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Growth and viability of mycorrhizal extraradical mycelia associated with three temperate orchid species

Martina Čuříková[1], Aleš Látr[1] & Miroslav Vosátka[2]

[1]*Department of Botany, Faculty of Science, Palacký University, Šlechtitelů 11, CZ-78371 Olomouc-Holice, Czech Republic; e-mail: M.Curikova@seznam.cz*
[2]*Institute of Botany, Academy of Sciences of the Czech Republic, CZ-25243 Průhonice, Czech Republic*

Abstract: Growth and enzymatic activities of extraradical mycelia (ERM) of native mycorrhizal symbionts associated with three orchid species, *Dactylorhiza fuchsii*, *D. majalis* and *Platanthera bifolia,* were studied. ERM extracted from the mycorrhizosphere of these species showed features typical for fungi that form orchid mycorrhiza. In the first pot experiment, three different treatments were applied on tubers of *D. fuchsii* transplanted from a natural site: control (no specific treatment), reinoculated (surface-sterilized tubers reinoculated with mycorrhizal fungi-colonised roots), and benomyl (non-sterilized tubers treated with fungicide). However, no significant differences in ERM growth and intensity of root mycorrhizal colonisation at harvest were observed among these treatments. ERM associated with reinoculated *D. fuchsii* plants showed significantly higher alkaline phosphatase (ALP) enzymatic activity at week 36 than at week 24, but no differences were observed for NADH diaphorase activity. Benomyl application significantly reduced ALP activity in comparison with reinoculated plants at week 36. In the second experiment, plants of all three species were either untreated (control), or repeatedly treated with benomyl. Similarly to the results of the first experiment, benomyl application did not reduce the ERM growth of mycorrhizal symbionts associated with *D. majalis* and *D. fuchsii.* The low ERM growth associated with benomyl-treated *P. bifolia* was probably caused by poor root system development in this treatment. Significantly higher mycorrhizal colonisation was found for *D. fuchsii* compared to *P. bifolia* in control treatments at the end of cultivation. The ERM of native symbionts of the three orchid species studied seemed to have a different growth pattern over time and responded differently to fungicide application.

Key words: benomyl; enzyme staining; extraradical mycelium; orchid mycorrhiza

Abbreviations: ALP, alkaline phosphatase; CITES, Convention on International Trade in Endangered Species of Wild Fauna and Flora; ERM, extraradical mycelium; INT, iodonitrotetrazolium chloride; NADH, reduced form of nicotinamide adenine dinucleotide; OM, orchid mycorrhiza; SE, standard error

Introduction

Roots of most orchid species are acknowledged to be associated with fungal partners forming orchid mycorrhiza (Burgeff 1909). The fungal partner in OM is mostly a basidiomycete and the isolates generally are sterile mycelia with very little tendency to sporulate in culture (Rasmussen 2002). Fungal symbionts are reported to belong mainly to genus *Rhizoctonia* (Bernard 1909; Warcup & Talbot 1967; Andersen 1996; Rasmussen & Whigham 2002). However, a few studies revealed that the mycorrhizal fungi associated with orchids also belong to the ascomycetes group (Currah et al. 1987; Currah et al. 1990; Bidartondo et al. 2004; Selosse et al. 2004). Molecular methods (PCR) could be used to identify fungi directly within the roots (Gardes & Bruns 1993; Cullings et al. 1996; Kjøller & Rosendahl 2000; Kristiansen et al. 2001). Orchid mycorrhizal fungi have traditionally been studied using the isolation and establishment of pure cultures from colonised root tissue (Warcup 1981; Currah et al. 1997). Some fungi, however, may not be able to grow on the media and therefore reinoculation of plants with a fungal partner *in vitro* cannot be used to study mycorrhizal association. Another possibility is to study mycorrhizal symbiosis on naturally occurring specimens. The most promising way to study the biology of orchid mycorrhizal fungi is to eliminate mycorrhizal fungi using fungicides (Bayman et al. 2002). This approach also has its limitations because no method for creating non-mycorrhizal controls exists, and because all naturally occurring individuals of the studied orchid species are usually colonised by symbiotic fungi (Čuříková, unpublished; Látr et al. 2008). Due to high mortality, it is difficult to transfer living specimens to pot cultures and to carry out cultivation experiments. Another limitation is that most orchids in the Czech Republic are rare and protected species. Sampling and destruction of plants must be thus kept at a necessary minimum.

The ERM of orchid mycorrhizal fungi consists mainly of narrow septate hyphae with abundant anastomoses and monilioid cells, which occur as more-or-

less branched chains that are considered as precursors of sclerotia (Tu & Kimbrough 1975). The extraradical hyphae spread in the soil and form the surface area for fungal uptake of organic C compounds (Smith 1966; Hadley 1984; Alexander & Hadley 1985), phosphate (Smith 1966), nitrogen (Burgeff 1936) or water (Yoder et al. 2000), which they then pass to the host plant.

When OM plants were treated with the fungicide thiabendazole, the growth of external mycelium stopped and a significant decrease in growth rate, P and N content, intensity of intraradical mycorrhizal colonisation, and net assimilation rate were observed (Alexander & Hadley 1984). The benomyl fungicide was previously reported to be highly effective in eliminating arbuscular mycorrhizal fungi (Menge 1982; Fitter & Nichols 1988; Merryweather & Fitter 1996). Kahiluoto et al. (2000 a,b) used benomyl to create a control with suppressed mycorrhiza for assessing the effectiveness of field arbuscular mycorrhizal communities in a bioassay, in terms of plant growth and P uptake. Benomyl, however, was also used in the isolation and cultivation of OM fungi as a part of selective medium, because basidiomycetes are only slightly responsive to benomyl (Boosalis & Scharen 1959; Bayman et al. 2002).

This study was conducted to contribute to the knowledge on the occurrence and ecology of native mycorrhizal symbionts associated with three orchid species, *Dactylorhiza fuchsii*, *D. majalis* and *Platanthera bifolia*. The study aimed to isolate ERM from the mycorrhizosphere of three orchid species using the inserted membrane technique and to study the influence of benomyl on the growth and development of extraradical and intraradical mycelium of mycorrhizal symbionts associated with orchids. The experiments were also established to assess enzymatic activity (ALP and INT) of mycelia based on vital staining. Although the identification of mycorrhizal extraradical mycelia associated with the three studied orchid species is an interesting topic, it was not the subject of this study.

Material and methods

Plant materials

In June 2005, tubers of adult specimens of *Dactylorhiza fuchsii* (Druce) Soó, *D. majalis* (Reichenbach pat.) P. F. Hunt & Summerhayes and *Platanthera bifolia* (L). Rich., grown under natural conditions in the Zlin region (the Hostynsko-vsetinske hills), the Czech Republic, were collected at the maximum amounts permitted by the Regional Authority of the Zlin Region (KUZL 5898/2003 and KUZL 5947/2003) due to the protected status of these CITES species. *D. fuchsii* is listed among taxa requiring further attention (C4), and *D. majalis* and *P. bifolia* are endangered species (C3) listed in the Red list of vascular plants of the Czech Republic (Holub & Procházka 2000).

All three studied orchid species are summer-green, producing green leafy shoots and aerial inflorescence in the spring and surviving winter underground as tubers. They flower from May to June.

Experiment 1

The experiment with *D. fuchsii* consisted of three treatments, each with 5 plants as replicates. In the first treatment (control), tubers were only washed with deionised water and transplanted to pots to observe the natural development of mycorrhizal fungi. In the second treatment (reinoculated), the adventitious roots and the root-like extensions were removed and the tubers were surface sterilized in 70% ethanol for 1 minute, then in 5% sodium hypochlorite for 3 minutes and in 70% ethanol for 30 seconds. When transplanted into pots, each tuber was reinoculated with approximately 3-cm-long pieces of adventitious roots collected from the non-sterilized plants. In the third treatment (benomyl), the tubers were prepared as in the control treatment but after transplantation 100 mL of benomyl [Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, Sigma Aldrich, Czech Republic] solution, 25 mg of benomyl per kg of substrate was applied to the growing substrate.

Experiment 2

The tubers of the three orchid species (*D. fuchsii*, *D. majalis* and *P. bifolia*) were treated according to the following design: for each plant species the first tuber was only washed with deionised water (control); in the second treatment 100 mL of benomyl solution (25 mg/kg of substrate) was repeatedly added every two weeks, starting at transplanting date (benomyl). Due to the very limited number of plants allowed for destruction, there was only one plant (replicate) per treatment.

Sample harvesting and analysis

In experiment 1 and 2, plants were grown in pots with zeolite as a substrate (Zeocem Inc., Bystré, Slovakia) in a temperate greenhouse under natural light conditions with supplementary 12-h illumination provided by mercury lamps (400 W) for 36 weeks. ERM development in the substrate in both experiments was investigated non-destructively using the inserted membrane technique (Baláž & Vosátka 2001) to preserve plant individuals throughout the experiments. Four cellulose nitrate membrane filters (Pragopore, Pragochema Ltd., Czech Republic, 0.6 μm pore size, 3.5 cm in diameter) were inserted vertically into the substrate in a position radial to each plant, with the upper edge of the filter 2–3 cm below the substrate surface. At each sampling (8, 16, 24 and 36 weeks after inserting the filters), one membrane filter with adhered hyphae was carefully removed from each plot using forceps, and gently washed with distilled water. Membrane filters were then placed into Petri dishes, stained with either trypan blue in lactoglycerol (Phillips & Hayman 1970), iodonitrotetrazolium chloride (INT) for NADH diaphorase activity (Sylvia 1988), or fast blue RR for alkaline phosphatase (ALP) activity (Tisserant et al. 1993). After staining, the membrane filters were mounted on microscope slides and examined using an Olympus BX-40 microscope at 200× magnification. Viable hyphae with dye depositions after staining for NADH diaphorase and alkaline phosphatase were assessed as the percentage of the total hyphal lengths determined using an eyepiece graticule.

At the last sampling, the plant roots were carefully removed from the substrate, washed in distilled water, fixed in FAA for 48 hours and transferred to a mixture of glycerol and 90% ethanol (1:1, v/v). Temporary mounts were made free hand for the mycorrhizal colonisation assessment. Cross sections of adventitious roots and root-like extensions were taken from three zones – the apical (5–11 mm behind the root tip), middle (in the middle of the root), and basal zone

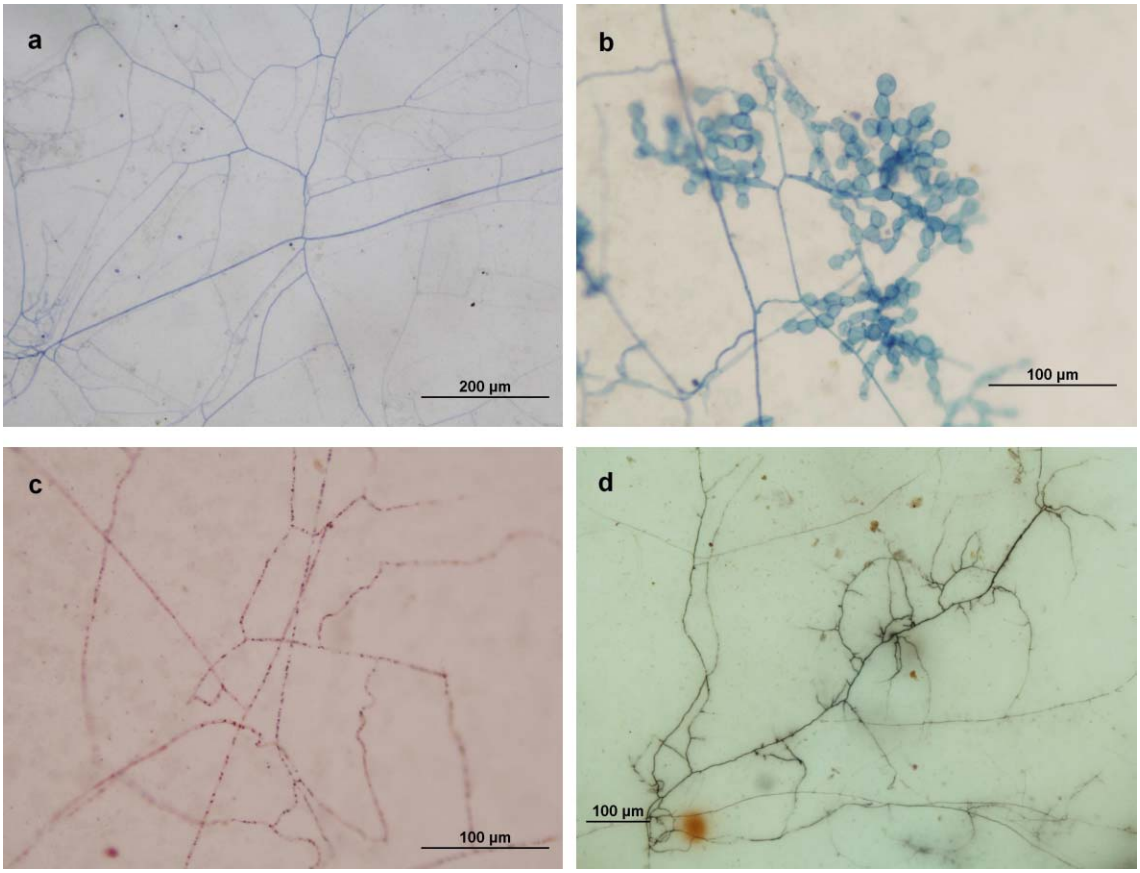

Fig. 1. Extraradical mycelium (ERM) of orchid mycorrhizal symbionts. a – morphology of ERM extracted from the mycorrhizosphere of *Dactylorhiza fuchsii* plant stained with trypan blue; b – detail of monilioid cells of the same fungus, stained with trypan blue; c – ERM extracted from the *Dactylorhiza majalis* after staining with iodonitrotetrazolium chloride (INT); d – ERM from mycorrhizosphere of *Dactylorhiza fuchsii* plant after staining for alkaline phosphatase (ALP) activity.

(5–11 mm from the tuber proper). One thin and complete section from each zone was then randomly picked for analysis. The sections were mounted in distilled water or glycerol and observed using an Olympus BX-40 compound microscope. Intensity of mycorrhizal colonisation was quantified as the proportion of the colonised cortical cells to all cortical cells assessed by counting in the whole section (Jurčák 2003). The overall intensity of mycorrhizal colonisation was assessed as a mean of intensities of mycorrhizal colonisation of all roots, root-like extensions, and tubers proper per plant per sampling ± SE.

Statistical treatment of the data

Differences in the overall intensities of mycorrhizal colonisation as well as the growth parameters of ERM were statistically analysed using one-way ANOVA in Statistica 6 software package with Fisher's LSD multiple-comparison test ($P \leq 0.05$) to assess significance levels.

Results and discussion

The inserted membrane technique proved to be suitable for extracting and quantifying the length and enzymatic activities of extraradical mycelium radiating from orchid roots grown in zeolite. Extraradical mycelium that adhered to the membrane filters showed typical features of orchid mycorrhizal fungi, i.e., narrow septate hyphae, branching mostly in right angles, with abundant anastomoses and chains of monilioid cells (Figs 1a, b). Similar morphological ERM features were observed by Baláž & Vosátka (2001).

After a relatively long lag phase, the ERM growth rate in Experiment 1 steadily increased and not even the surface sterilisation of tubers (reinoculation treatment) managed to slow the rate. Beau (1913), Fuchs & Ziegenspeck (1927), Mitchell (1989) and Čuříková (unpublished) assumed that fungal growth could not start from the cortical layers of the tubers, particularly when the fungal symbionts of *D. fuchsii* mainly colonised the adventitious roots and root-like extensions that were removed in this treatment. The mycorrhizal symbionts of the control and reinoculated plants showed steady growth between week 16 and 36 in Experiment 1, whereas no growth was observed until week 16 (Fig. 2). Overall intensity of mycorrhizal colonisation was 23.3 ± 2.6% in reinoculated treatment and 17.2 ± 4.5% in the control treatment. The differences in the overall intensities of mycorrhizal colonisation in all three treatments (control, reinoculated, benomyl) were not significant in Experiment 1.

The overall intensity of mycorrhizal colonisation in the benomyl treatment (17.3 ± 1.6%) also confirmed that benomyl application did not have any significant effects on ERM development at all sampling times. The

Table 1. Metabolic activity of ERM of mycorrhizal fungi associated with *Dactylorhiza fuchsii* after staining for NADH diaphorase and alkaline phosphatase (ALP); vital hyphae were assessed as the percentage of the total hyphal lengths (mean ± SE, n = 5); Data marked with the same letters are not significantly different according to Fisher's LSD multiple-comparison, ($P \leq 0.05$); * significantly different between week 24 and week 36 (experiment 1).

Sampling time	Enzyme	Control	Reinoculated	Benomyl
Week 24	NADH	20.9 ± 9.0 a	24.4 ± 5.4 a	17.3 ± 11.8 a
	ALP	10.6 ± 9.5 a	2.7 ± 1.6 a*	1.2 ± 1.1 a
Week 36	NADH	7.9 ± 7.1 a	2.3 ± 2.0 a	20.0 ± 10.0 a
	ALP	21.6 ± 5.9 ab	31.4 ± 9.4 a*	2.4 ± 1.9 b

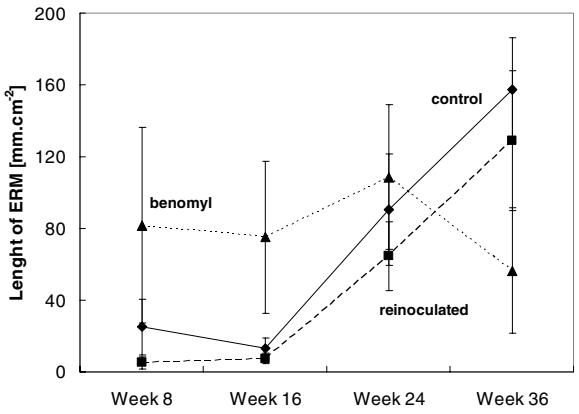

Fig. 2. Development of the extraradical mycelium of native mycorrhizal symbiont of *Dactylorhiza fuchsii* over time (mean of 5 membranes per sampling time ± 1 SE). Control treatment (◆), reinoculated treatment (■), benomyl treatment (▲), (experiment 2).

lack of negative effects of the fungicide is surprising, but confirms similar observations for ectomycorrhizal fungal symbionts (Manninen et al. 1998). The mycorrhizal symbionts seemed insensitive to benomyl application, which is in contrast to observations of inhibitory effects of benomyl on arbuscular mycorrhizal fungi in current studies (Hartnett & Wilson 1998; Wilson et al. 2001; O'Connor et al. 2002). Some earlier studies on the effect of different fungicides on the development of arbuscular mycorrhizal symbiosis did not show a consistent overall trend (Trappe et al. 1984). For example Němec (1980) and Jabaji-Hare & Kendrick (1987) reported a stimulating effect of some systemic fungicides on the development of arbuscular mycorrhiza. ERM growth associated with benomyl-treated *P. bifolia* was low, but it was caused by poor root system development in this treatment. Different responses to benomyl have been previously observed for various strains of arbuscular mycorrhizal fungi (Hartnett & Wilson 1998; Wilson et al. 2001; O'Connor et al. 2002) or ectomycorrhizal fungi (Manninen et al. 1998; Teste et al. 2006). Bayman et al. (2002) used benomyl and propiconazole to reduce colonisation of mycorrhizal and other fungi in the tissue of the epiphytic orchid *Lepanthes rupestris*. In their study, benomyl was more effective at inhibiting fungal growth in *L. rupestris* leaves than propiconazole, suggesting that benomyl may have restricted the growth of deleterious fungi more than that of beneficial fungi. Benomyl might have had a positive impact by inhibiting pathogens but not myccorhizal fungi and ERM development.

The mycorrhizal ERM showed activity of evaluated enzymes, NADH diaphorase and alkaline phosphatase. Staining with INT for NADH diaphorase activity resulted in the deposition of red formazan precipitates in viable hyphae (Fig. 1c). After staining for ALP activity, russet-to-black precipitates were observed (Fig. 1d). The presence of ALP contradicts earlier observation of Baláž & Vosátka (2001), who did not detect any ALP activity in the ERM of mycorrhizal fungi associated with the host plant *Ophrys vernixia*. Látalová et al. (2003) confirmed that benzimidazol-based fungicides are suitable to form control, semi-mycorrhizal plants and showed that benomyl reduced ERM spreading but did not affect ERM viability. These authors, however, did not apply benomyl directly to the plants. The percentage of hyphae showing NADH diaphorase activity was comparable in all three treatments at both sampling times in Experiment 1 (Table 1). Reinoculated plants showed significantly higher ALP activity at week 36 than at week 24. ERM viability in the control and benomyl treatments was comparable at both sampling times. Treatment with benomyl significantly reduced the ALP activity in comparison with reinoculated plants at week 36 (Table 1).

The results obtained in Experiment 2 are considered only illustrative because no replications were made. In Experiment 2, the ERM development of the studied orchid species has reached maximal levels at different sampling times. Mycorrhizal symbionts associated with *D. fuchsii* roots showed the highest ERM growth activity after benomyl application (Fig. 3) in the later part of the cultivation period. Although no statistical treatment could be applied on the data, the control treatment showed the trend of higher values compared to benomyl application. Overall intensity of mycorrhizal colonisation of *D. fuchsii* roots (given as a mean of all roots at the harvest ± 1 SE) was 15.2 ± 2.3% in the benomyl treatment and 20.7 ± 4.2% in the control treatment. Mycorrhizal symbionts of *D. fuchsii* showed low or even zero metabolic activity of NADH diaphorase and ALP after benomyl application (Table 2). In comparison with corresponding values for other species and treatments, the ALP value found for benomyl-treated *D. majalis* was very high.

ERM growth of the symbiotic fungus associated

Table 2. Enzymatic activity of ERM of three orchid species after staining with iodonitrotetrazolium chloride (INT) for NADH diaphorase and alkaline phosphatase (ALP). Viable hyphae assessed as the percentage of the total hyphal lengths (experiment 2).

Species	Sampling time	Enzyme	Control	Benomyl
Dactylorhiza fuchsii	Week 24	NADH	60.9	4.4
		ALP	8.7	0
	Week 36	NADH	0	0
		ALP	72	0
Dactylorhiza majalis	Week 24	NADH	34.1	50
		ALP	12.7	28.8
	Week 36	NADH	13.8	nd
		ALP	10.5	nd
Platanthera bifolia	Week 24	NADH	42.1	0
		ALP	0	0
	Week 36	NADH	17.1	0
		ALP	27.6	0

nd – not determined

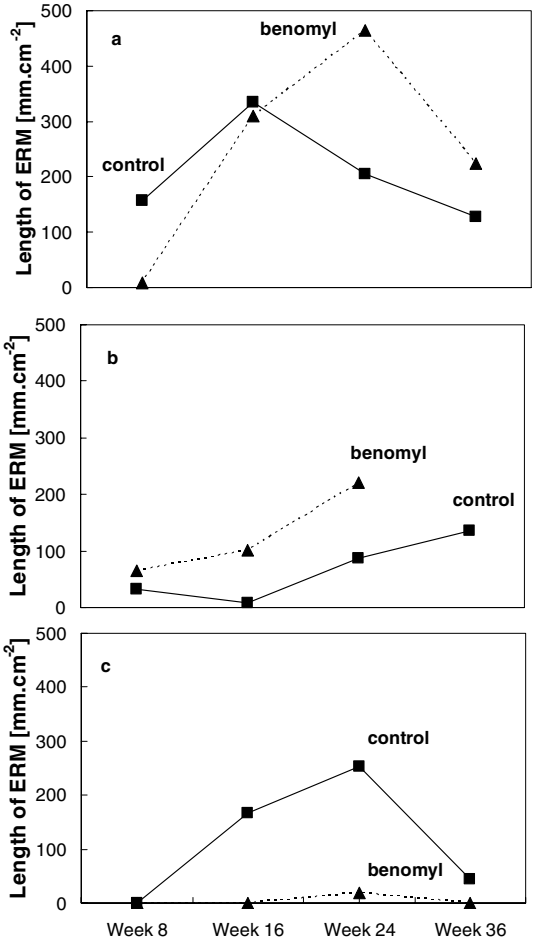

Fig. 3. Development of extraradical mycelium of orchid mycorrhizal symbionts associated with *Dactylorhiza fuchsii* (a), *D. majalis* (b) and *Platanthera bifolia* (c). Control treatment, i.e., tubers washed with deionised water (■); benomyl treatment (▲) (experiment 2).

with *D. majalis* gradually peaked at week 36. The benomyl application did not reduce mycorrhizal symbiont development (Fig. 3). ERM of the control and benomyl-treated plants showed both NADH and ALP activity (Table 2). The application of benomyl on *P. bifolia*, however, almost completely reduced ERM growth (Fig. 3) and the ERM of benomyl-treated plant showed neither NADH nor ALP activity at either sampling time. The fungal hyphae in the mycorrhizosphere of the control treatment plant, however, were vital and enzymatically active (Table 2). This low ERM growth and enzymatic activity was related to poor root-system development. At harvest, the control plant of *D. majalis* and benomyl-treated plant of *P. bifolia* did not have sufficient amounts of the roots to assess the intensity of mycorrhizal colonisation. The overall intensity of benomyl-treated *D. majalis* roots' mycorrhizal colonisation was 19.0 ± 2.3% and for *P. bifolia* 8.81 ± 1.7%. The differences in the intensities of mycorrhizal colonisation between control treatments of *P. bifolia* and *D. fuchsii* plants were significant according to Fisher's LSD multiple-comparison test, ($P \leq 0.05$, df = 17).

The study showed that the development of extraradical mycelium of orchid mycorrhizal symbionts can differ among species as well as in the infectivity of benomyl in the attempt to form the plants with reduced mycorrhizal development.

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