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INOCULATION OF *RHODODENDRON* CV. BELLE-HELLER WITH TWO STRAINS OF *PHIALOCEPHALA FORTINII* IN TWO DIFFERENT SUBSTRATES

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Abstract: The growth response of an ornamental *Rhododendron* hybrid to the inoculation with *Phialocephala fortinii* was studied in two pot experiments in order to decide about the effectiveness of the inoculation of young rhododendron microplants. Two different substrates were used in both experiments, either sterilized or non-sterilized: a horticultural substrate and a soil collected from a field site with dominant ericoid vegetation. Two fungal isolates were used for an inoculation: *P. fortinii* strain P (UAMH 8433) and *P. fortinii* strain F, a dark septate endophyte (DSE) previously isolated from naturally-infected roots of *Vaccinium myrtillus*. Both *Phialocephala* strains successfully colonized the roots of the host plants forming typical DSE (=pseudomycorrhizal) colonization pattern including the formation of intracellular microsclerotia. However, pseudomycorrhizal colonization did not affect the growth parameters of the host rhododendrons. The results from both experiments indicate a neutral effect of the inoculation with DSE fungi on the growth of *Rhododendron* cv. Belle-Heller.

Keywords: Dark septate endophytes, Pseudomycorrhiza, Ericoid mycorrhiza

INTRODUCTION

Dark septate endophytes (DSE) are conidial or sterile fungi inhabiting roots of a wide range of higher plants. They appear ubiquitous with worldwide distribution and represent a heterogeneous group, functionally and ecologically interacting with other soil biota (JUMPPONEN & TRAPPE 1998a). It is believed that they replace arbuscular mycorrhizal fungi at climatically and edaphically extreme sites (HASELWANDTER & READ 1980, READ & HASELWANDTER 1981). Among DSE fungi, *Phialocephala fortinii* WANG et WILCOX (1985) is the most studied due to its frequent isolation from roots.

Although DSE fungi are frequently isolated from roots or rhizosphere of higher plants, their identification has been difficult for a long time. The relationship between fungal strains s now often analyzed using ribosomal DNA (rDNA) amplified by the polymerase chain reaction (PCR) (MCLEAN et al. 1999). The universal primers are used to amplify the region of rDNA containing the two highly variable internal transcriber spacers (ITS) and the 5.8S subunit between them. These sequences can be analyzed phylogenetically in order to

elucidate the relationship between isolates (McLean et al. 1999, Monreal et al. 1999, Sharples et al. 2000).

The form of the coexistence between DSE fungi and roots of plants is often called pseudomycorrhiza (Wilcox & Wang 1987) or DSE-association (Jumpponen 2001). Similar to other root-fungus associations, DSE-associations vary from negative to neutral and positive when evaluated by host performance. The whole spectrum of possible interactions was reported for *P. fortinii* with a variety of hosts (Wilcox & Wang 1987, Stoyke & Currah 1993, O'Dell et al. 1993, Fernando & Currah 1996, Jumpponen et al. 1998, Jumpponen & Trappe 1998b).

The pattern of DSE colonization is similar in the roots of different plant species (Currah et al. 1993, O'Dell et al. 1993, Stoyke & Currah 1993). DSE colonization was described mainly for conifers (species of *Pinus*) and *P. fortinii*. Although several reports about the isolation of *P. fortinii* from the roots of rhododendrons exist (Hambleton & Currah 1997, Currah et al. 1993b), colonization was described mostly at the ultrastructural level and morphological studies of DSE colonization in *Ericaceae* are missing.

Phialocephala fortinii have been frequently isolated from ericaceous plants (Hambleton & Currah 1997). This indicates that aside from the true ericoid mycorrhizal symbionts (*Hymenoscyphus ericae*, *Oidiodendron* genera; see Straker 1996), DSE fungi colonizing roots perform a significant ecological function in the *Ericaceae*.

In the *Ericaceae*, the so-called ornamental species are of great horticultural interest, being commercially produced and grown in parks and gardens. The observations of Moore-Parkhurst & Englander (1982) revealed that different cultivars of rhododendrons are already being colonized by ericoid mycorrhiza in nurseries. Taking into account the wide distribution of DSE fungi it can be expected that they are present in ornamental ericoid species, too. This expectation was confirmed by checking *Rhododendron* roots from the nursery at the Institute of Botany in Průhonice (Vohník 2002). It was shown that despite intensive fungicide treatments during the cultivation of rhododendrons (Lemoine et al. 1992), root colonizing fungi are not eliminated from the substrate. Therefore, besides the research on ericoid mycorrhiza (Lemoine et al. 1992, Jansa & Vosátka 2000, Starrett et al. 2001) and on fungal pathogens (Litterick et al. 1995), morphological and physiological studies of DSE-associations in ornamental ericoid species could be of great interest.

MATERIALS AND METHODS

Fungal strains used for inoculation

One unidentified DSE strain was chosen from the group of endophytic fungi previously isolated from naturally-infected roots of ericoid species and deposited in the fungal collection at the Institute of Botany, Academy of Sciences of the Czech Republic, Průhonice (DSE strain F). It was isolated from the roots of *Vaccinium myrtillus*, Jeseníky Mts., Czech Republic (Jansa & Vosátka 2000). The endosymbiotic capabilities of the chosen strain were proven by re-synthesis of a DSE association in vitro with seedlings of *Vaccinium vitis-idaea* (Fig. 1).

Since the sampled strain did not produce reproductive structures during conventional cultivation on 4% maltose agar (MEA) or 4% potato dextrose agar (PDA), it was cultivated at 0.5% PDA in the dark at low temperatures, and irradiated with UV several times during

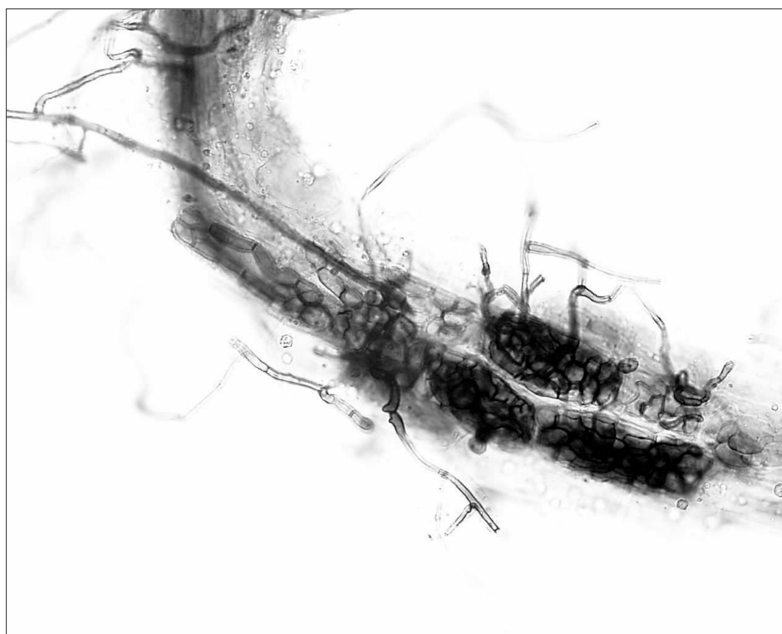

Fig. 1. Abundant intra- and extracellular colonization of *Vaccinium vitis-idaea* root by *Phialocephala fortinii* strain F (magnification 1000×).

cultivation. After 11 months, the fungal mycelium started to produce conidiophores with phialides and conidia similar to those of *P. fortinii* (see WANG & WILCOX 1985, CURRAH & TSUNEDA 1993). The ITS1-5.8S-ITS2 rDNA region was sequenced from this strain. The edited alignment of the sampled rDNA was compared with several alignments of *P. fortinii* strains reported in the literature. The sequence of DSE strain F showed 99.3% homology with the sequence of *P. fortinii* strain CBS 554.86 (FAP7 – holotype; GIRLANDA & GHIGNONE, unpubl.). For the list of procedures, the sequence similarity table and a parsimony tree see VOHNÍK (2002). In the following, this strain is referred to as *P. fortinii* strain F.

In the experimental part of the study another strain of *P. fortinii* was used for the comparison with *P. fortinii* strain F. It was *P. fortinii* UAMH 8433, previously isolated from roots of *Erica herbacea*, Smrjene, Slovenia (VODNIK et al. 1997). This strain is in the following referred to as *P. fortinii* strain P.

Plant material

Rooted stem cuttings of *Rhododendron* cv. Belle-Heller were used in both experiments. Cuttings were produced from tissue cultures (Vitrolab Ltd., Prague). They were rooted and pre-cultivated in Lignocel (coconut fibres with tree bark) amended with complex slow-releasing fertilizer.

Substrates

Rhododendrons were in both experiments cultivated in two substrates (1) commercially produced garden substrate (AGRO Ltd., Czech Republic) and (2) natural substrate collected in December 2000 in the Lužické Mts. (northern Czech Republic) from an area with a dominant occurrence of *Vaccinium myrtillus* and *V. vitis-idaea* (*Ericaceae*). Due to the high inhomogeneity of the natural substrate, it was sieved through a 5-mm sieve, which resulted in about half of the original substance remaining on the sieve (roots, pieces of wood, old branches). This part was substituted by mixing the sieved substrate with Lignocel (1 : 1). For the first experiment, substrates were left non-sterilized, for the second experiment, substrates were sterilized by autoclaving. Analysis of nutrients in the substrates before setting up the first experiment revealed comparable levels of NO_3 in both substrates (in mg/kg: 20 in garden and 17 in natural substrate), a higher level of NH_4 in the natural substrate (21 garden / 33 natural) and considerably higher levels of PO_4 (373 garden / 26 natural) and total P (904 garden / 292 natural) in the garden substrate.

Design of experiment 1

Both substrates were treated with hot steam under atmospheric pressure for 5 hours according to a procedure common in horticulture to eliminate weeds, mosses and other soil inhabitants. Rooted cuttings of the rhododendrons were transplanted into ∅ 10 cm pots containing approx. 300 ml of the substrate. Fungal cultures of both strains were maintained on PDA (39 $g.l^{-1}$, Difco). An inoculum homogenate was prepared by blending the content of three 9-cm Petri dishes in 300 ml sterile water. Inoculation was performed by pipetting 5 ml of the homogenate directly under the cuttings. Five ml of non-inoculated PDA suspension was pipetted under the control plants. There were 3 treatments (2 fungal strains + control) and 12 replicates for each treatment. Pots were randomly placed in the growth chamber (21 °C/18 °C day/night temperature, 150 µmol $m^{-2}s^{-1}$, 70 ± 5% relative humidity) and watered with distilled water.

Design of experiment 2

Both substrates were sterilized by autoclaving two times for 60 minutes at 121 °C in a 24-hour interval. In order to exclude the possibility that plant material is a source of infection (hyphal coils similar to ericoid mycorrhiza were found in controls in the first experiment), rhododendrons were treated three times with the fungicide benomyl (1.5 $g.l^{-1}$) every week at the end of the rooting period. Three weeks after the last fungicide application, new cuttings were obtained from plantlets and were surface-sterilized in 0.3% sodium hypochlorite for 10 min. Cuttings were washed in sterilized water, treated with commercial rooting powder (IAA) and transferred into autoclaved Lignocel in a 2 l plastic container for re-rooting. Cuttings were rooted at 21 °C/18 °C day/night temperature, 100 µmol $m^{-2}s^{-1}$, 70 ± 5% RH for 10 weeks. A screening of re-rooted cuttings showed no fungal infection. Experiment 2 was then set up as described for Experiment 1. Light conditions were changed to lower light intensity (100 µmol $m^{-2}s^{-1}$) considering the experience from the first experiment.

Measurements and analyses

Both experiments were harvested after six months. The fresh weight of shoots and roots was measured and the dry weight of shoots was estimated after oven drying at 120 °C. Roots were carefully washed under tap water, cleared in 10% KOH at 90 °C for 75 min, washed with tap water and then stained in trypan blue for 15 min at 90 °C. The staining method was modified according to PHILIPS & HAYMAN (1970). For each plant 10 root segments of approx. 1 cm length were mounted on microscopic slides in lactoglycerol and screened for infection. The colonization pattern of established DSE association was evaluated and compared with that obtained from roots of *V. vitis-idaea*. The infection rate was estimated by dividing fragments into three classes: (1) non-infected, (2) roots with scarce infection units and (3) more heavily colonized roots (see Table 1). The measured growth parameters were analyzed using two-way ANOVA.

RESULTS AND DISCUSSION

Colonization pattern and infection rate

Root systems of all inoculated seedlings were colonized by dark septate mycelium. Colonization patterns of both *P. fortinii* strains were very similar and corresponded to patterns obtained from *in vitro* synthesized DSE associations with *V. vitis-idaea*. Most frequently, hyphae grew along the surface of the root, generally following the sulcae between adjacent epidermal cells, penetrated the root cells and formed intraradical infection units of limited size (Fig. 2). Microsclerotia were formed within individual epidermal cells. Frequently the cells surrounding the point of emergence of lateral roots were invaded. Hyphae did not invade the stele. No growth of hyphae over the root tips, as reported for *P. fortinii-Rhododendron brachycarpum* association (CURRAH et al. 1993), was found. Still, single hyphae attaching to the tips of the lateral roots were observed. No infection with DSE was found in the non-inoculated control. However in the first experiment, most of the roots including those from controls were colonized by blue-stained hyphal coils presumably belonging to an ericoid root-associated fungus. Obviously, steaming of the substrate did not entirely eliminate the inoculation potential of ericoid endobionts present in both substrates. It was also not possible to exclude micro-cuttings as the source of this infection. Since ericoid endobionts were observed in all plants and no DSE colonization was found in the control, we can consider the growth response to inoculation as a response to colonization with *P. fortinii* strains. On the other hand, we cannot ignore possible interactions between the inoculated *P. fortinii* strains and ericoid strains while interpreting the results. In the second experiment no fungal colonization was found in the control samples and only DSE colonization was found in the roots of inoculated plants. The colonization pattern of inoculated plants was very similar to the pattern found in the first experiment.

In the first experiment the infection rate was higher with the F-strain than with the P-strain (Table 1). There was no evident difference between substrates, with the exception of the P-strain treatment for which a higher proportion of most heavily infected root fragments was measured in the garden substrate. In the second experiment, however, the estimation of the infection rate revealed a higher infectivity of both fungi in the garden substrate (Table 1).

Because the garden substrate was richer in P content, it is obvious that the high P amount did not limit the rate of DSE colonization. The same experiment showed no clear differences

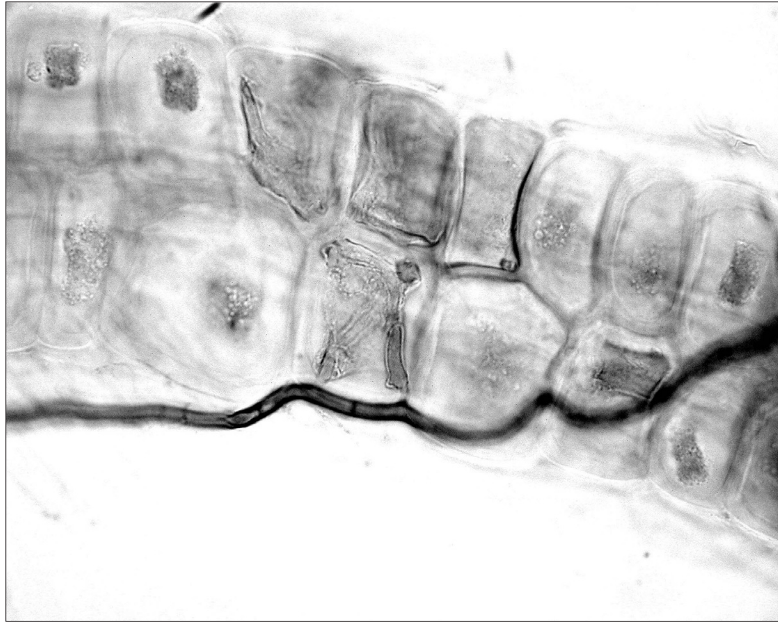

Fig. 2. Intracellular hyphal loops formed by *Phialocephala fortinii* strain F in roots of *Rhododendron* cv. Belle-Heller (magnification 1000×).

in the colonization rate between both strains. The reason for the different results observed in both experiments can be differences in nutrient levels and conditions of cultivation, especially light intensity. Still, in *Pinus contorta* the degree of root colonization by *P. fortinii* was not significantly affected by the application of N or organic matter (JUMPPONEN et al. 1998).

Generally the quantification of DSE infection is not straightforward. Mainly rough estimations of colonization rates are therefore given for DSE fungi in the literature. For *P. fortinii*-inoculated *Pinus contorta*, JUMPPONEN et al. (1998) reported 4–20% root length colonization. In the studies with inoculated ericoid species, colonization is often described only qualitatively (CURRAH et al. 1993, JANSA & VOSÁTKA 2000). The huge variability in root colonization found in some studies (JUMPPONEN & TRAPPE 1998b) could also be a result of the polymorphic nature of DSE fungi. It was reported that DSE produce structurally different hyphae, melanized and hyaline and particularly hyaline internal hyphae can easily be overlooked using conventional fungus staining methods and microscopy (BARROW & AALTONEN 2001).

It is therefore possible that the infection rate was underestimated in our case, too. However, taking into account at least the presence of melanized hyphae in the roots of the inoculated plants, we can conclude that the inoculation was successful in both experiments. The colonization rate was comparable to rates from studies in which a significant physiological effect of the fungus was observed (e.g. JUMPPONEN et al. 1998).

Growth response to inoculation

Table 1. The colonization rates of *Phialocephala fortinii* strain F and strain P in the roots of *Rhododendron* cv. Belle-Heller in both experiments. The amount of infection found in the roots in the both substrates is scored into three classes (no colonization – medium colonization – high colonization) and the percentage of screened root fragments belonging to each class is given.

Fungus	Substrate	Infection (% of screened root fragments) None	Medium	High
Experiment 1				
P. fortinii P	natural	56	27	17
	garden	49	18	33
P. fortinii F	natural	27	24	54
	garden	30	19	51
Experiment 2				
P. fortinii P	natural	22	70	8
	garden	8	50	42
P. fortinii F	natural	26	67	7
	garden	16	54	30

The growth promoting effects in inoculated ericoid plants have been studied mainly using the ericoid mycorrhizal fungus *Hymenoscyphus ericae*. In the study of LEMOINE et al. (1992) series of experiments was carried out to determine whether the production of *Rhododendron* hybrids could be improved through controlled mycorrhization. For some fungal strains and *Rhododendron* clones an increase up to 200% in fresh weight of inoculated plants was observed. However, differences between isolates regarding growth effects indicated a great specificity between fungi and plants. The growth response of rhododendrons was dependent on the substrate composition and pH.

In the study of JANSA & VOSÁTKA (2000) 26 strains of root endophytes isolated from different ericoid hosts were screened in an inoculation experiment with a *Rhododendron* hybrid. No negative influence on the growth of host plants has been observed for any inoculated isolate, while about 10 of the tested strains exhibited positive effects on the growth of *Rhododendron* micro-cuttings grown in peat-based media.

In both experiments of the present study only minor inoculation effects on the growth of *Rhododendron* plants were observed. Results were more prominent in the first experiment in which ANOVA revealed a significant interaction (substrate × fungus) for shoot fresh and dry weight, root fresh weight and total fresh weight (Table 2). Fresh shoot weight and total fresh weight of F-strain-inoculated plants grown in natural substrate were significantly lower than in control plants from the same substrate. The same fungus showed a neutral or even a beneficial effect in the garden substrate. There were no differences observed among P-strain-inoculated plants in both substrates.

A strain-specific effect of *P. fortinii* was reported by CURRAH et al. (1993). One strain of fungus had a significant negative effect on dry weight accumulation in *Rhododendron brachycarpum*, whereas the second had no effect. For *P. fortinii*-inoculated *Pinus resinosa* JUMPPONEN et al. (1998) observed no significant response under a low-nitrogen regime,

Table 2. The growth parameters of *Rhododendron* cv. Belle-Heller plants planted in two nonsterilized substrates (natural and garden) and inoculated with two strains of *Phialocephala fortinii* (P and F). Control plants were not inoculated (ANOVA results: ns – not significant at $\alpha = 0.05$).

Experiment 1

Treatment	Substrate	Growth parameters Shoot FW (g)	Shoot DW (g)	Root FW (g)	Total FW (g)	Root/Shoot
Control	natural	1.66 ± 0.37	0.51 ± 0.11	0.34 ± 0.06	2.00 ± 0.40	0.28 ± 0.06
	garden	1.34 ± 0.18	0.42 ± 0.05	0.54 ± 016	1.89 ± 0.26	0.44 ± 0.11
P. fortinii P	natural	1.26 ± 0.28	0.38 ± 0.08	0.43 ± 0.08	1.65 ± 0.35	0.54 ± 0.22
	garden	1.32 ± 0.22	0.39 ±0.07	0.30 ± 0.05	1.62 ± 0.27	0.26 ± 0.03
P. fortinii F	natural	0.73 ± 0.17	0.25 ± 0.05	0.24 ± 0.06	0.88 ± 0.17	0.63 ± 0.23
	garden	2.10 ± 0.47	0.69 ± 0.15	0.58 ± 0.12	2.67 ± 0.58	0.30 ± 0.04
ANOVA	substrate	ns	ns	ns	ns	ns
(*P*-value)	fungus	ns	ns	ns	ns	ns
	substrate × fungus	0.025	0.018	0.046	0.019	ns

Table 3. The growth parameters of *Rhododendron* cv. Belle-Heller plants planted in two sterilized substrates (natural and garden) and inoculated with two strains of *Phialocephala fortinii* (P and F). Control plants were not inoculated (ANOVA results: ns – not significant at $\alpha = 0.05$).

Experiment 2

Treatment	Substrate	Growth parameters Shoot FW (g)	Shoot DW (g)	Root FW (g)	Total FW (g)	Root/Shoot
Control	natural	11.12 ± 2.12	3.32 ± 0.67	3.52 ± 0.93	16.36 ± 2.71	0.30 ± 0.05
	garden	13.73 ± 1.11	3.95 ± 0.30	4.35 ± 0.55	18.08 ± 1.45	0.32 ± 0.04
P. fortinii P	natural	8.43 ± 3.02	2.34 ± 0.80	2.08 ± 0.75	10.51 ± 3.66	0.24 ± 0.03
	garden	12.05 ± 1.02	3.33 ± 0.41	4.53 ± 0.85	16.58 ± 1.80	0.32 ± 0.06
P. fortinii F	natural	10.13 ± 2.79	3.14 ± 0.87	3.44 ± 1.22	13.58 ± 3.99	0.28 ± 0.04
	garden	13.64 ± 0.33	4.01 ± 0.14	4.44 ± 0.45	18.08 ± 0.40	0.33 ± 0.04
ANOVA	substrate	ns	ns	0.048	ns	ns
(*P*-value)	fungus	ns	ns	ns	ns	ns
	substrate × fungus	ns	ns	ns	0.019	ns

while inoculation increased the host biomass by approximately 50% when nitrogen fertilizer was applied.

In our study the growth enhancement by the F-strain could also be related to the higher nutrient level in the garden substrate. No effect of different nutrient levels was observed for the P-strain. For ericoid mycorrhizal fungi it is known that nutrient acquisition varies depending on isolates and the conditions of cultivation (Bajwa & Read 1986). In order to relate observed growth response and possible improvements in mineral nutrition (F-strain, garden substrate) analyses of mineral nutrients in the plant tissues would be needed.

Due to different plant material (older cuttings were used for the second experiment) and growth conditions (light intensity, sterilization of substrates) it is difficult to compare the growth effects observed in the first and in the second experiment. Optimization of growth conditions resulted in the much better growth of rhododendrons in the second experiment as

revealed by biomass measurements. In spite of the relatively good colonization rate no effect of inoculation was detected (Table 3). Generally the growth in the garden substrate was much better than in the natural substrate. However, this difference was significant only for the root fresh weight.

To conclude, both experiments showed relatively neutral effects of inoculated DSE fungi on the growth of *Rhododendron* cv. Belle-Heller irrespective of cultivation conditions. Therefore, only limited growth effects of DSE fungi are to be expected during practical cultivation of *Rhododendron* cultivars.

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