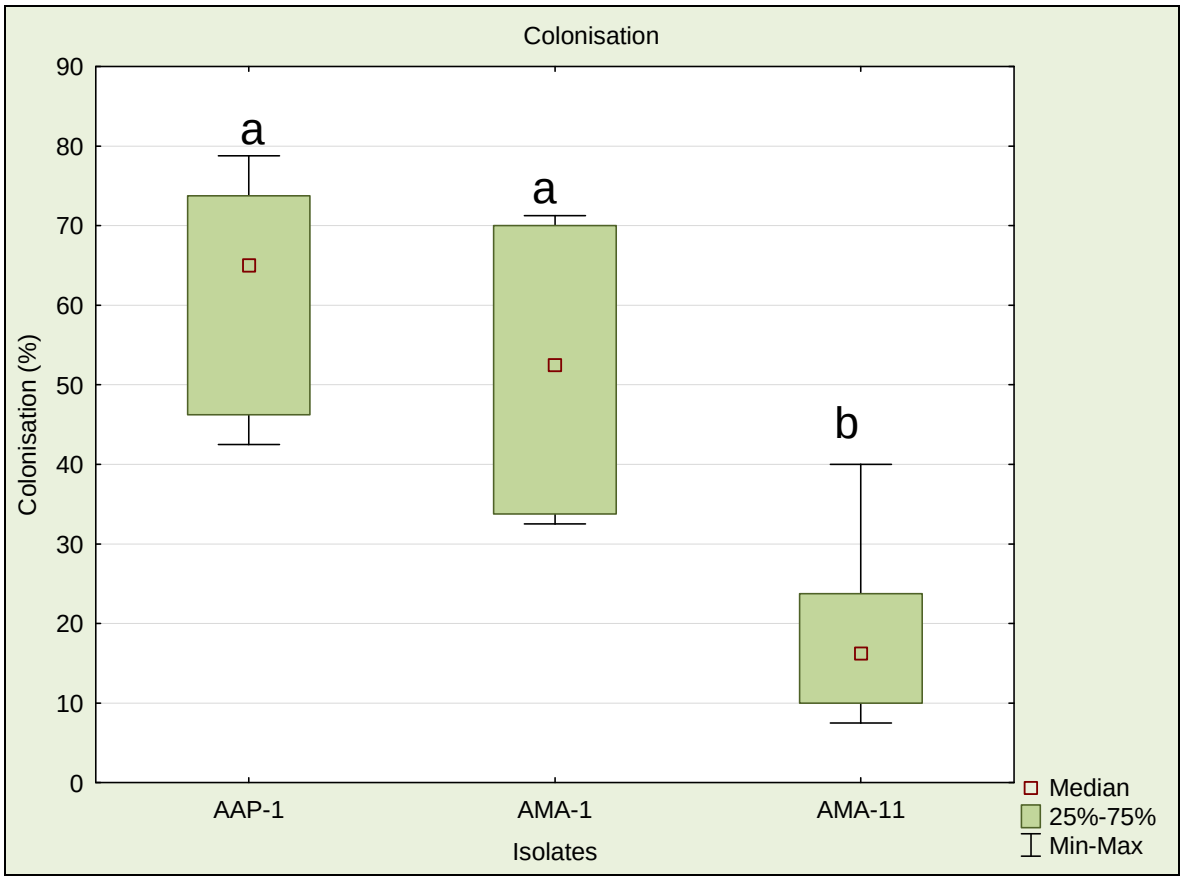

Figure 20 Percentage of colonized root sections in roots of *B. pendula* colonized by three different *Acephala* species (AAP-1, AMA-1 and AMA-11). Different letters correspond to significant differences at $P < 0.05$

No EcM structures formed by *A. macroscletoriorum* were observed but roots were colonized intracellulary by loose hyphal loops and intercellulary by melanised running hyphae. *A. applanata* formed microsclerotia and sclerotia on the root surface. In the roots of all plants inoculated by *P. involutus* a hyphal mantle and the Hartig net were observed (see Fig. 21). The control plants did not show any signs of fungal infection.

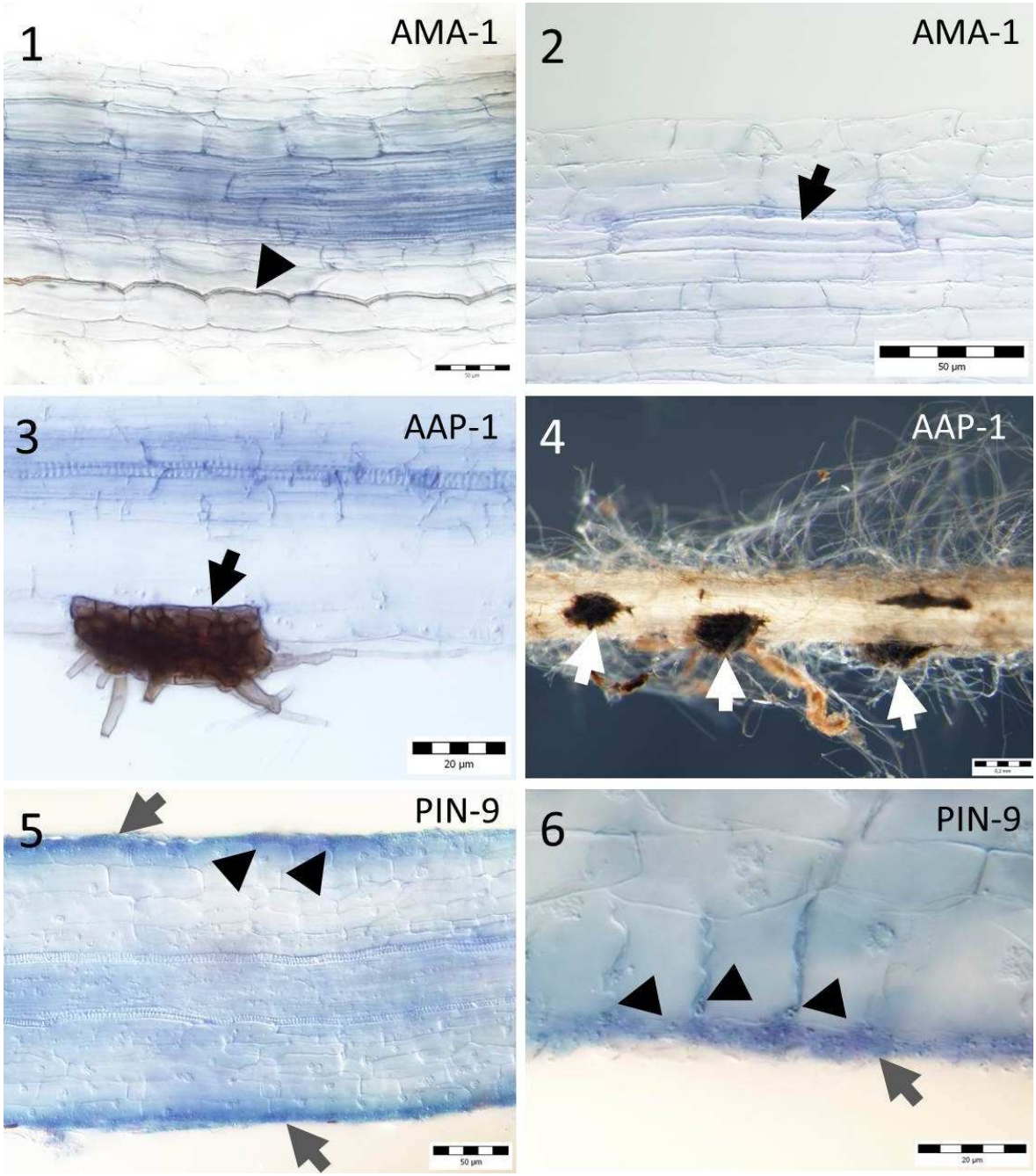

Figure 21 Structures formed in roots of *B. pendula* by *A. macrosclerotiorum* (1,2), *A. applanata* (3,4) and *P. involutus* (5,6) 1 – Intercellular colonization by melanised hyphae (black arrowhead) 2 – Intracellular colonization by hyaline hyphae (black arrow) 3 – Intercellular microsclerotium (black arrow) 4 – Sclerotia on the root surface (white arrows) 5,6 – Hyphal mantle on the root surface (grey arrows) and Hartig net (black arrowheads). 1-3, 5 and 6 – light microscopy: bright field 4 – dissecting microscopy. 1-3, 5 and 6 stained by trypan blue. Bars correspond in 1, 2 and 6 to 50 µm, in 3 to 20 µm and in 4 to 0.2 mm.

The inoculation had an influence on dry shoot weight. All inoculated plants except positive mycorrhizal control had significantly higher shoot biomass than control plants. Significant differences were also observed in influence of the isolate – plants inoculated by AAP-1 had significantly higher shoot dry weight than those inoculated by both isolates of *A. macrosclerotiorum* and *P. involutus*. For more information see Fig. 22)

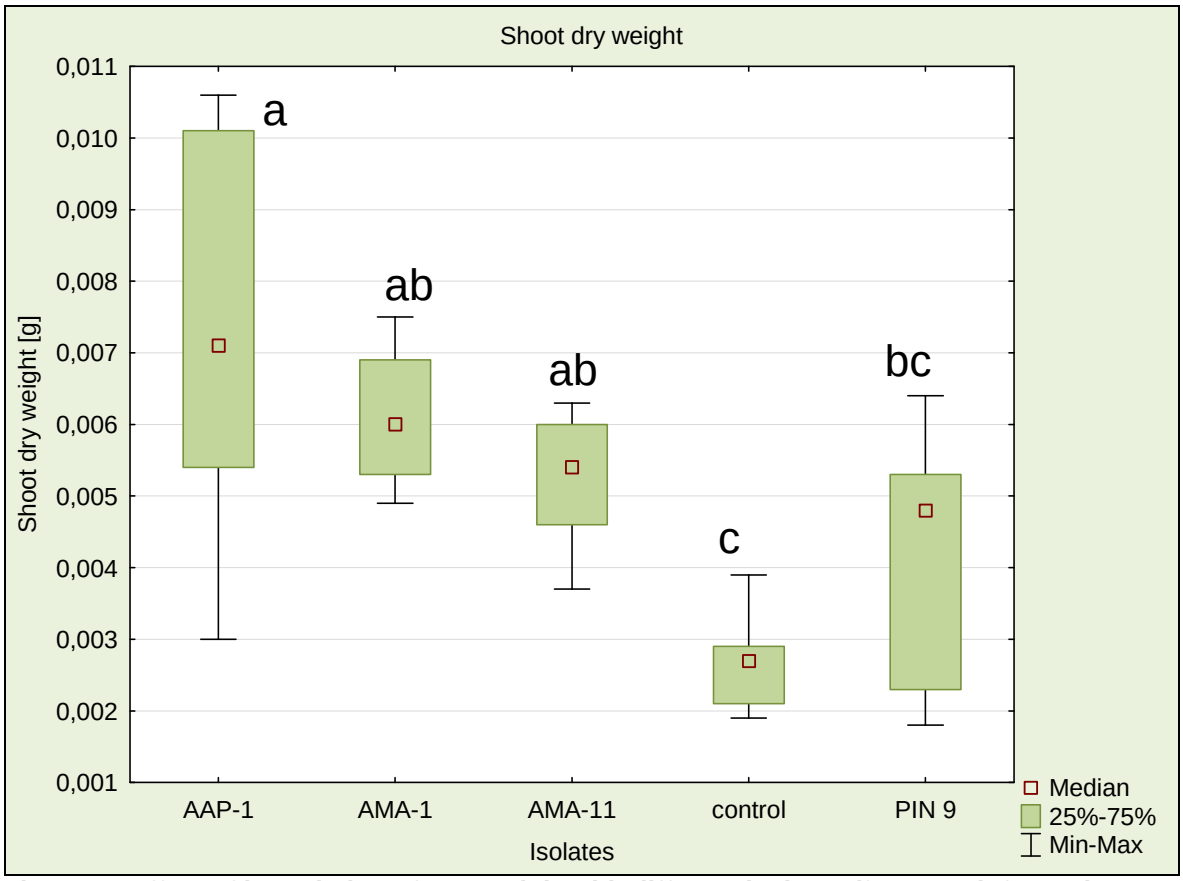

Figure 22 Effect of inoculation of *B. pendula* with different isolates (for more information see Table 1) on dry shoot weight. Different letters correspond to significant differences at $P < 0.05$

Differences were also observed in fresh root weight (see Table 23) – all of the inoculated plants had higher root biomass than the uninoculated control plants. An effect of specific isolates was not observed.

Correlation between colonization and dry shoot weight and fresh root weight was tested, but no significant correlation was found.

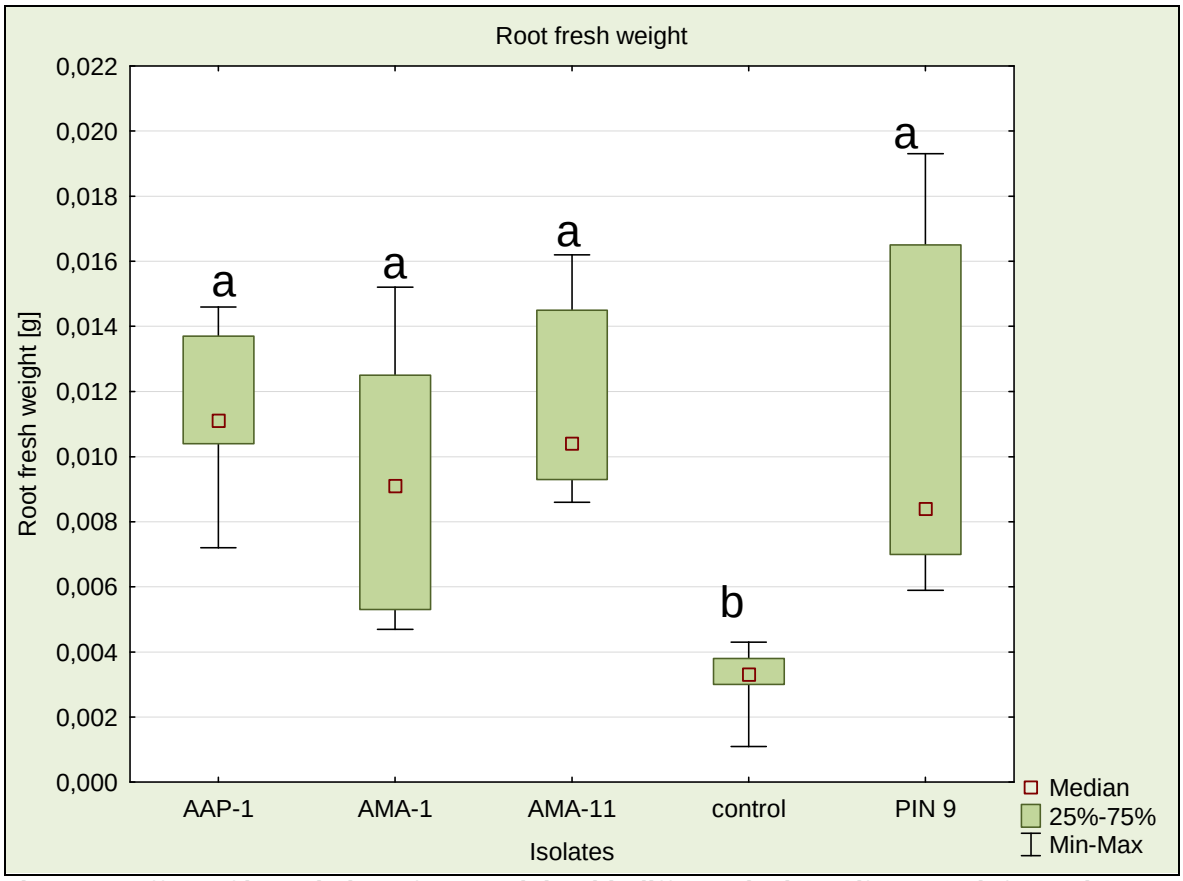

Figure 23 Effect of inoculation of *B. pendula* with different isolates (for more information see Table 1) on fresh root weight. Different letters correspond to significant differences at $P < 0.05$

Concentration of CO_2 and O_2 was measured to show whether the plant growth is not limited by source of C and O in the experimental system. No significant differences were noted in concentration of O_2. The concentration of CO_2 is shown in Fig. 24. Only one isolate (AMA-1) showed higher values of CO_2. No correlations between elevated CO_2 concentration and fresh root weight or dry shoot weight were observed.

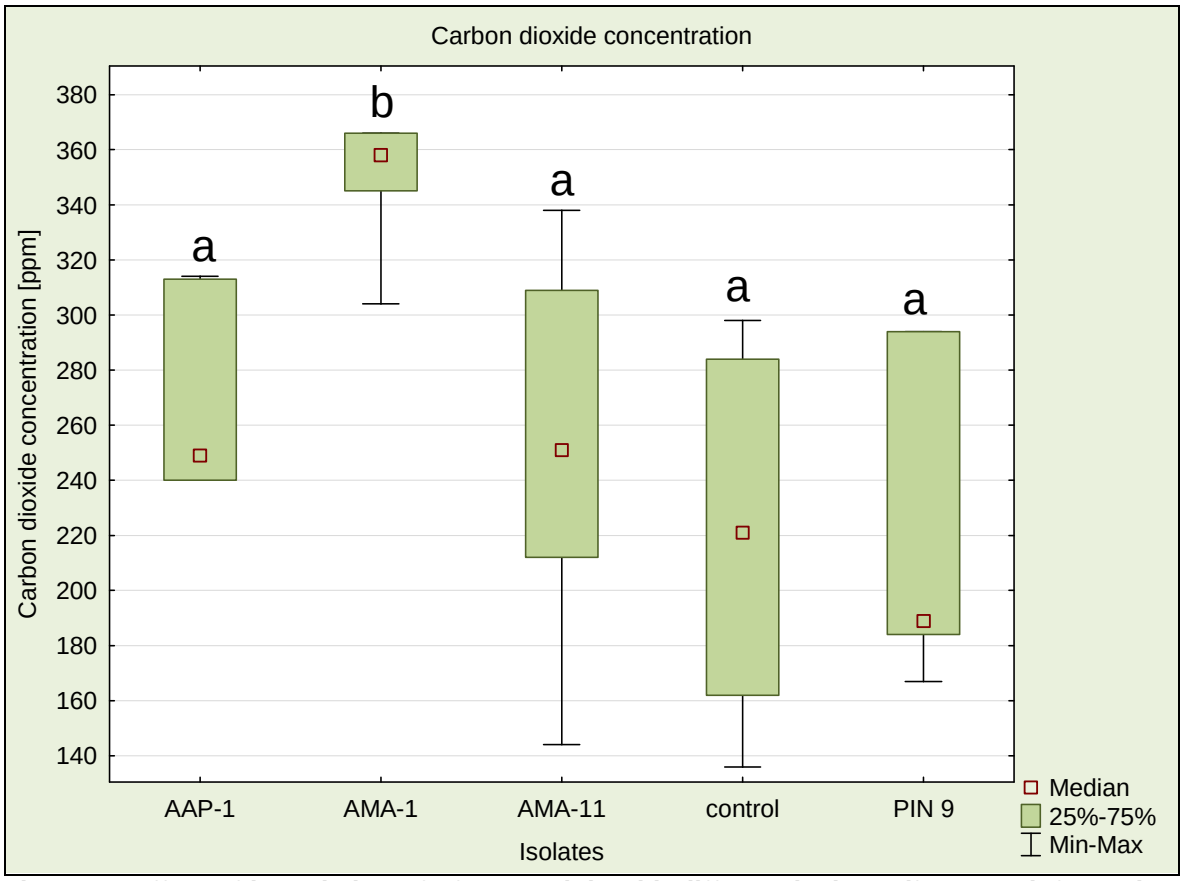

Figure 24 Effect of inoculation of of *B. pendula* with different isolates (for more information see Table 1) on CO_2 concentration. Different letters correspond to significant differences at $P < 0.05$

5. Discussion

My work is focused on interactions between dark septate endophytes (DSE), ubiquitous symbionts of plant roots, and two host plants which often coexist in forest ecosystems not only in Central Europe – *P. abies* and *V. myrtillus*. DSE were detected in virtually every plant species screened (Jumpponen & Trappe, 1998a) and their ecophysiological role has been intensively discussed since their discovery in 1922 (Melin, 1922) up to last work published not a month ago (Zhang *et al.*, 2013). I wanted to contribute to the knowledge of this elusive group of fungi; thanks to international collaboration a unique collection of DSE was completed and thus experiments with the group of the most frequent plant root endophytes could be conducted.

5.1 Methodical approaches

5.1.1 *Picea abies* and *Vaccinium myrtillus* as model plants

P. abies was chosen as a model plant for our experiments because it is one of the most common forest conifer species in the Czech Republic and Central Europe. It is able to form ectomycorrhizal symbiosis with various asco- and basidiomycetes but *P. abies* ectomycorrhizae regularly host a wide range of co-associated endophytes including DSE (Vohník *et al.*, 2013) Moreover DSE were first described to form associations with *P. abies* and *P. sylvestris* (Melin, 1922). Not least, its seeds germinate in *in vitro* conditions successfully.

Co-occurrence of *P. abies* and members of the Ericaceae in Central European forests is very common (Kohout *et al.*, 2011). Germination in *in vitro* system was successfully performed for *V. myrtillus* and in spite of its slow growth in sterile conditions (Grelet & Johnson, 2009) culture of aseptic plants was established in our department. DSE often associate with roots of Ericaceae (Vohník & Albrechtová, 2011) and thus DSE colonization was reported from both model plants used in our study. Therefore various features such as CMN between EcM and ErM plants could have been studied using these model plants.

5.1.2 Experimental design of *in vitro* experiments

In contrast to AMF which live as obligatory symbionts of vascular plants (Smith & Read, 2008) DSE could be cultured aseptically on agar media. Experimental system can be thus designed to observe behaviour of one individual fungal isolate and one individual plant in controlled *in vitro* conditions.

Due to limited knowledge of such a complex system as soils, aseptical *in vitro* systems are our best chance to understand physiological effects of fungal species on their host performance. The *in vitro* experimental approach enables us to filter effects of other soil microorganisms on the plan-fungus symbiosis and observe its formation in conditions of constant irradiation and humidity. It brings many advantages – ability to limit adverse effects of contaminating microorganisms, to observe the formation of symbiosis constantly and to decrease possibility of damaging the plant during harvest process (Kottke *et al.*, 1987). But the *in vitro* approach is not without disadvantages – experimental plants can suffer by morphological and physiological anomalies such as hyperhydration (Ziv, 1990). And by separating one fungal isolate from plethora of soil biota we have to be aware that observed results are to some extent artificial and their application in *in situ* system may be disputable. To overcome this limitation positive mycorrhizal controls were used in Experiments 1, 2 and 6 were used to make sure the environment in the microcosms was favourable for formation of typical mycorrhizal structures and structures observed in *in vitro* grown roots were compared to roots from natural ecosystems.

The design of the *in vitro* systems reported in this thesis underwent changes during the course of the experiments. Due to high mortality of *V. myrtillus* seedlings in Experiment 1 (unpublished data) where plants and fungi were grown on MMN agar in Petri dishes it was decided to use peat as a growth substrate in Experiment 2. Peat substrate reflects more natural conditions on sites where *V. myrtillus* seeds germinate and it also shades the roots but during the sterilisation process compounds can be released from the substrate that have adverse effects on the plants (Stribley *et al.*, 1975). Although the survival rates of the seedling in Experiment 2 were better than in Experiment 1 we decided to use peat diluted by vermiculite to reduce the possible adverse influence of autoclaved peat in Experiments 5 and 6.

The germination process was also adjusted - in the beginning MMN medium was used but the germination rates were repeatedly lower in MMN than in water agar. Although water agar is a limited source of nutrients for plants the seedlings were able to undergo the germination process successfully and malformations (such as lack of geotrophy) were observed rarely.

5.1.3 Use of stable and radioactive isotopes in study of plant-fungal relationship

Stable and radioactive isotope tracking can be very useful in studying relationships between plants and their fungal associates. It enables us to study nutrient transports from plant to fungus and vice versa. Microscopic observation of mycorhizal structures (such as arbuscules, Hartig net or hyphal coils) is not a sufficient proof of a mutualistic symbiosis – the structures do not have to be functional. The detection of changes of isotopic composition enables us to observe the crucial feature of any mutualistic root-fungus symbiosis – the nutrient flow.

The ^{33}P imagining by digital autoradiography is a very interesting technique of visualising nutrient transport. It enables us to observe a flow of radioactive elements (such as ^{33}P) in flat experimental systems in time. This ability can be very useful in studying plant-fungus symbioses in sterile and/or semi sterile *in vitro* systems.

Detection of the nutrient flow itself is not an evidence for mutualism either – the transport does not have to be caused by direct flow through mycelia but by exchanging of exudates in the substrate. Microscopic and isotopic analyses together with molecular methods of molecular detection can give a better picture of processes that take place in mycorrhizal associations.

5.2 Discussion of the results

5.2.1 Colonization patterns of ten DSE species in roots of *P. abies* and *V. myrtillus*

In the Experiment 1 the colonization of roots of *P. abies* and *V. myrtillus* by members of PAC and *P. glacialis* and *A. macrosclerotiourm* was observed in *in vitro* conditions. Cryptic species (CPS) from the *P. fortinii* s.l. complex are not only morphologically indistinguishable (Grünig *et al.*, 2008a) but in our Experiment 1

showed also similar colonization patterns in roots of both tested hosts. All of the CSP formed microsclerotia in root cells of both hosts and melanised intracellular hyphae were present in the roots as same as in plants colonized by *A. applanata*.

In contrast to all tested members of PAC, *A. macrosclerotiorum* formed ectomycorrhizal structures in roots of *P. abies*. Although DSE species were repeatedly isolated from ectomycorrhizal root tips (Melin, 1922, Kaldorf *et al.*, 2004, Urban *et al.*, 2008) it is not an evidence of their ectomycorrhizal behaviour. DSE can live in root tips as EcM co-associated endophytes and thanks to their fast growth can overgrow the slower growing EcM fungi during the isolation process. The ability of *A. macrosclerotiorum* to form EcM structures was first proved in an axenic *in vitro* experiment on *P. sylvestris* (Münzenberger *et al.*, 2009). Similarly in our experiment the fungus and host plant formed root tips with sclerotia, the Hartig net and a thin hyphal mantle. *A. macrosclerotiorum* is thus able to form ectomycorrhizae not only with *P. sylvestris* but also with another conifer *P. abies* and thus can be due to this ability an important mycorrhizal symbiont in Middle European forest ecosystems, which are formed in almost 50% by large-scale *P. abies* monocultures. Whether the observed mycorrhizal structures are functional, i.e. if they enable and enhance nutrient transfer, was tested in the Experiments 3 and 4.

Although DSE species are usually thought to have low host specificity (Jumpponen *et al.*, 1998), *A. macrosclerotiorum* was not yet observed to form EcM with any of broadleaved tree species. Similarly, in an *in vitro* experiment with hybrid *P. tremula* L.× *P. tremuloides* Michx. no EcM root tips were observed (Münzenberger *et al.*, 2009). To test this, we conducted the *in vitro* Experiment 6 to decide whether *B. pendula* can be an ectomycorrhizal host plant of *A. macrosclerotiorum*. We observed that it did not form any EcM structures and *A. macrosclerotiorum* colonized the roots intra- and intercellulary. These results are further discussed in Chapter 5.2.5.

P. glacialis and *A. macrosclerotiorum* were able to form hyphal loops resembling ErM coils in roots of *V. myrtillus*. Mycorrhizal colonization is typical for forming structures that increase surface area (i.e., Hartig net, hyphal coils, arbuscules, etc.) to enhance nutrient transport (Brundrett, 2004). Though, typical DSE colonization lacks such structures – it mainly comprises microsclerotia and

melanised hyphae (Grünig *et al.*, 2008b). Formation of hyphal loops observed in the Experiment 1 can signify that there is an “effort” to enhance nutrient adsorption surface, which can imply, if bidirectional, a sign of a mutualistic symbiosis.

DSE species are common associates of roots of plants forming ErM (Hambleton & Currah, 1997) and are dominant associates of conifers and ericaceous shrubs in heathlands, forests and alpine ecosystems (Sieber & Grünig, 2006). All of the DSE isolates used in our experiments were able to colonize the ErM plant *V. myrtillus* and formed typical structures in the roots of both host plants. Their mycelium might possibly be a mediator of transfer of nutrients between individuals of different plant species and could play an important role in forming of forest ecosystems.

5.2.2 Influence of colonization by ten different DSE species on *V. myrtillus* growth

The inoculation by DSE affected *V. myrtillus* performance in *in vitro* conditions. With this respect the isolates used in the Experiment 2 can by divided into two groups – Group 1 comprises *A. macrosclerotium*, one isolate of *A. applanata* and *P. glacialis*. Isolates from Group 1 did not differ from positive mycorrhizal control in both plant biomass parameters measured and their colonization rates were lower than those of the secong group. Group 2 was formed by P*. europea*, *P. fortinii*, *P. letzii*, *P. turiciensis*, *P. uotolensis* and one isolate of *P. helvetica* and *P. subalpina.* They all varied from positive mycorrhizal control and their colonization was higher than Group 1.

A possibility to discuss our results with previous findings of other authors is rather limited because of the fact that all dark septate fungi isolated from plant roots were considered as *P. fortinii* before approx. year 2000. It may have led to inconsistent results from resythesis *in vitro* experiments because different reaction of a host plant to inoculation could have been caused by different CSP used in the experiments. In our Experiment 2, the behavior of the member of PAC *A. applanata* in roots of *V. myrtillus* and its effect on plant growth were significantly different from those of other members of the PAC species complex. *A. applanata* was virtually unidentifiable from other PAC until it was described by molecular methods (Grünig & Sieber, 2005b).

Differences between isolates of the same species were also observed – especially for species *A. applanata*, *P. glacialis, P. helvetica* and *P. subalpina.* Isolate dependent variation of effects on plant growth was also reported in a recent study with *P. abies* in *in vitro* conditions (Tellenbach, 2011). The authors reported higher intraspecific than interspecific variation but only one of the species used was highly virulent (Tellenbach, 2011). High among-isolate variability occurs not only in PAC but has been reported for many mutualistic AMF (. e.g. Koch *et al.*, 2006) and parasitic fungi (Rowe & Kliebenstein, 2010). We used only two isolates per each cryptic species and thus, the influence of species x isolate on plant performance is barely comparable but a trend of high variability between isolates of the same fungal species can be seen.

5.2.3 ^{33}P transport to *P. abies* via mycelium of DSE *A. macrosclerotiorum*

Transport of ^{33}P isotope to *P. abies* shoot mediated by *A. macrosclerotiorum* was observed in one of three tested plants in a pilot *in vitro* experiment conducted in the lab of prof. D. Johnson in Aberdeen. The remaining two plants did not show any increase of ^{33}P content in shoot after 26 hours.

What is surprising is that although one isolate was used to inoculate all of the plants and the experimental design was the same for all of the plants the behaviour of the plant-fungus symbiosis was different. The reason for this inconsistency may be explained by the ability of endophytes to change their life strategy according their host plant and conditions (Rodriguez & Redman, 2008). The environmental conditions were almost identical (two of the tested plants even shared the same Petri dish and were approx. 3 cm apart) but the fungi may have adapted their behaviour to the physiological state of the plant or its genotype. The seedlings were all from different seeds not of uniform origin. If the fitness of the particular host plant was lower the endophyte may have acted as a parasite similarly to *D. quercina* endophyte in leaves of Mediterranean Oaks (Moricca & Ragazzi, 2008). To test this hypothesis it would be essential to use more replicates and test the host fitness for example indirectly by measuring N and P content in the needles before and after the experiment. Using genetically identical host plants may help prevent variant responses of the fungus.

The hypothesis that the endophyte reacts on plant fitness can be tested by using a stress factor (for example drought or lack of nutrients) and observing response of the fungus to plants cultivated under different stressor intensity.

5.2.4 Nutrient transport between *P. abies* and *A. macrosclerotiorum*

Although *A. macrosclerotiorum* formed typical mycorrhizal structures in roots of the host plant a bidirectional flow of nutrients was not recorded. The fungus did not supply the plant with N and C assimilated by the plant was traced in fungal structures. Higher amount of ^{13}C in sclerotia can be caused by direct flow of glucose and fructose from interfacial apoplast between plant and fungal cell walls as in the EcM symbiosis (Smith & Read, 2008) or it may have been absorbed from root exudates. The function of sclerotia is to store proteins, lipids, polysaccharides, and polyphosphates (Moore *et al.*, 1991). The outer layer is formed by melanised hyphae with thickened cell walls (see Fig. 15 and 16) which help to protect the inner core where lipid bodies are being stored (Münzenberger *et al.*, 2009). The structure of sclerotia is similar to intracellular microsclerotia except their size is much larger.

In contrast to sclerotia the increased amount of ^{13}C was not detected in the fungal compartment. This can be caused by the speed of movement of the isotope through the mycelium – sclerotia are placed straight on the surface of the root and thus the isotopes may have accumulated there quicker. The path of ^{13}C to fungal soil compartment was much longer and moreover not only the mycelium was collected from the soil and thus the concentration of ^{13}C there was much lower. The main function of sclerotia is to store nutrients and withstand poor environmental conditions in quiescent state and after change of conditions to re-establish the mycelial growth (Willetts & Bullock, 1992). High flow of C to these structures can be caused by their preferential supplying by nutrients.

Although typical mycorrhizal structures were formed in the roots of *P. abies*, *A. macrosclerotiorum* was behaving as a weak parasite. The total N content in needles of the control nonmycorrhizal plants was higher than in those inoculated. Similarly to Experiment 4 this response can be caused by genetical incompatibility of the host plant and the endophyte. Alternatively, the experimental conditions were not suitable to form mutualistic symbiosis (for example because of low a concentration of essential nutrients in the experimental system) and due to low

fitness of the host the endophyte started to exploit it. Adverse conditions would also explain the flow of C to sclerotia – as to form structures that can endure until change of conditions. Nevertheless the plants did not show any signs of disease or lack of nutrients or water.

The ^{15}N content in the root compartment was significantly higher in control plants than in the plants inoculated by *A. macrosclerotiorum*. The diffusion of the $(NH_4)(NO_2)$ solution from the fungal to the root compartment was anticipated but there should not be a reason why in systems with fungus the flow should be higher. The difference may have been caused by utilization of N by the fungus and its transfer to the fungal compartment in microcosms with *A. macrosclerotiorum*.

5.2.5 Colonization patterns of selected DSE species in roots of *B. pendula* and their effect on growth

The isolates used in Experiment 6 were chosen for their colonization pattern and effect on plant growth observed in Experiments 1 and 2 - *A. applanata* as a member of PAC with mild effect on host plant performance and *A. macrosclerotiorum* as a potential EcM symbiont of *B. pendula*. *P. involutus* was used for its good ability to form EcM in *in vitro* systems.

Neither of the two isolates of *A. macrosclerotiorum* used formed EcM structures although hyphal mantle and Hartig net were observed in the roots colonized by *P. involutus*. The conditions in the experimental system were very similar to those used in Experiment 5 where *A. macrosclerotiorum* formed typical EcM structures on roots of *P. abies*. Therefore, the reason why *A. macrosclerotiorum* did not form similar structures in roots of *B. pendula* was likely not due to unfavourable conditions in the microcosm. The endophyte was not forming EcM with another broad-leaf tree P*. tremula* L.× *P. tremuloides* Michx. hybrid in *in vitro* conditions (Münzenberger *et al.*, 2009) and was to our knowledge not yet reported from any other host plant than *P. sylvestris*. Possible explanation could be that the endophyte *A. macrosclerotiorum* is EcM host specific to conifers and that the other species are colonized only intracellulary. Or it may be restricted to dry and sand soils as hypothesized by authors of the first (and until now) only resysthesis experiment with *A. macrosclerotiorum* (Münzenberger *et al.*, 2009) and be able to form EcM only with plant species which favor these environments.

A. applanata formed sclerotia and typical DSE structures in roots of *B. pendula*. Its colonization rates were similar to those of *A. macrosclerotiorum* but the positive influence on host shoot dry weight was slightly larger. The positive influence of fungal inoculation on plant growth in *in vitro* systems does not have to be caused by direct nutrient flow or by releasing nutrients from the substrate, but also by fungal respiration increase of CO_2 concentration in closed systems. In resynthesis experiment with *P. fortinii* s.l. in aseptic culture system the inoculation had positive influence on *Pinus contorta* Dougl. ex Loud. biomass but foliar nutrient concentrations were lowered (Jumpponen & Trappe, 1998b). The authors hypothesized that the positive effect of fungal infection was indirectly caused by elevated CO_2 concentration in the closed system which was affected by fungal respiration. The system used in Experiment 6 was also closed, to prevent contaminations by airborn fungi and other organisms (bacteria, mites). The levels of CO_2 in the microcosms were checked but positive correlations between plant performance parameters and concentration of CO_2 were not detected. Microcosms with *A. applanata* did not have significantly elevated levels of CO_2 and thus the increased shoot dry weight was not due to concentration of CO_2. Whether *A. applanata* had positive influence on plant growth because of direct supplying of the host by nutrients, of making nutrients from peat available for the plant or by some other cause is a question for further investigation.

5.3 DSE species and their role in forest ecosystems

Every DSE isolate used in our experiments was able to colonize EcM and ErM forest plant species in *in vitro* conditions in similar manner as under field conditions. Microsclerotia and intracelullar colonization were the most often observed structures in the roots, but also structures with increased surface area (Hartig net, intracellular coils) were detected in *P. abies* and *V. myrtillus* roots. These structures are typical for mutualistic mycorrhizal symbioses and increased interface absorption surface between host plant and a fungus may help to enhance nutrient and water exchange between the plant and the fungus. We were not able to detect transport of N from the fungus to the plant and the endophyte acted as a mild parasite under our experimental conditions but on the other hand, it was able to enhance P uptake. DSE can influence their hosts not only by mediating nutrient uptake but can also increase host tolerance to abiotic stresses

(Zhang *et al.*, 2013) or protect the host against biotic stressors (Tellenbach *et al.*, 2013).

Except *A. applanata*, DSE from PAC in aseptic cultures did not enhance plant biomass but nevertheless, they are still common part of the endofytic fungi assemblages detected in roots under natural conditions and they do not cause strong plant defense response. Under field conditions of temperate *P. abies* forests they coexist with other fungal species – especially with ectomycorrhizal fungi. It has been reported that when DSE colonize roots together with mycorrhizal fungi their adverse effects on the host can be reduced. This phenomenon has been described for both ErM (Vohník *et al.*, 2005) and EcM hosts (Reininger & Sieber, 2012). Mechanisms remain unclear but mycorrhizal fungi can act as a barrier against DSE and thus reduce DSE colonization rates (Reininger & Sieber, 2012).

Thanks to the ability to colonize plants of different mycorrhizal types, DSE maight potentially be able to link species that cannot be connected by mycorrhizal networks. DSE do not usually form structures that would enhance nutrient transport and thus transport of substances between plants does not seem to be very likely. But the network can function as a mediator of spreading of hydraulically lifted water as reported by Warren & Brooks, 2008 in the case of drought. Since hyphae of DSE grow inside of mature forest trees roots they can easily absorb water from adjoining cells when hydraulic redistribution of water occurs and transport it to soil and other plants they colonize. Or the network can be used by the host plants for signalling – recently it has been described that individuals affected by aphid attack were able to warn other plants trough CMN formed by AMF (Babikova *et al.*, 2013). But the real importance of linking different plant species in ecosystems by DSE still waits to be described.

6. Conclusions

DSE have been reported to act in symbiosis with plants as mutualists, commensals and/or parasites. This inconsistency could have been caused by different influence of morphologically undistinguished cryptic species (CSP) on their host plant performance. In my thesis we used duplicates of 10 different molecularly identified DSE species (out of which 7 belonged to PAC) to inoculate

V. myrtillus and *P. abies* in *in vitro* conditions; we were thus able to describe and compare colonization patterns of 20 different DSE isolates in roots of both host plants. Although DSE are often isolated from EcM and ErM roots, none of the isolates tested formed mycorrhizal structures. We did not observe differences in life strategy of the tested CSP – all of them had negative influence on host shoot biomass and had the same colonization pattern. Interspecific variability thus does not explain the inconsistency of results in the DSE research with respect to host plant responses; it seems that the true reason is in different combinations of particular DSE strains with particular host plants.

The only DSE that formed true mycorrhizal structures was *A. macrosclerotiorum*. It was known to be mycorrhizal with *P. sylvestris* but we showed for the first time that it was able to form EcM also in roots of *P. abies*. Although DSE are not thought to be host specific, no mycorrhizal structures were observed in roots of *B. pendula*. Both isolates of *A. macrosclerotiorum* had positive influence on shoot biomass of *V. myrtillus* and *B. pendula*. The established root-DSE symbioses varied from mutualistic to weak parasitic and we were able to detect a nutrient transport to the plant mediated by DSE. Many aspects of the symbiosis between plants and DSE remain still unclear and especially studying the coexistence and interactions between DSE and true mycorrhizal fungi could bring new insight into the topic.

7. Bibliography

Addy H. **2005**. Microfungal endophytes in roots. *Canadian Journal of Botany* **13**: 1–13.

Aly AH, Debbab A, Proksch P. **2011**. Fungal endophytes: unique plant inhabitants with great promises. *Applied microbiology and biotechnology* **90**: 1829–45.

Arnold A, Maynard Z, Gilbert G. **2001**. Fungal endophytes in dicotyledonous neotropical trees: patterns of abundance and diversity. *Mycological Research* **105**: 1502–1507.

Arnold A, Mejía L, Kyllo D. **2003**. Fungal endophytes limit pathogen damage in a tropical tree. *Proceedings of the …* **100**: 15649–15654.

Babikova Z, Gilbert L, Bruce TJ a, Birkett M, Caulfield JC, Woodcock C, Pickett J a, Johnson D. **2013**. Underground signals carried through common mycelial networks warn neighbouring plants of aphid attack. *Ecology letters* **16**: 835–43.

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

Barrow J, Aaltonen R. **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.

Berch SM, Massicotte HB, Tackaberry LE. **2005**. Re-publication of a translation of “The vegetative organs of Monotropa hypopitys L.” published by F. Kamienski in 1882, with an update on Monotropa mycorrhizas. *Mycorrhiza* **15**: 323–32.

Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ. **2004**. Changing partners in the dark: isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees. *Proceedings. Biological sciences / The Royal Society* **271**: 1799–806.

Bidartondo MI, Read DJ, Trappe JM, Merckx V, Ligrone R, Duckett JG. **2011**. The dawn of symbiosis between plants and fungi. *Biology letters* **7**: 574–7.

Brundrett M. **2002**. Coevolution of roots and mycorrhizas of land plants. *New phytologist*: 275–304.

Brundrett M. **2004**. Diversity and classification of mycorrhizal associations. *Biological reviews of the Cambridge Philosophical Society* **79**: 473–95.

Cairney J, Ashford A. **2002**. Biology of mycorrhizal associations of epacrids (Ericaceae). *New Phytologist*: 305–326.

Cameron DD, Leake JR, Read DJ. **2006**. Mutualistic mycorrhiza in orchids: evidence from plant-fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid Goodyera repens. *The New phytologist* **171**: 405–16.

Clay K, Schardl C. **2002**. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *The American naturalist* **160 Suppl** : S99–S127.

Craven KD. **2012**. Population studies of native grass-endophyte symbioses provide clues for the roles of host jumps and hybridization in driving their evolution. *Molecular ecology* **21**: 2562–4.

Croll D, Giovannetti M, Koch AM, Sbrana C, Ehinger M, Lammers PJ, Sanders IR. **2009**. Nonself vegetative fusion and genetic exchange in the arbuscular mycorrhizal fungus Glomus intraradices. *New phytologist* **181**: 924–37.

Dearnaley JDW. **2007**. Further advances in orchid mycorrhizal research. *Mycorrhiza* **17**: 475–86.

Dickson S. **2004**. The Arum-Paris continuum of mycorrhizal symbioses. *New Phytologist* **163**: 187–200.

Duò A, Bruggmann R, Zoller S, Bernt M, Grünig CR. **2012**. Mitochondrial genome evolution in species belonging to the Phialocephala fortinii s.l. - Acephala applanata species complex. *BMC genomics* **13**: 166.

Egerton-Warburton LM, Querejeta JI, Allen MF. **2007**. Common mycorrhizal networks provide a potential pathway for the transfer of hydraulically lifted water between plants. *Journal of experimental botany* **58**: 1473–83.

Faeth S. **2002**. Are endophytic fungi defensive plant mutualists? *Oikos* **98**: 25–36.

Feddermann N, Finlay R, Boller T, Elfstrand M. **2010**. Functional diversity in arbuscular mycorrhiza – the role of gene expression, phosphorous nutrition and symbiotic efficiency. *Fungal Ecology* **3**: 1–8.

Frank AB. **1885**. Ueber die auf Wurzelsymbiose beruhende Ernahrung gewisser Baume durch unterirdische Pilze. *Ber. dt. Bot. Ges.*: (3): 128–45.

- **Freeman S, Rodriguez R**. **1993**. Genetic conversion of a fungal plant pathogen to a nonpathogenic, endophytic mutualist. *Science*. **260** (5104): 75-78

Grelet G, Johnson D. **2009**. Reciprocal carbon and nitrogen transfer between an ericaceous dwarf shrub and fungi isolated from Piceirhiza bicolorata ectomycorrhizas. *New Phytologist* **182**: 359–366.

Grünig CR, Duò A, Sieber TN, Holdenrieder O. **2008a**. Assignment of species rank to six reproductively isolated cryptic species of the Phialocephala fortinii s.1.-Acephala applanata species complex. *Mycologia* **100**: 47–67.

Grünig C, McDonald B. **2004**. Evidence for subdivision of the root-endophyte Phialocephala fortinii into cryptic species and recombination within species. *Fungal Genetics and Biology* **41**: 676–687.

Grünig C, Queloz V, Sieber T, Holdenrieder O. **2008b**. Dark septate endophytes (DSE) of the Phialocephala fortinii sl-Acephala applanata species complex in tree roots: classification, population biology, and ecology. *Botany* **86**: 1355–1369.

Grünig C, Sieber T. **2002**. Genetic variability among strains of Phialocephala fortinii and phylogenetic analysis of the genus Phialocephala based on rDNA ITS sequence comparisons. *Canadian Journal of Botany* **1249**: 1239–1249.

Grünig CR, Sieber TN. **2005a**. Molecular and phenotypic description of the widespread root symbiont Acephala applanata gen. et sp. nov., formerly known as dark-septate endophyte type 1. *Mycologia* **97**: 628–40.

Grünig CR, Sieber TN. **2005b**. Molecular and phenotypic description of the widespread root symbiont Acephala applanata gen. et sp. nov., formerly known as dark-septate endophyte type 1. *Mycologia* **97**: 628–640.

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

Heijden M Van Der, Horton T. **2009**. Socialism in soil? The importance of mycorrhizal fungal networks for facilitation in natural ecosystems. *Journal of Ecology* **97**: 1139–1150.

Hibbett DS, Gilbert LB, Donoghue MJ. **2000**. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* **407**: 506–8.

Hibbett DS, Matheny PB. **2009**. The relative ages of ectomycorrhizal mushrooms and their plant hosts estimated using Bayesian relaxed molecular clock analyses. *BMC biology* **7**: 13.

James TY, Kauff F, Schoch CL, Matheny PB, Hofstetter V, Cox CJ, Celio G, Gueidan C, Fraker E, Miadlikowska J, *et al.* 2006. Reconstructing the early evolution of Fungi using a six-gene phylogeny. *Nature* **443**: 818–22.

Johnson C a, Amarasekare P. **2013**. Competition for benefits can promote the persistence of mutualistic interactions. *Journal of theoretical biology* **328**: 54–64.

Johnson NC, Graham JH. **2013**. The continuum concept remains a useful framework for studying mycorrhizal functioning. *Plant and Soil* **363**: 411–419.

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

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

Kaldorf M, Renker C, Fladung M, Buscot F. **2004**. Characterization and spatial distribution of ectomycorrhizas colonizing aspen clones released in an experimental field. *Mycorrhiza* **14**: 295–306.

Kaska D, Myllylä R, Cooper J. **1999**. Auxin transport inhibitors act through ethylene to regulate dichotomous branching of lateral root meristems in pine. *New phytologist*: 49–58.

Kohout P, Sýkorová Z, Bahram M, Hadincová V, Albrechtová J, Tedersoo L, Vohník M. **2011**. Ericaceous dwarf shrubs affect ectomycorrhizal fungal community of the invasive Pinus strobus and native Pinus sylvestris in a pot experiment. *Mycorrhiza* **21**: 403–412.

Kohout P, Sýkorová Z, Ctvrtlíková M, Rydlová J, Suda J, Vohník M, Sudová R. **2012**. Surprising spectra of root-associated fungi in submerged aquatic plants. *FEMS microbiology ecology* **80**: 216–35.

Koch AM, Croll D, Sanders IR. **2006**. Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. *Ecology letters* **9**: 103–10.

Koch AM, Kuhn G, Fontanillas P, Fumagalli L, Goudet J, Sanders IR. **2004**. High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. *Proceedings of the National Academy of Sciences of the United States of America* **101**: 2369–74.

Kottke I, Guttenberger M, Hampp R, Oberwinkler F. **1987**. An in vitro method for establishing mycorrhizae on coniferous tree seedlings. *Trees* **1**: 191–194.

Krings M, Taylor TN, Hass H, Kerp H, Dotzler N, Hermsen EJ. **2007**. Fungal endophytes in a 400-million-yr-old land plant: infection pathways, spatial distribution, and host responses. *The New phytologist* **174**: 648–57.

Kumar D, Hyde K. **2004**. Biodiversity and tissue-recurrence of endophytic fungi in Tripterygium wilfordii. *Fungal Diversity* **17**: 69–90.

Kumar S, Kaushik N. **2013**. Metabolites of endophytic fungi as novel source of biofungicide: a review. *Phytochemistry Reviews* **11**: 507–522.

Leake J. **1994**. The biology of mycoheterotrophic ('saprophytic') plants. *New Phytologist* **127**: 171–216.

Macia-Vicente J. **2008**. Colonization of barley roots by endophytic fungi and their reduction of take-all caused by Gaeumannomyces graminis var. tritici. *Canadian Journal Microbiology* **609**: 600–609.

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

Márquez LM, Redman RS, Rodriguez RJ, Roossinck MJ. **2007**. A virus in a fungus in a plant: three-way symbiosis required for thermal tolerance. *Science (New York, N.Y.)* **315**: 513–5.

Melin E. **1922**. On the mycorrhizas of Pinus silvestris L. and Picea abies Karst: a preliminary note. *Journal of Ecology* **9**: 254–257.

Moore AEP, Ashford AE, Peterson RL. **1991**. Reserve substances in Paxillus involutus sclerotia. *Protoplasma* **163**: 67–81.

Moricca S, Ragazzi a. **2008**. Fungal endophytes in Mediterranean oak forests: a lesson from Discula quercina. *Phytopathology* **98**: 380–6.

Münzenberger B, Bubner B, Wöllecke J. **2009**. The ectomycorrhizal morphotype Pinirhiza sclerotia is formed by Acephala macrosclerotiorum sp. nov., a close relative of Phialocephala fortinii. *Mycorrhiza* **19**: 481–492.

Nara K. **2006**. Ectomycorrhizal networks and seedling establishment during early primary succession. *The New phytologist* **169**: 169–78.

Narisawa K, Usuki F, Hashiba T. **2004**. Control of Verticillium yellows in Chinese cabbage by the dark septate endophytic fungus LtVB3. *Phytopathology* **94**: 412–418.

Navarro-Ródenas A, Pérez-Gilabert M, Torrente P, Morte A. **2012**. The role of phosphorus in the ectendomycorrhiza continuum of desert truffle mycorrhizal plants. *Mycorrhiza* **22**: 565–75.

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.

Newsham K. **2011**. A metaanalysis of plant responses to dark septate root endophytes. *New Phytologist* **190**: 783–793.

Newsham K, Upson R, Read D. **2009**. Mycorrhizas and dark septate root endophytes in polar regions. *Fungal ecology* **2**: 10–20.

Peterson R, Farquhar M. **1994**. Mycorrhizas: integrated development between roots and fungi. *Mycologia* **86**: 311–326.

Peterson R, Wagg C, Pautler M. **2008**. Associations between microfungal endophytes and roots: do structural features indicate function? *Botany* **86**: 445–456.

Petrini O. **1991**. Fungal Endophytes of Tree Leaves. In: Andrews J, Hirano S, eds. Microbial Ecology of Leaves SE - 9. Springer New York, 179–197.

Pirozynski KA, Malloch DW. **1975**. The origin of land plants: A matter of mycotrophism. *Biosystems* **6**: 153–164.

Prieto I, Armas C, Pugnaire FI. **2012**. Water release through plant roots: new insights into its consequences at the plant and ecosystem level. *New phytologist* **193**: 830–41.

Queloz V, Sieber TN, Holdenrieder O, McDonald B a., Grünig CR. **2011**. No biogeographical pattern for a root-associated fungal species complex. *Global Ecology and Biogeography* **20**: 160–169.

R M, J PG. **1982**. *Isolation, maintenance and pure culture manipulation of ectomycorrhizal fungi (S NC, Ed.). Am. Phytopathol. Soc*., St. Paul, MN.

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

Rasmussen H. **2002**. Recent developments in the study of orchid mycorrhiza. *Plant and soil*: 149–163.

Redecker D, Kodner R, Graham LE. **2000**. Glomalean Fungi from the Ordovician. *Science* **289** : 1920–1921.

Redman RS, Dunigan DD, Rodriguez RJ. **2001**. Fungal symbiosis from mutualism to parasitism: who controls the outcome, host or invader? *New Phytologist* **151**: 705–716.

Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM. **2002**. Thermotolerance Generated by Plant/Fungal Symbiosis. *Science* **298**: 1581–1581.

Reininger V, Sieber TN. **2012**. Mycorrhiza reduces adverse effects of dark septate endophytes (DSE) on growth of conifers. *PloS one* **7**: e42865.

Remy W, Taylor TN, Hass H, Kerp H. **1994**. Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proceedings of the National Academy of Sciences* **91** : 11841–11843.

Robertson D, Robertson J. **1982**. Ultrastructure of Pterospora andromedea Nuttall and Sarcodes sanguinea Torrey mycorrhizas. *New Phytologist*: 539–551.

Rodriguez R, Redman R. **2008**. More than 400 million years of evolution and some plants still can't make it on their own: plant stress tolerance via fungal symbiosis. *Journal of experimental botany* **59**: 1109–14.

Rodriguez RJ, White JF, Arnold a E, Redman RS. **2009**. Fungal endophytes: diversity and functional roles. *New phytologist* **182**: 314–30.

Rowe HC, Kliebenstein DJ. **2010**. All mold is not alike: the importance of intraspecific diversity in necrotrophic plant pathogens. *PLoS pathogens* **6**: e1000759.

Saikkonen K, Ion D, Gyllenberg M. **2002**. The persistence of vertically transmitted fungi in grass metapopulations. *Proceedings. Biological sciences / The Royal Society* **269**: 1397–403.

Selosse M-A, Richard F, He X, Simard S. **2006**. Mycorrhizal networks: des liaisons dangereuses? *Trends in Ecology & Evolution* **21**: 621–628.

Selosse M-A, Roy M. **2009**. Green plants that feed on fungi: facts and questions about mixotrophy. *Trends in plant science* **14**: 64–70.

Selosse M-A, Le Tacon F. **1998**. The land flora: a phototroph–fungus partnership? *Trends in ecology & evolution (Personal edition)* **13**: 15–20.

Schulz B, Boyle C. **2005**. The endophytic continuum. *Mycological Research* **109**: 661–686.

Schulz B, Römmert A, Dammann U. **1999**. The endophyte-host interaction: a balanced antagonism? *Mycological Research* **103**: 1275–1283.

Schüβler A, Schwarzott D, Walker C. **2001**. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological research* **105**: 1413–1421.

Sieber T, Grünig C. **2006**. Biodiversity of Fungal Root-Endophyte Communities and Populations, in Particular of the Dark Septate Endophyte Phialocephala fortinii s. l. In: Schulz BE, Boyle CC, Sieber T, eds. Microbial Root Endophytes SE - 7. Springer Berlin Heidelberg, 107–132.

Simard S, Perry D, Jones M. **1997**. Net transfer of carbon between ectomycorrhizal tree species in the field. *Nature* **388**.

Smith S, Read D. **2008**. *Mycorrhizal Symbiosis*. Academic Press, London.

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

Stoyke G, Currah R. **1993**. Resynthesis in pure culture of a common subalpine fungus-root association using Phialocephala fortinii and Menziesia ferruginea (Ericaceae). *Arctic and Alpine Research*.**25**: 189-193

Stribley D, Read D, Hunt R. 1975. The Biology of Mycorrhiza in the Ericaceae. V. The Effects of Mycorrhizal Infection, Soil Type and Partial Soil-Sterilization (by Gamma-Irradiation) on Growth of Cranberry (Vaccinium macrocarpon Ait.) *New Phytologist.75: 119-130*

Taylor DL, Bruns TD. **1997**. Independent, specialized invasions of ectomycorrhizal mutualism by two nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 4510–5.

Taylor TN, Krings M, Galtier J, Dotzler N. **2012**. Fungal endophytes in Astromyelon-type (Sphenophyta, Equisetales, Calamitaceae) roots from the Upper Pennsylvanian of France. *Review of Palaeobotany and Palynology* **171**: 9–18.

Tedersoo L, May TW, Smith ME. **2010**. Ectomycorrhizal lifestyle in fungi: global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza* **20**: 217–63.

Tedersoo L, Pellet P, Kõljalg U, Selosse M-A. **2007**. Parallel evolutionary paths to mycoheterotrophy in understorey Ericaceae and Orchidaceae: ecological evidence for mixotrophy in Pyroleae. *Oecologia* **151**: 206–17.

Tejesvi M V., Mahesh B, Nalini MS, Prakash HS, Kini KR, Subbiah V, Shetty HS. **2005**. Endophytic Fungal Assemblages from Inner Bark and Twig of Terminalia arjuna W. & A. (Combretaceae). *World Journal of Microbiology and Biotechnology* **21**: 1535–1540.

Tellenbach C. **2011**. Negative effects on survival and performance of Norway spruce seedlings colonized by dark septate root endophytes are primarily isolate dependent. *Environmental Microbiology* **13**: 2508–2517.

Tellenbach C, Sieber TN. **2012**. Do colonization by dark septate endophytes and elevated temperature affect pathogenicity of oomycetes? *FEMS microbiology ecology* **82**: 157–68.

Tellenbach C, Sumarah MW, Grünig CR, Miller JD. **2013**. Inhibition of Phytophthora species by secondary metabolites produced by the dark septate endophyte Phialocephala europaea. *Fungal Ecology* **6**: 12–18.

Trappe JM. **1994**. What is a mycorrhiza? Proceedings of the Fourth European Symposium on Mycorrhizae, Granada, Spain.

Trevor E, Egger K, Peterson L. **2001**. Ectendomycorrhizal associations–characteristics and functions. *Mycorrhiza*: **19 (1)**:167–177.

Urban A, Puschenreiter M, Strauss J, Gorfer M. **2008**. Diversity and structure of ectomycorrhizal and co-associated fungal communities in a serpentine soil. *Mycorrhiza* **18**: 339–354.

Usuki F, Narisawa K. **2007**. A mutualistic symbiosis between a dark septate endophytic fungus, Heteroconium chaetospira, and a nonmycorrhizal plant, Chinese cabbage. *Mycologia* **99**: 175–184.

Vincenot L, Tedersoo L, Richard F. **2008**. Fungal associates of Pyrola rotundifolia, a mixotrophic Ericaceae, from two Estonian boreal forests. *Mycorrhiza* **19 (1)**: 15–25.

Vohník M, Albrechtová J. **2011**. The co-occurrence and morphological continuum between ericoid mycorrhiza and dark septate endophytes in roots of six European Rhododendron species. *Folia Geobotanica* **46**: 373–386.

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. *Symbiosys* **40**: 87–96.

Vohník M, Mrnka L, Lukešová T, Bruzone MC, Kohout P, Fehrer J. **2013**. The cultivable endophytic community of Norway spruce ectomycorrhizas from microhabitats lacking ericaceous hosts is dominated by ericoid mycorrhizal Meliniomyces variabilis. *Fungal Ecology* **6**: 281–292.

Vohník M, Sadowsky JJ, Kohout P, Lhotáková Z, Nestby R, Kolařík M. **2012**. Novel root-fungus symbiosis in Ericaceae: sheathed ericoid mycorrhiza formed by a hitherto undescribed basidiomycete with affinities to Trechisporales. *PloS one* **7**: e39524.

Wang B, Qiu Y-L. **2006**. Phylogenetic distribution and evolution of mycorrhizas in land plants. *Mycorrhiza* **16**: 299–363.

Wang CJK, Wilcox HE, Dec NN. **1985**. NEW SPECIES OF ECTENDOMYCORRHIZAL AND PSEUDOMYCORRHIZAL FUNGI□: Phialophora finlandia, Chloridium pucisporum, and Phialocephala fortinii. *Mycologia* **77**: 951–958.

Wang B, Yeun LH, Xue J-Y, Liu Y, Ané J-M, Qiu Y-L. **2010**. Presence of three mycorrhizal genes in the common ancestor of land plants suggests a key role of mycorrhizas in the colonization of land by plants. *New Phytologist* **186**: 514–525.

Warren J, Brooks J. **2008**. Hydraulic redistribution of water from Pinus ponderosa trees to seedlings: evidence for an ectomycorrhizal pathway. *New Phytologist* **178**: 382–94.

Warren JM, Brooks JR, Meinzer FC, Eberhart JL. **2008**. Hydraulic redistribution of water from Pinus ponderosa trees to seedlings: evidence for an ectomycorrhizal pathway. *New phytologist* **178**: 382–94.

Wilcox H, Wang C. **1987**. Mycorrhizal and pathological associations of dematiaceous fungi in roots of 7-month-old tree seedlings. *Canadian Journal of Forest Research*.**17(8)**: 884-899

Willetts HJ. **1972**. THE MORPHOGENESIS AND POSSIBLE EVOLUTIONARY ORIGINS OF FUNGAL SCLEROTIA. *Biological Reviews* **47**: 515–536.

Willetts HJ, Bullock S. **1992**. Developmental biology of sclerotia. *Mycological Research* **96**: 801–816.

Wu L, Lv Y, Meng Z, Chen J, Guo S-X. **2010**. The promoting role of an isolate of dark-septate fungus on its host plant Saussurea involucrata Kar. et Kir. *Mycorrhiza* **20**: 127–35.

Yonezawa M, Usuki F, Narisawa K. **2004**. Anatomical study on the interaction between the root endophytic fungus Heteroconium chaetospira and Chinese cabbage. *Mycoscience* **45**: 367–371.

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

Zhang Y, Li T, Zhao Z. **2013**. Colonization Characteristics and Composition of Dark Septate Endophytes (DSE) in a Lead and Zinc Slag Heap in Southwest China. *Soil and Sediment Contamination: An International Journal* **22**: 532–545.

Ziv M. **1990**. Vitrification: morphological and physiological disorders of in vitro plants. In: Debergh PC, Zimmerman RH, eds. Micropropagation SE - 4. Springer Netherlands, 45–69.

8. Attachments

Attachment 1 Multiple comparison of means. Significances are marked in red according to the Tuckey HSD test.

Tukey HSD test for colonisation, differences marked in red are significant at p < 0,05000																									
isolate	AAP-1	AAP-2	AMA-1	AMA-11	PF-EU-1	PF-EU-2	PFO-F	PFO-9	PF-HE-1	PF-HE-2	PF-LE-1	PF-LE-2	PF-SU-1	PF-SU-2	PFO-2	PFO-6	PF-UO-1	PF-UO-2	RER-2	RER-6	PF-GL-1	PF-GL-2	control
AAP-1		0,999956	1,000000	1,000000	0,001052	0,000170	0,000170	0,000170	0,999904	0,009183	0,000258	0,012561	0,000704	0,000199	0,027614	0,000707	0,000170	0,000170	0,314063	0,479947	1,000000	0,000175	0,927387
AAP-2	0,999956		0,983303	0,968427	0,052744	0,000170	0,000235	0,000200	1,000000	0,251858	0,009781	0,303694	0,036201	0,004280	0,464966	0,036335	0,000299	0,000299	0,971941	0,994712	1,000000	0,001309	0,212501
AMA-1	1,000000	0,983303		1,000000	0,000233	0,000170	0,000170	0,000170	0,976168	0,001102	0,000175	0,001510	0,000205	0,000171	0,003362	0,000205	0,000170	0,000170	0,075025	0,142832	0,999014	0,000170	0,998856
AMA-11	1,000000	0,968427	1,000000		0,000208	0,000170	0,000170	0,000170	0,957052	0,000781	0,000173	0,001052	0,000191	0,000170	0,002439	0,000191	0,000170	0,000170	0,055469	0,109096	0,997252	0,000170	0,999633
PF-EU-1	0,001052	0,052744	0,000233	0,000208		0,475038	0,956490	0,958069	0,062302	1,000000	1,000000	1,000000	1,000000	1,000000	0,999999	1,000000	0,994594	0,981387	0,965276	0,892112	0,018186	0,999983	0,000170
PF-EU-2	0,000170	0,000170	0,000170	0,000170	0,475038		1,000000	1,000000	0,000170	0,135653	0,838335	0,107192	0,568227	0,935817	0,054927	0,567326	0,999954	1,000000	0,002410	0,001028	0,000170	0,990891	0,000170
PF-EU-F	0,000170	0,000235	0,000170	0,000170	0,956490	1,000000		1,000000	0,000254	0,664639	0,998948	0,600258	0,977742	0,999924	0,430729	0,977587	1,000000	1,000000	0,053872	0,026474	0,000184	1,000000	0,000170
PFO-9	0,000170	0,000200	0,000170	0,000170	0,958069	1,000000	1,000000		0,000209	0,649009	0,999246	0,581004	0,979443	0,999958	0,404758	0,979290	1,000000	1,000000	0,042414	0,019707	0,000175	1,000000	0,000170
PF-HE-1	0,999904	1,000000	0,976168	0,957052	0,062302	0,000170	0,000254	0,000209		0,283488	0,011897	0,338898	0,043060	0,005255	0,506921	0,043216	0,000336	0,000334	0,980076	0,996714	1,000000	0,001597	0,186640
PF-HE-2	0,009183	0,251858	0,001102	0,000781	0,135653	0,664639	0,649009	0,283488			0,999966	1,000000	1,000000	0,999358	1,000000	1,000000	0,849994	0,766335	0,999814	0,997511	0,112172	0,988091	0,000171
PF-LE-1	0,000258	0,009781	0,000175	0,000173	1,000000	0,838335	0,998948	0,999246	0,011897	0,999966		0,999882	1,000000	1,000000	0,998268	1,000000	0,999987	0,999799	0,735767	0,558922	0,002913	1,000000	0,000170
PF-LE-2	0,012561	0,303694	0,001510	0,001052	1,000000	0,107192	0,600258	0,581004	0,338898	1,000000	0,999882		1,000000	0,998358	1,000000	1,000000	0,799245	0,707925	0,999944	0,998971	0,141649	0,978357	0,000171
PF-SU-1	0,000704	0,036201	0,000205	0,000191	1,000000	0,568227	0,977742	0,979443	0,043060	1,000000	1,000000	1,000000		1,000000	0,999988	1,000000	0,998199	0,991802	0,934792	0,831655	0,011971	0,999998	0,000170
PF-SU-2	0,000199	0,004280	0,000171	0,000170	1,000000	0,935817	0,999924	0,999958	0,005255	0,999358	1,000000	0,998358	1,000000		0,988124	1,000000	1,000000	0,999992	0,565659	0,388417	0,001268	1,000000	0,000170
PFO-2	0,027614	0,464966	0,003562	0,002439	0,999999	0,054927	0,430729	0,404758	0,506921	1,000000	0,998268	1,000000	0,999988	0,988124		0,999989	0,635947	0,539340	0,999999	0,999942	0,246788	0,924055	0,000176
PFO-6	0,000707	0,036335	0,000205	0,000191	1,000000	0,567326	0,977587	0,979290	0,043216	1,000000	1,000000	1,000000	1,000000	1,000000	0,999989		0,998174	0,991732	0,935156	0,832309	0,012020	0,999998	0,000170
PF-UO-1	0,000170	0,000299	0,000170	0,000170	0,994594	0,999954	1,000000	1,000000	0,000336	0,849994	0,999987	0,799245	0,998199	1,000000	0,835947	0,998174		1,000000	0,103523	0,052317	0,000196	1,000000	0,000170
PF-UO-2	0,000170	0,000299	0,000170	0,000170	0,981387	1,000000	1,000000	1,000000	0,000334	0,766335	0,999799	0,707925	0,991802	0,999992	0,539340	0,991732	1,000000		0,082126	0,041895	0,000198	1,000000	0,000170
RER-2	0,314063	0,971941	0,075025	0,055469	0,965276	0,002410	0,053872	0,042414	0,980076	0,999814	0,735767	0,999944	0,934792	0,565659	0,999999	0,935156	0,103523	0,082126		1,000000	0,865155	0,330447	0,000655
RER-6	0,479947	0,994712	0,142832	0,109096	0,892112	0,001028	0,026474	0,019707	0,996714	0,997511	0,558922	0,988971	0,831655	0,388417	0,999942	0,832309	0,052317	0,041895	1,000000		0,952421	0,198317	0,001456
PF-GL-1	1,000000	1,000000	0,999014	0,997252	0,018186	0,000170	0,000184	0,000175	1,000000	0,112172	0,002913	0,141649	0,011971	0,001268	0,246788	0,012020	0,000196	0,000198	0,865155	0,952421		0,000438	0,416003
PF-GL-2	0,000175	0,001309	0,000170	0,000170	0,999983	0,990891	1,000000	1,000000	0,001597	0,988091	1,000000	0,978357	0,999998	1,000000	0,924055	0,999998	1,000000	1,000000	0,330447	0,198317	0,000438		0,000170
control	0,927387	0,212501	0,998856	0,999633	0,000170	0,000170	0,000170	0,000170	0,186640	0,000171	0,000170	0,000171	0,000170	0,000170	0,000176	0,000170	0,000170	0,000170	0,000655	0,001456	0,416003	0,000170	

Attachment 2 Multiple comparison of means. Significances are marked in red according to the Tuckey HSD test.

Tukey HSD test for shoot dry weight, differences marked in red are significant at p < 0,05000																						
isolate	AAP-1	AAP-2	AMA-1	AMA-11	PF-EU-1	PF-EU-2	PF-O-F	PF-O-9	PF-HE-1	PF-HE-2	PF-LE-1	PF-LE-2	PF-SU-1	PF-SU-2	PFO-2	PFO-6	PF-UO-1	PF-UO-2	RER-2	RER-6	PF-GL-1	PF-GL-2	control
AAP-1		0,604798	0,649147	0,557218	0,466679	0,002418	0,097489	0,244920	0,000259	0,543803	0,411574	0,085807	0,996263	0,973989	0,494812	0,677524	0,698232	0,066632	0,191770	0,046119	0,883773	0,999954	0,999496
AAP-2	0,604798		1,000000	1,000000	0,000275	0,000169	0,000172	0,000190	0,000169	0,000338	0,000245	0,000172	0,013768	0,005345	0,000295	0,000554	0,000603	0,000171	1,000000	0,999943	1,000000	0,048216	0,026846
AMA-1	0,649147	1,000000		1,000000	0,000308	0,000169	0,000174	0,000197	0,000169	0,000389	0,000269	0,000173	0,016828	0,006600	0,000334	0,000667	0,000729	0,000171	1,000000	0,999873	1,000000	0,057554	0,032411
AMA-11	0,557218	1,000000	1,000000		0,000248	0,000169	0,000172	0,000185	0,000169	0,000296	0,000226	0,000171	0,011113	0,004254	0,000264	0,000451	0,000494	0,000170	1,000000	0,999977	1,000000	0,039829	0,021908
PF-EU-1	0,466679	0,000275	0,000308	0,000248		0,968230	1,000000	1,000000	0,577689	1,000000	1,000000	1,000000	0,999902	0,999999	1,000000	1,000000	1,000000	1,000000	0,000174	0,000170	0,000916	0,994145	0,998953
PF-EU-2	0,002418	0,000169	0,000169	0,000169	0,968230		0,999968	0,997360	1,000000	0,945328	0,979323	0,999985	0,268194	0,441499	0,960850	0,881571	0,868186	0,999997	0,000169	0,000169	0,000169	0,104577	0,168818
PF-EU-2	0,002418	0,000169	0,000169	0,000169	0,968230		0,999968	0,997360	1,000000	0,945328	0,979323	0,999985	0,268194	0,441499	0,960850	0,881571	0,868186	0,999997	0,000169	0,000169	0,000169	0,104577	0,168818
PFO-F	0,097489	0,000172	0,000174	0,000172	1,000000	0,999968		1,000000	0,962176	1,000000	1,000000	1,000000	0,951107	0,990694	1,000000	0,999996	0,999993	1,000000	0,000169	0,000169	0,000197	0,778628	0,881128
PFO-9	0,244920	0,000190	0,000197	0,000185	1,000000	0,997360	1,000000		0,816012	1,000000	1,000000	1,000000	0,996006	0,999750	1,000000	1,000000	1,000000	1,000000	0,000170	0,000169	0,000333	0,947850	0,982115
PF-HE-1	0,000259	0,000169	0,000169	0,000169	0,577689	1,000000	0,962176	0,816012		0,499875	0,632085	0,970877	0,036598	0,081330	0,548934	0,371870	0,352688	0,983231	0,000169	0,000169	0,010094	0,019146
PF-HE-2	0,543803	0,000338	0,000389	0,000296	1,000000	0,945328	1,000000	1,000000	0,499875		1,000000	1,000000	0,999977	1,000000	1,000000	1,000000	1,000000	0,999998	0,000177	0,000170	0,001300	0,997589	0,999662
PF-LE-1	0,411574	0,000245	0,000269	0,000226	1,000000	0,979323	1,000000	1,000000	0,632085	1,000000		1,000000	0,999754	0,999995	1,000000	1,000000	1,000000	1,000000	0,000173	0,000170	0,000726	0,989873	0,997870
PF-LE-2	0,085807	0,000172	0,000173	0,000171	1,000000	0,999985	1,000000	1,000000	0,970877	1,000000	1,000000		0,938535	0,987019	1,000000	0,999990	0,999985	1,000000	0,000169	0,000169	0,000192	0,748322	0,858965
PF-SU-1	0,996263	0,013768	0,016828	0,011113	0,999902	0,266194	0,951107	0,996006	0,036598	0,999977	0,999754	0,938535		1,000000	0,999942	0,999999	0,999999	0,908387	0,001422	0,000296	0,055977	1,000000	1,000000
PF-SU-2	0,973989	0,005345	0,006600	0,004254	0,999999	0,441499	0,990694	0,999750	0,081330	1,000000	1,000000	0,987019	1,000000		0,999999	1,000000	1,000000	0,976779	0,000582	0,000205	0,024232	1,000000	1,000000
PFO-2	0,494812	0,000295	0,000334	0,000264	1,000000	0,960850	1,000000	1,000000	0,548934	1,000000	1,000000	1,000000	0,999942	0,999999		1,000000	1,000000	0,999999	0,000175	0,000170	0,001039	0,995707	0,999301
PFO-6	0,677524	0,000554	0,000667	0,000451	1,000000	0,881571	0,999996	1,000000	0,371870	1,000000	1,000000	0,999990	0,999999	1,000000	1,000000		1,000000	0,999961	0,000189	0,000171	0,002461	0,999621	0,999968
PF-UO-1	0,698232	0,000603	0,000729	0,000494	1,000000	0,868186	0,999993	1,000000	0,352688	1,000000	1,000000	0,999985	0,999999	1,000000	1,000000	1,000000		0,999941	0,000192	0,000171	0,002733	0,999730	0,999979
PF-UO-2	0,066632	0,000171	0,000171	0,000170	1,000000	0,999997	1,000000	1,000000	0,983231	0,999908	1,000000	1,000000	0,908387	0,976779	0,999999	0,999961	0,999941		0,000169	0,000169	0,000184	0,685981	0,810252
RER-2	0,191770	1,000000	1,000000	1,000000	0,999997	0,000169	0,000169	0,000169	0,000177	0,000177	0,000173	0,000169	0,001422	0,000582	0,000175	0,000189	0,000192	0,000169		1,000000	0,999985	0,005829	0,002940
RER-6	0,046119	0,999943	0,999873	0,999977	0,000170	0,000169	0,000169	0,000169	0,000170	0,000170	0,000170	0,000169	0,000296	0,000205	0,000170	0,000171	0,000171	0,000169	1,000000		0,993642	0,000885	0,000481
PF-GL-1	0,883773	1,000000	1,000000	1,000000	0,000916	0,000169	0,000197	0,000333	0,000169	0,001300	0,000726	0,000192	0,055977	0,024232	0,001039	0,002461	0,002733	0,000184	0,999985	0,993642		0,160754	0,099058
PF-GL-2	0,999954	0,048216	0,057554	0,039829	0,994145	0,104577	0,778628	0,947850	0,010094	0,997589	0,988973	0,748322	1,000000	1,000000	0,995707	0,999621	0,999730	0,685981	0,005829	0,000885	0,160754		1,000000
control	0,999496	0,026846	0,032411	0,021908	0,998953	0,168818	0,881128	0,982115	0,019146	0,999662	0,997870	0,858965	1,000000	1,000000	0,999301	0,999968	0,999979	0,810252	0,002940	0,000481	0,099058	1,000000	

Attachment 3 Multiple comparison of means. Significances are marked in red according to the Tuckey HSD test.

Tukey HSD test for root log fresh weigh, differences marked in red are significant at p < 0,05000																							
isolate	AAP-1	AAP-2	AMA-1	AMA-11	PF-EU-1	PF-EU-2	PF-O-F	PFO-9	PF-HE-1	PF-HE-2	PF-LE-1	PF-LE-2	PF-SU-1	PF-SU-2	PFO-2	PFO-6	PF-UO-1	PF-UO-2	RER-2	RER-6	PF-GL-1	PF-GL-2	control
AAP-1		0,999875	0,999994	0,998128	0,086341	0,002317	0,033009	0,001921	0,000393	0,146932	0,001533	0,045271	0,544620	0,857811	0,122463	0,520046	0,002480	0,002137	1,000000	1,000000	1,000000	0,520121	0,989801
AAP-2	0,999875		1,000000	1,000000	0,001453	0,000178	0,000597	0,000176	0,000170	0,003008	0,000174	0,000692	0,030236	0,116653	0,002320	0,027259	0,000179	0,000177	0,999948	1,000000	0,999996	0,027267	0,364208
AMA-1	0,999994	1,000000		1,000000	0,002996	0,000193	0,001114	0,000188	0,000171	0,006235	0,000183	0,001347	0,055948	0,191844	0,004831	0,050802	0,000196	0,000191	0,999998	1,000000	0,050817	0,508258	
AMA-11	0,998128	1,000000	1,000000		0,000653	0,000172	0,000319	0,000171	0,000170	0,001266	0,000171	0,000344	0,013659	0,059740	0,000991	0,012230	0,000172	0,000172	0,999026	1,000000	0,999834	0,012234	0,222843
PF-EU-1	0,086341	0,001453	0,002996	0,000653		0,999980	1,000000	0,999957	0,992188	1,000000	0,999897	1,000000	1,000000	0,999516	1,000000	1,000000	0,999985	0,999972	0,072064	0,009074	0,045674	1,000000	0,968486
PF-EU-2	0,002317	0,000178	0,000193	0,000172	0,999980		1,000000	1,000000	1,000000	0,999694	1,000000	1,000000	0,941298	0,703602	0,999874	0,949965	1,000000	1,000000	0,001836	0,000272	0,001066	0,949940	0,334504
PF-EU-F	0,033009	0,000597	0,001114	0,000319	1,000000	1,000000		1,000000	0,999987	1,000000	1,000000	1,000000	0,999600	0,980780	1,000000	0,999721	1,000000	1,000000	0,027155	0,003182	0,016738	0,999721	0,820392
PFO-9	0,001921	0,000176	0,000188	0,000171	0,999957	1,000000	1,000000		1,000000	0,999447	1,000000	0,999999	0,925310	0,666186	0,999758	0,935622	1,000000	1,000000	0,001526	0,000250	0,000896	0,935592	0,302238
PF-HE-1	0,000393	0,000170	0,000171	0,000170	0,992188	1,000000	0,999987	1,000000		0,970172	1,000000	0,998923	0,649864	0,315404	0,980649	0,673716	1,000000	1,000000	0,000337	0,000177	0,000252	0,673644	0,095293
PF-HE-2	0,146932	0,003008	0,006235	0,001266	1,000000	0,999694	1,000000	0,999447	0,970172		0,988911	1,000000	0,999964	1,000000	1,000000	0,999755	0,999603	0,124717	0,018106	0,082273	1,000000	0,991605	
PF-LE-1	0,001533	0,000174	0,000183	0,000171	0,999897	1,000000	1,000000	1,000000	1,000000	0,998911		0,999997	0,902175	0,619220	0,999496	0,914577	1,000000	0,999999	0,001222	0,000229	0,000732	0,914540	0,265553
PF-LE-2	0,045271	0,000692	0,001347	0,000344	1,000000	1,000000	1,000000	0,999999	0,998923	1,000000	0,999997		0,999980	0,999678	1,000000	0,999988	1,000000	0,999999	0,037147	0,004091	0,022687	0,999988	0,906729
PF-SU-1	0,544620	0,030236	0,055948	0,013659	1,000000	0,941298	0,999600	0,925310	0,649864	1,000000	0,902175	0,999980		1,000000	1,000000	1,000000	0,946412	0,934740	0,495978	0,130954	0,384214	1,000000	0,999997
PF-SU-2	0,857811	0,116653	0,191844	0,059740	0,999516	0,703602	0,980780	0,666186	0,315404	0,999964	0,619220	0,999678	1,000000		0,999902	1,000000	0,716749	0,687651	0,823393	0,364896	0,725515	1,000000	1,000000
PFO-2	0,122463	0,002320	0,004831	0,000991	1,000000	0,999874	1,000000	0,999758	0,980649	1,000000	0,999496	1,000000	1,000000	0,999902		1,000000	0,999902	0,999832	0,103323	0,014229	0,067200	1,000000	0,986005
PFO-6	0,520046	0,027259	0,050802	0,012230	1,000000	0,949965	0,999721	0,935622	0,673716	1,000000	0,914577	0,999988	1,000000	1,000000	1,000000		0,954518	0,944109	0,471824	0,120440	0,362282	1,000000	0,999995
PF-UO-1	0,002480	0,000179	0,000196	0,000172	0,999985	1,000000	1,000000	1,000000	1,000000	0,999755	1,000000	1,000000	0,946412	0,716749	0,999902	0,954518		1,000000	0,001964	0,000281	0,001136	0,954495	0,346564
PF-UO-2	0,002137	0,000177	0,000191	0,000172	0,999972	1,000000	1,000000	1,000000	1,000000	0,999603	1,000000	0,999999	0,934740	0,687651	0,999832	0,944109	1,000000		0,001695	0,000262	0,000988	0,944082	0,320393
RER-2	1,000000	0,999948	0,999998	0,999026	0,072064	0,001836	0,027155	0,001526	0,000337	0,124717	0,001222	0,037147	0,495978	0,823393	0,103323	0,471824	0,001964	0,001695		1,000000	1,000000	0,471898	0,984067
RER-6	1,000000	1,000000	1,000000	1,000000	0,009074	0,000272	0,003182	0,000250	0,000177	0,018106	0,000229	0,004091	0,130954	0,364896	0,014229	0,120440	0,000281	0,000262	1,000000		1,000000	0,120471	0,735880
PF-GL-1	1,000000	0,999996	1,000000	0,999834	0,045674	0,001066	0,016738	0,000896	0,000252	0,082273	0,000732	0,022687	0,384214	0,725515	0,067200	0,362282	0,001136	0,000988	1,000000	1,000000		0,362348	0,959684
PF-GL-2	0,520121	0,027267	0,050817	0,012234	1,000000	0,949940	0,999721	0,935592	0,673644	1,000000	0,914540	0,999988	1,000000	1,000000	1,000000	1,000000	0,954495	0,944082	0,471898	0,120471	0,362348		0,999995
control	0,989801	0,364208	0,508258	0,222843	0,968486	0,334504	0,820392	0,302238	0,095293	0,991605	0,265553	0,906729	0,999997	1,000000	0,986005	0,999995	0,346564	0,320393	0,984067	0,735880	0,959684	0,999995	