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

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Abstract

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

Keywords: Ericoid mycorrhiza, dark septate endophytes, DSE-association, split root system, colonization level, pseudomycorrhiza, co-inoculation

1. Introduction

Members of Ericaceae dominate vast areas in both Northern and Southern hemisphere, which are often characterized by "harsh edaphic conditions" (Cairney and Meharg, 2003). These conditions include low pH, low nutrient availability, high soil C:N ratio and high contents of phenolic compounds and toxic elements (Smith and Read, 1997). The roots of members of Ericaceae are colonized by ErM fungi, which are regarded as important mutualistic associates. ErM fungi positively influence growth, survival and competitiveness of their host species by enhancing nutrient uptake (Read, 1996; Read and Perez-Moreno, 2003) and alleviating heavy metal toxicity (Perotto et al., 2002).

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

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uptake in ErM is based on experiments with *H. ericae* (Bajwa and Read, 1985; Bajwa et al., 1985; Kerley and Read, 1995; Kerley and Read, 1997; Kerley and Read, 1998).

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

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

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

2. Materials and Methods

Fungal Compatibility Experiment

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

After 3 months from the inoculation, the roots of the cuttings were separated from the shoots, divided into halves and stained either with trypan blue or chlorazol black, respectively, according to the method described by Brundrett et al. (1996). Roots were then observed at high magnification (400–1,000×) with DIC, using Olympus BX60 microscope. Pictures were taken with Olympus DP70 camera.

Fungal Efficacy Experiment

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

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

Mycelium of *O. maius* B, *P. fortinii* F and *P. fortinii* H was pre-cultivated for two months on PDA media (39 g of

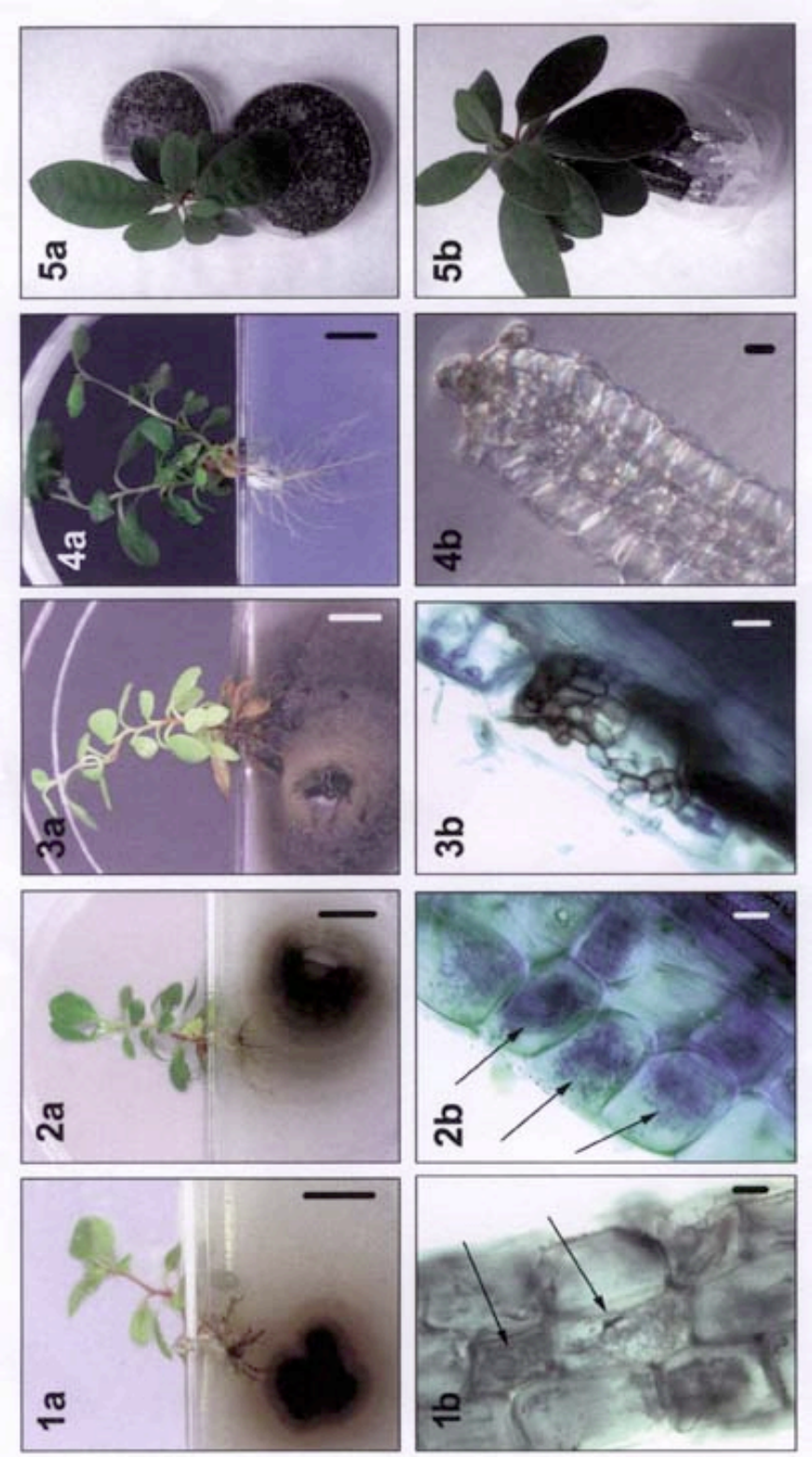
PDA powder, Difco, per 1 l of de-ionized water) at room temperature in the dark. Plants in the split root systems were inoculated i) separately with single fungal strains onto one dish (then the other dish remained non-inoculated) – treatments B = *O. maius* B, F = *P. fortinii* F and H = *P. fortinii*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

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

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

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

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



Figures 1–5. 1a: Inoculation of *Rhododendron* cuttings with *O. maius* B resulted in reduction of the root development; 1b: Intracellular structures (arrows) formed by *O. maius* B in *Rhododendron* root; 2a: Rhododendrons inoculated with *H. ericae* developed branched root systems; 2b: Intracellular structures (arrows) formed by *H. ericae* in *Rhododendron* roots; 3a: Rhododendrons inoculated with both strains of *P. fortinii* developed branched root systems; 3b: Intracellular microsclerotium formed by *P. fortinii* H; 4a: Also non-inoculated rhododendrons had branched, well developed root systems; 4b: a non-colonized root of a non-inoculated control plant; 5: A *Rhododendron* cv. Azurro rooted cutting in the split root system; 5a: before the inoculation, 5b: after the inoculation. The fungal structures were stained with chorazol black (Fig. 1b) or trypan blue (Figs. 2b and 3b). Bars in the upper row correspond to 1 cm, in the lower row correspond to 10 μm.

3. Results

Fungal Compatibility Experiment

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

Fungal Efficacy Experiment

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

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

Screening of the mycorrhizal colonization revealed very low levels of intracellular colonization by fungal hyphae. If present, *O. maius* B hyphae grew around the roots and were attached to the root surface, only very rarely penetrating rhizodermal cells to form sparse intracellular coils. Hyphae of *O. maius* B failed to stain with trypan blue and were only lightly stained with chlorazol black. *P. fortinii* H failed to produce abundant DSE-colonization. Its hyphae grew mostly on the root surface, and if they penetrated the root, they grew along the central axis producing mostly lightly stained (better with chlorazol black than in trypan blue) hyaline hyphae. The hyphae of *P. fortinii* H scarcely formed microsclerotia. Relatively higher colonization occurred in the dishes inoculated with *P. fortinii* F, where dark septate hyphae formed well-developed hyaline microsclerotia. However, even in the dishes inoculated with *P. fortinii* F,

the colonization was low, and we estimate it did not reach more than 5% (counting both the presence of hyphae and microsclerotia) of the total root length colonized. The colonization level of *O. maius* B and *P. fortinii* H belonged to the first class, with that of *P. fortinii* F within the second class as described in Materials and Methods.

4. Discussion

Fungal Compatibility Experiment

The Fungal Compatibility Experiment showed that the selected fungi were able to form both ErM and DSE in the roots of *Rhododendron* micro-cuttings. *O. maius* B proved to be the least efficient colonizer, exhibiting poor intracellular colonization compared to the typical ErM fungus *H. ericae*. The presence of *O. maius* B also reduced the development of the root system of the inoculated plants compared to the non-inoculated and the inoculated with *H. ericae* or either of the two strains of *P. fortinii*. Similar root growth depression by *O. tenuissimum* (Peck) Hughes was observed by Dalpé (1986) in *Vaccinium angustifolium* Ait., where *O. tenuissimum* failed to form ErM.

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

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

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

Fungal Efficacy Experiment

In different habitats, different fungi usually inhabit plant roots, as is the case with ericaceous species (Midgley et al., 2004), even though the dominant role of *H. ericae* is stressed. McLean and Lawrie (1996) stated, on the base of the different colonization patterns, that more than one fungus was involved in the screened ericoid mycorrhizae in the roots of epacridaceous plants from different natural sites. Each fungus may make different contribution to the ecophysiology of its host plant depending on the large scale of the environmental factors. This was also revealed in Fungal Compatibility Experiment, where ErM fungi *O. maius* B and *H. ericae*, grown on the same media, exhibited different effects on host plants. In the Fungal Efficacy Experiment, the direct comparison of the effects of *O. maius* and both *P. fortinii* strains on the nutrient uptake by the host plants showed a higher efficiency of *O. maius* than *P. fortinii*. The results of our previous unpublished experiments, performed under the conditions similar to those presented here, repeatedly indicated that *O. maius* B was more efficient in the terms of improved plant growth of rhododendrons than *H. ericae*, which we had not expected. Unfortunately, studies employing *O. maius*, and especially both *O. maius* and *H. ericae* simultaneously are missing, and the effect of both fungi is therefore difficult to compare. Such studies would elucidate whether oidiodendrons could be as efficient in facilitating nutrient uptake by host plants as *H. ericae* and thus play the same or similar role in the environments dominated by ericaceous species. Results of our study indicate that oidiodendrons might have such potential.

Phosphorus and especially nitrogen are primarily transported to host plants by ericoid mycorrhizal fungi, according to experiments using *H. ericae* as a mycorrhizal partner of ericaceous plants (Smith and Read, 1997). Our study shows positive effects of inoculation with *O. maius*, in particular, on P uptake but also indicates a positive trend

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

	Total weight of the dried biomass (g) NS	Weight of the dried shoot biomass (g) NS	Weight of the dried root biomass (g) NS	P (μg/g) p=0.00011	N (%) p=0.053	C:N ratio p=0.032
B (n=6)	1.24 (0.91–1.57)	1.13 (0.85–1.41)	0.11 (0.06–0.17)	1,288 (913–1,662)c	1.39 (1.19–1.57)c	34.6 (29.7–39.6)a
F (7)	1.51 (1.25–1.77)	1.36 (1.15–1.57)	0.15 (0.10–0.20)	818 (725–913)ab	1.21 (1.13–1.29)ab	39.4 (36.4–42.4)abc
H (7)	1.55 (1.28–1.81)	1.40 (1.17–1.63)	0.15 (0.10–0.19)	1,060 (849–1,272)bc	1.19 (1.06–1.32)ab	40.5 (36.0–44.9)bc
BF (7)	1.36 (1.13–1.58)	1.23 (0.85–1.41)	0.12 (0.09–0.16)	745 (609–882)a	1.26 (1.16–1.36)abc	37.8 (34.6–41.1)ab
BH (7)	1.29 (1.04–1.53)	1.15 (0.91–1.40)	0.13 (0.08–0.19)	1,138 (815–1,460)c	1.30 (1.16–1.44)bc	36.7 (32.8–40.4)ab
Control (7)	1.50 (1.15–1.84)	1.35 (1.06–1.64)	0.15 (0.09–0.20)	720 (552–888)a	1.12 (0.93–1.32)a	43.6 (36.8–50.3)c

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

	0–0	B–0	F–0	H–0	B–F	B–H
WDR (mg)	73.9±32a	64.2±33a	74.1±28a	81.4±22a	64.7±31a	61.9±21a
	71.8±33a	49.9±23a	75.7±25a	65.4±32a	58.7±23a	73.0±45a
Ratio	1.04 (0.92–1.16)a	1.34 (0.81–1.86)ab	0.99 (0.77–1.22)a	1.61 (0.62–2.60)b	1.22 (0.67–1.77)a	0.99 (0.64–1.35)a

in N uptake by inoculated plants. To our knowledge, this is the first report about such effects observed for ericaceous plants inoculated with *O. maius*.

The plants inoculated with *O. maius* B had higher foliar N content when compared to both control and either of two *P. fortinii* strains and also higher foliar P content when compared to control and *P. fortinii* F. On the other hand, *P. fortinii* H also increased foliar P content compared to the control, which confirms its beneficial effect for the inoculated plants and at the same time highlights the strain specificity, since *P. fortinii* F failed to facilitate P uptake by the inoculated plants. In regard to nutrient availability for plants inoculated with DSE, Jumpponen et al. (1998) reported enhanced phosphorus uptake by *Pinus contorta* when inoculated with *P. fortinii*. Studies addressing similar questions with ericaceous host plants are missing. In our

previous study (Vohník et al., 2003) we reported no effect of two *P. fortinii* strains (one of them was *P. fortinii* F from this study) on the growth of *Rhododendron* cv. Belle-Heller in two different substrates, either sterilized or non-sterilized.

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

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

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

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

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

Both *O. maius* B and *P. fortinii* H failed to form extensive intracellular root colonization in the Fungal Efficacy Experiment. Intracellular structures, such as ericoid mycorrhizal coils and loops and DSE intraradical hyaline

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

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

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

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

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REFERENCES

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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