Original research article

Interactions of ericoid mycorrhizal fungi and root pathogens in Rhododendron: *In vitro* tests with plantlets in sterile liquid culture

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Abstract: Plant protection against soil-borne diseases has been a challenge in horticultural production of Ericaceae for a long time. We tested the hypothesis that ericoid mycorrhizal fungi (ERMF) control root pathogens. Isolates of ERMF previously obtained from *Calluna vulgaris* and *Rhododendron hirsutum* were selected based on growth inhibiting activity against Oomycetes in dual agar plate tests. In addition, we assessed their impact on economically important Pythium spec. and *Phytophthora cinnamomi* in sterile Rhododendron plantlets in a liquid culture system, which is suitable for continuous observations of the infection process. For this purpose, rooted cuttings of micro-propagated Rhododendron plantlets were transferred to a mineral solution and subsequently inoculated with either *Oidiodendron maius* or the ERMF isolates. Before the root pathogens were applied to the experimental system, the symbioses were established over a four-week period. Mycorrhizal development, pathogen infection sites and development in mycorrhizal plants and non-mycorrhizal control plants were assessed microscopically. The root-colonising intensity of the tested ERMF differed considerably, but each of the applied ERMF impaired external pathogen mycelium and reduced pathogenic infections. A complete reduction was achieved at higher ERMF colonisation levels. The failure of symbiosis formation allowed pathogens to infect and spread. The quantification data concerning mycorrhiza frequencies and extramatrical hyphal nets provided details for a discussion on the suppressive effects of ERMF on the pathogens. The tested ERMF confer at least a localized protection from pathogen attack through suppression prior to infection. It is now to prove, whether these biocontrol effects will also be expressed in pot experiments.

Keywords: antibiosis, biocontrol, *Leohumicola* sp., *Oidiodendron maius*, *Phytophthora* spp., *Pythium* spec.

Abbreviations: CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAOM, National Mycological Herbarium, Biosystematics Research Institute, Department of Agriculture, Ottawa, Ontario, Canada; DSMZ, DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; JKI, Julius Kuehn-Institute, Federal Research Centre for Cultivated Plants, Institute for Plant Protection in Horticulture and Forests, Braunschweig, Germany.

Introduction

Ericoid mycorrhizal fungi (ERMF) are known to promote plant growth in nutrient deficient soils. The most essential function of ERMF is to support the host plants through the absorption and translocation of nitrogen from various biogenic sources that are otherwise limited or even unavailable for plants without mycorrhizal associations (Cairney et al. 2000, Emmerton et al. 2001, Kosola et al. 2007, Grelet et al. 2009). In addition, ericoid mycorrhizae (ERM) buffer or detoxify site-specific abiotic stress factors, such as organic acids or heavy metals (Martino et al. 2003). However, little is known about the mechanisms of the

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symbiotic interactions between ERMF and host plants (Peterson et al. 2004, Kosola et al. 2007, Smith and Read 2008, Grelet et al. 2009). The impact of ERMF and other fungal root endophytes on the protection against plant pathogens is considered as an optional feature (Mandyam and Jumpponen 2005, Girlanda et al. 2006, Barrow et al. 2008, Rodriguez et al. 2009, Newsham 2011, Porras-Alfaro and Bayman 2011). But there is only little knowledge about and less evidence of positive effects of ericoid mycorrhiza in plants under biotic stress.

In horticultural production systems of ericaceous plants, the pathogens *Phytophthora* spp. and *Pythium* spp. deserve special attention, as the root infections by these species cause severe losses in open-field nurseries and container cultures. Disease development of root rot and subsequent shoot decay may be diffuse, slow or sudden, depending on unregulated environmental factors (Coyier and Roane 1986, Erwin and Ribeiro 1996, Orlikowski and Szkuta 2002, Orlikowski and Szkuta 2003, Hardham 2005). Latent root infections, frequent failure in early diagnosis, problems in a sufficient protective or curative chemical treatment of older plants or sale products, and, e.g. in Germany, the availability of only three effective compounds within the registered oomycete specific fungicides (Federal Office of Consumer Protection and Food Safety 2012) make a chemical control of these soil-borne diseases difficult. As other mycorrhizal associations are known for protective effects against root diseases (Schönbeck et al. 1994, Whipps 2004), it was a challenge to investigate the potential role of ERMF for biocontrol purposes.

In our preliminary studies on the ericoid mycorrhizal symbiosis in Rhododendron, some ERMF isolates obtained from commercial peat moss substrate exhibited antifungal effects toward several root pathogens in dual agar cultures. This finding strengthened our hypothesis that ERMF could control root pathogens. In the biocontrol tests presented in this paper, we used ERMF isolated from *Erica carnea* and *Rhododendron hirsutum* plants that grew in the Karwendel mountain region (Austria) in native stands with soil pH > 5.0. The aim was to select fungal isolates which are adapted to soil reaction levels that are favourable for root invading Oomycetes. These target pathogens were chosen because of their economic relevance in the production of ericaceous plants, especially in the context of the commercial release of Rhododendron rootstocks and varieties with lime-tolerance in soil. Because such Rhododendrons exhibit growth without iron-deficiency at a higher pH range of up to 6.8, the rootstock selections have a widened suitability to various planting sites and thus enlarged production numbers and market segments (Preil and Ebinghaus 1997, Preil and Ebinghaus 1998). However, these culture conditions could favour pathogen epidemics. For example the incidence of *Phytophthora* spp. in Rhododendron might increase due to the support of sporulation and propagule survival at pH values > 4.3 in soil or nursery substrates (Coyier and Roane 1986, Erwin and Ribeiro 1996, Raaijmakers et al. 2009).

The observation of the interaction processes of symbionts and pathogens *in planta* need adapted test methods that are applied under standardized conditions with feasible assessments of colonisation and infection in the plant-symbiont-pathogen system. In this paper, we present evidence for the biocontrol potential of selected ERMF against *Pythium* spec. and *P. cinnamomi* from micro-propagated Rhododendron plantlets in a sterile liquid culture test system.

Materials and Methods

Host plant

Rhododendron caucasicum x ponticum hybrid cv. Cunningham's White micro-cuttings were propagated from axillary shoots on Anderson's Rhododendron medium, pH 5.7, according to Mordhorst et al. (1993). For *in vitro* rooting, a soft agar medium (8 g/L) containing 118 mg/L $Ca(NO_3)_2$ x 4 H_2O, 42.5 mg/L KNO_3, 32.5 mg/L KCl, 5 mg/L NaH_2PO4, 18 mg/L $MgSO_4$ x 7 H_2O, 1 mg/L $FeSO_4$ x 7 H_2O, 20 g/L sucrose was used.

ERMF

Five ericoid mycorrhizal fungi were used in the tests presented here. Four of these fungi were isolated from plants in the Karwendel mountain region in Tyrol, Austria: E 3-5, E 3-8, and E 3-24a originated from *Erica carnea* L., whereas Rh11-4 was isolated from *Rhododendron hirsutum* L. There sequences are annotated in GenBank (see below) and the fungal cultures are deposited at DSMZ Open Collection. For their isolation, microscopically examined mycorrhizal root segments were washed in sterile water, cut into pieces of 1-2 mm in length, and transferred to malt extract agar (MEA) plates (1.5% malt extract, Villa Natura Gesundprodukte GmbH, D-55606 Kirn, 20 g agar/L, pH 6.6±0.2) amended with chloramphenicol and streptomycin (100mg/L, each) to prevent bacterial growth. Only slow growing hyphae (<1 mm/d) emerging from root segment ends were chosen for subcultures on MEA plates without antibiotics (pH 6.6±0.2) and for resynthesis affirmation in Rhododendron. Four Karwendel isolates which showed antibiotic features in subsequent preliminary *in vitro* tests were selected for detailed

tests and subjected to further determination:

- E3-5 (GenBank accession No. JX912153; DSMZ collection No. DSM 26511) showed 98% identity with *Leohumicola verrucosa* strain DAOM 226889 and 97% identity with *L. minima* strain DAOM 232587, as determined with ITSI-5.8S-ITSII sequence analyses via BLAST search, but lacked conidia-formation for further morphological comparisons.
- E3-8 (GenBank accession No. JX912154; DSMZ collection No. DSM 26512) and E3-24a (GenBank accession No. JX912155; DSMZ collection No. DSM 26513) were determined at the CBS as probably two distinct species of *Leohumicola* N. L. Nickerson, but displayed cultural characters as quite similar and sterile.
- Rh11-4 (GenBank accession No. JX912156; DSMZ collection No. DSMZ 26577) is an unidentified member of the Leotiomycetes which could not be determined on the basis of the ITS sequence analysis. The proof for the identity with *Leohumicola* spp. failed in subcultures due to the sterility of the isolate.

The fifth of the tested fungi was *Oidiodendron maius* Barron, DAOM 184108, a proven strain from *Vaccinium corymbosum* L. that had been selected in Canada (Couture et al. 1983). This *O. maius* was cultured in the dark on potato dextrose agar (PDA; Merck 10130, 30 g/L, pH 5.4±0.2) and on 1.5% MEA plates (pH 5.5±0.2) at 22°C.

Pathogens

Phytophthora cinnamomi Rands, isolate 8/88/92, was kindly provided by JKI Braunschweig. *Pythium* spec., isolate Py1-2005, was supplied by Agroinnova (University of Torino, Italy). Inoculum agar plugs (0.8 cm in diameter) were prepared from 10-day-old stock cultures of the pathogens either on 1.5% MEA or PDA, (pH 5.5±0.2) at 22°C in the dark.

Dual agar plate test for plant pathogen growth inhibition

The tests on PDA and MEA plates (8.5 cm in diameter, prepared as for maintenance of *O. maius*, see above) were performed with four plates per test and three test repetitions. Each of the five mycorrhizal isolates was grown in a one-side single streak for 10 to 12 days before the pathogen was added to the opposite side with an agar plug. The growth data for the pathogen in the dual culture were recorded in two-day intervals, and the experiments lasted for 35 days with *Pythium* spec. and 57 days with *P. cinnamomi* according to the rate of pathogen growth and the development of an inhibition zone. The details of the mycelium impairment were analysed using bright field microscopy with Differential Interference Contrast (DIC; 400x, 1000x; Axiophot®, Carl Zeiss, Germany).

Liquid culture system for in planta tests

The rooted plantlets were transferred into a glass vessel containing a 25-ml mineral solution (pH 5.7) of $Ca(NO_3)_2$ x 4 H_2O (29.5 mg/L), KCl (8.15 mg/L), NaH_2PO_4 (1.25 mg/L), $MgSO_4$ x 7 H_2O (4.5 mg/L) and $FeSO_4$ x 7 H_2O (0.25 mg/L). Filter-sterilised and autoclaved bovine serum albumin (SA fraction V, Merck 112018, 87 mg/L) was added as a protein supply (Bajwa et al. 1985). To increase stability on the liquid surface, the plantlets were placed in sterilised Styrofoam rings, four plantlets per vessel. Two vessels per mycorrhizal treatment were inoculated each with four agar plugs (0.8 cm in diameter) of one single ericoid test fungus. In the liquid, the hyphae grew from the plugs towards the roots and colonised them. After four weeks of mycorrhiza establishment, the plantlets were transferred into fresh nutrient solution to exclusively test the present effect of active symbionts. Subsequently, agar plugs of the pathogen culture were positioned underneath the plantlets but without contact to the submerged roots. Accordingly, control plantlets cultured without symbionts were inoculated with the pathogens on agar plugs. Summarized, five mycorrhizal fungi were tested against two pathogenic fungi in the first trials named Pc I and Py I. In the following repetition trials Pc II and Py II, only the best performing three ERMF were tested. In these trials Pc II and Py II, two additional vessels (8 plantlets) per treatment were set up to visually record plant appearance and disease symptoms after a four-week test period with pathogens.

Microscopic assessments

For analyses of mycorrhiza and pathogen development, unfixed hair roots were stained with acid fuchsine (C.I. 42685, Merck; 1 g/100 mL *a. dest.*) mixed 1:10 with lacto phenol solution (80 g glycerol, 40 g lactic acid, 80 g phenol, dissolved in 40 mL *a. dest.*) in a stepwise rinsing procedure in lacto phenol (2 min), acid fuchsine-lacto phenol (2 min), and clearing and mounting in lacto phenol. Assessments were conducted using bright field microscopy (DIC; 400x, 1000x; Axiophot®, Carl Zeiss, Germany), and Confocal Laser Scanning Microscopy (CLSM, Leica TCS SP 2; at 543 nm excitation and 560-620 nm

emission wavelengths) was applied for the examination of cellular details.

Microscopic assessments were made at 1, 2, 3, 4 and 8 days after inoculation with *Pythium* spec. and at 5, 7, 10 and 12 days after inoculation with *P. cinnamomi.*

Stained root segments from the whole root system were rated with regard to the internal mycorrhizal root colonisation and the development of mycorrhizal extramatrical mycelium. Data on the root colonisation from 60 randomly chosen root segments of 1 cm in length per treatment (bulked from two plantlets) were obtained with frequency class ratings (0 = no mycorrhiza; 1 = <3%; 2 = >3 to 10%; 3 = >10 to 50%; 4 = > 50 to 100% colonisation and used to calculate the Myc-Index (according to a Wilt Index from scoring data, Grunewaldt-Stöcker et al. 2007). In total, index values were based on the assessment of n = 4 x 60 or n = 5 x 60 root segments per treatment and trial. Similarly, the mycelia attached outside the root segment were scored to calculate the extramatrical root surface colonisation (Extr-Index).

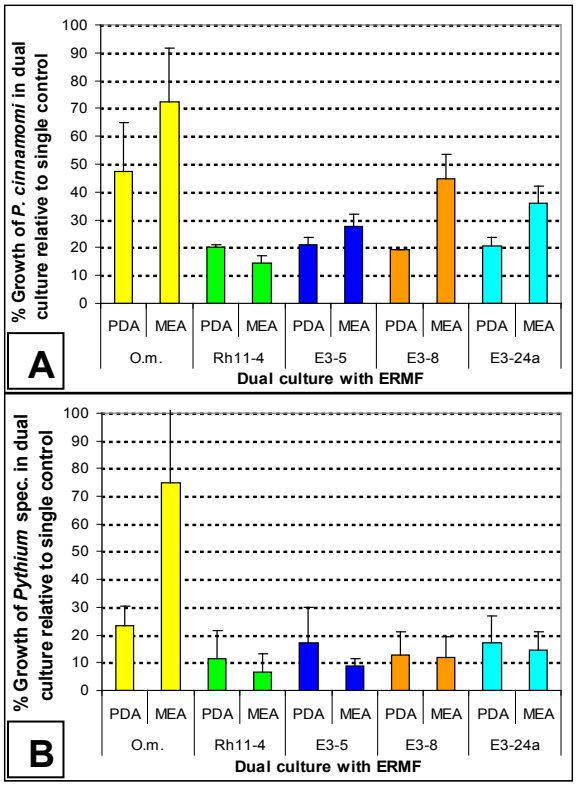

Fig. 1. Growth of pathogen mycelium in dual agar culture with ERMF on two different media (PDA, MEA), relative to the growth of single pathogen control (100%); means and SD (error bars) of bulked data of three independent repetitions, each with n=4 plates. **A:** *P. cinnamomi,* end of rating after 57 days; **B:** *Pythium* spec., end of rating after 35 days.

For pathogen assessments, red stained oomycete structures in the entry point and/or adjacent infected cells were counted as infection sites in the root segments that were previously prepared for the mycorrhiza assessments. The numbers of infection sites were summarized per treatment over the test period of 8 (*Pythium* spec.) or 12 days (*P. cinnamomi*). Additionally, extramatrical hyphae and vesicles of pathogens were investigated for visible damage or integrity.

Statistics

Data from mycorrhizal assessments, presented as frequencies in classes of mycorrhizal colonisation and extramatrical growth, were tested with two-sided permutation Wilcoxon-Mann-Whitney tests. If required, a multiplicity adjustment for all pairwise comparisons was performed with a standard (BH) correction (Benjamini and Hochberg 1995); the overall significance level was set to $\alpha = 0.05$. For comparisons against controls, a one-sided Wilcoxon permutation test was used with $\alpha = 0.05$. The statistical analyses were performed using the Open Source Software Programme R, version 2.12.0 (R Development Core Team 2010).

Results

Dual agar plate test for pathogen growth inhibition

In three independent tests, the suppressive effects of the five mycorrhizal fungi on pathogen development were observed. The growth rates of the ERMF and control pathogens were media dependent. *Pythium* spec., with an average daily rate of 2.5 cm (MEA) and 2.0 cm (PDA), was the faster growing pathogen, whereas *P. cinnamomi*, with an average rate of 1 cm/d on MEA and 0.6 cm/d on PDA, required a longer period of assessment. The growth rates of the ERMF isolates differed, but were 10-fold lower than that of *P. cinnamomi*. In dual culture, all mycorrhizal fungi impaired the growth of *Pythium* spec. and *P. cinnamomi* mycelium (Fig. 1 A, B). *O. maius* was the least effective isolate, especially on MEA plates, whereas isolate Rh11-4 showed steady, long lasting and strong suppression in all trials. The clearly visible inhibition zones for *P. cinnamomi* caused by the Karwendel isolates lasted for the whole 57-day observation period, whereas *Pythium* spec. could reach the ERMF colonies with thinned hyphae after more than one week of strong growth inhibition (Fig. 2 A, B). The PDA test medium supported the suppressive efficiency of the ERMF better than MEA. As the experiments progressed, the four Karwendel solates induced browning of the PDA medium in dual

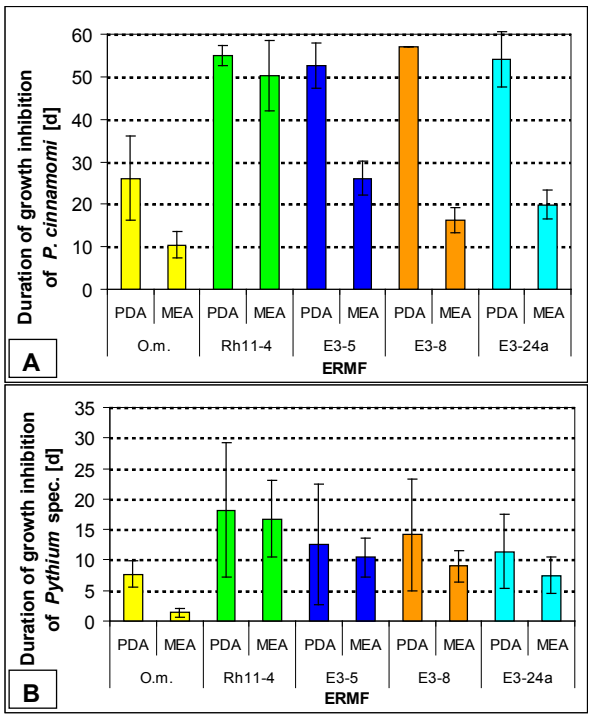

Fig. 2. Duration of growth inhibition of pathogen mycelium in dual agar culture with ERMF on two different media (PDA, MEA); means and SD (error bars) of bulked data of three independent repetitions, each with n=4 plates. **A:** *Phytophthora cinnamomi,* end of rating after 57 days; **B:** *Pythium* spec., end of rating after 35 days.

cultures (Fig. 3).

The microscopic examination revealed the impairing effects of the ERMF isolates on the mycelia of *Pythium* spec. in MEA and PDA culture as morphological alterations with hyphal distortions and lysis at the hyphal tips. In addition, the isolate E3-8 caused increased branching of *Pythium* hyphae. The hyphal morphology of *P. cinnamomi* was primarily affected by all five mycorrhizal fungi, typically leading to extended loop formation and lysis in both types of culture media. Occasionally, stunting, excessive branching and increased vesicle formation occurred (Fig. 4). There was no recovery in mycelium alterations of *P. cinnamomi* which lasted for the whole observation period (57 d). *Pythium* spec. was less affected. At least a few and thinned hyphae continued spreading towards the ERMF colonies in the end of the experiments (35 d). Hyphal parasitism was not observed under any circumstance.

In planta test in liquid culture

To demonstrate the biocontrol potential of the mycorrhizal endophytes in association with the host plant, the dual culture test was extended to a sterile system with Rhododendron plants in liquid culture.

Morphology and development of ERMF: Hyphae of *O. maius* and the Karwendel isolates colonised the hair root surface and invaded the epidermal layer. The hyphae penetrated from the exterior of the root but could also pass from cell to cell. Early infections with intracellular coil formation were observed at five days after inoculation. However, the pattern of hair root colonisation depended on the distribution of active host cells. During growth and development of the hair root, several epidermal cells exhibited senescence and collapse (Fig. 5) and did not harbour ERMF; mature cells, whether colonised or not, were typically separate and slough from the core.

The colonisation pattern was unevenly distributed within the hair root segments. Sectors with dense

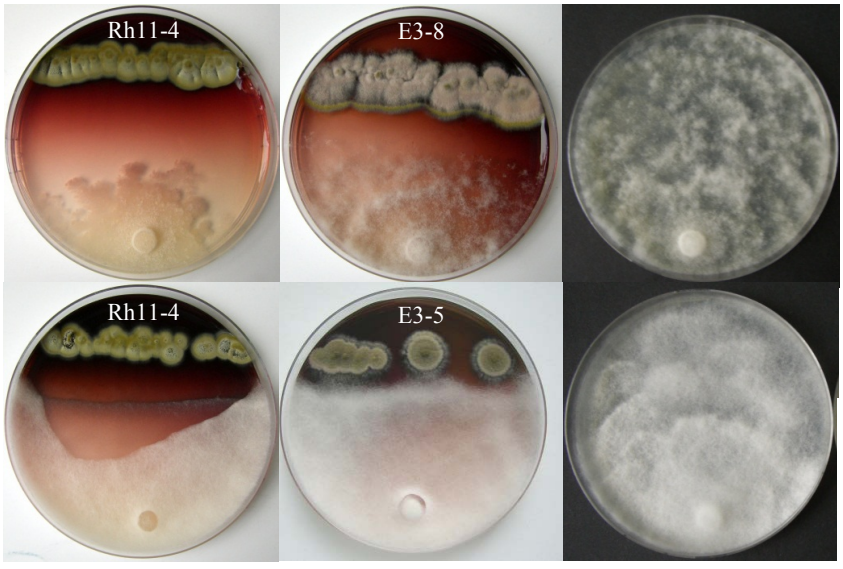

Fig. 3. Growth inhibition of *P. cinnamomi* (above) and *Pythium* spec. (below) by ERMF on PDA, 12 days of ERMF single culture plus 22 days of dual culture; right: control plates of pathogens in single culture, 22 days.

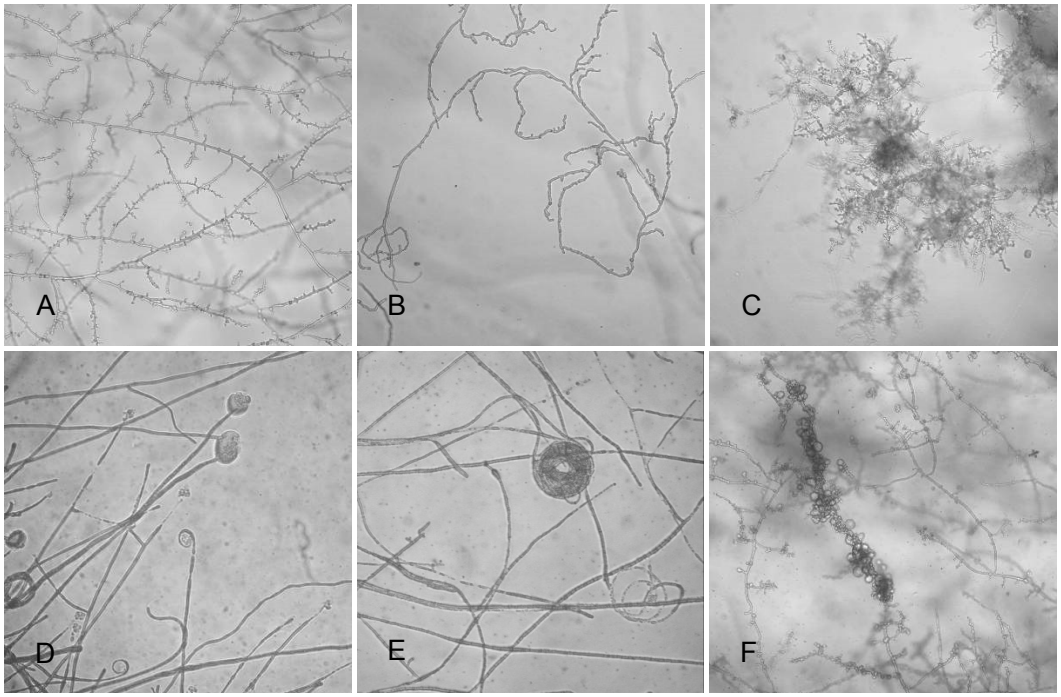

Fig. 4. Morphological alterations in *P. cinnamomi* in dual agar culture with ERMF. **A**: *P. cinnamomi* control in single culture (PDA), **B**: hyphal distortion (*O. maius,* PDA), **C**: excessive branching (E3-24a, PDA), **D**: lyses of hyphal tips (E3-8, MEA), **E**: hyphal loops and lyses (Rh11-4, MEA), **F**: stunting and anomalous vesicle formation (E3-8, PDA).

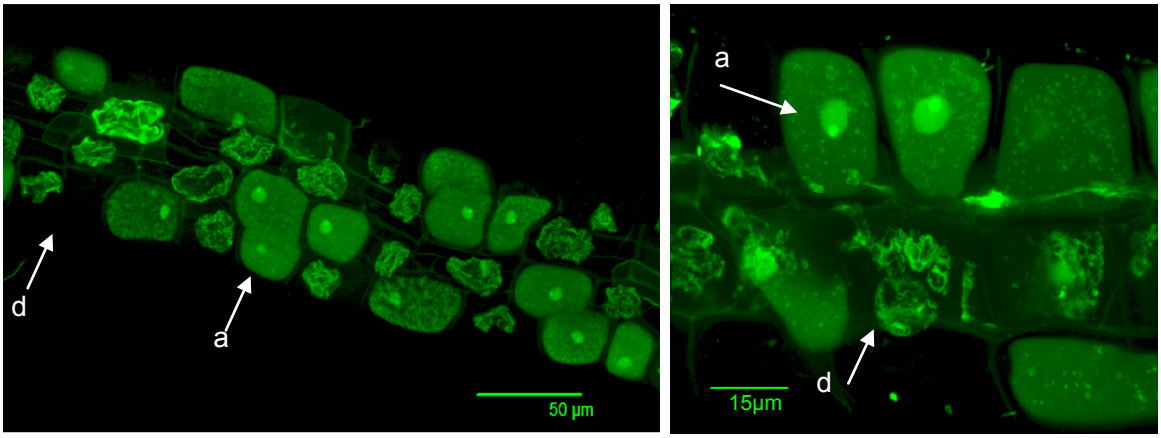

Fig. 5. Non-mycorrhizal hair root-segments of Rhododendron in liquid culture with epidermal cells of different physiological state from highly active (a) to totally degenerated (d); CLSM-technique after acid fuchsine-lactophenol staining, maximum projections of serial sections with a total depth of 19.53 µm (left) and 5.98 µm (right).

colonisation up to 100% as well as non-mycorrhizal zones were observed within the root system of one plantlet, comprising approximately 35-50 cm in total length. The hyphal morphology of the Karwendel isolates and of *O. maius* differed in cell size and vacuolisation (Fig. 6). *O. maius* sporulated, forming conidiophores on heavily colonised root segments. As development progressed, the *O. maius* and Karwendel isolates produced extramatrical mycelia with dark brown thickened cell walls. In cases of dispersed colonisation, the Karwendel isolates formed dark microsclerotia-like cells that were closely packed in the basal region of emerging lateral roots (Fig. 7 and Fig. 8). All of the tested ERMF were able to colonise the hair root tissue up to the apex.

Pathogen development: In the control plantlets

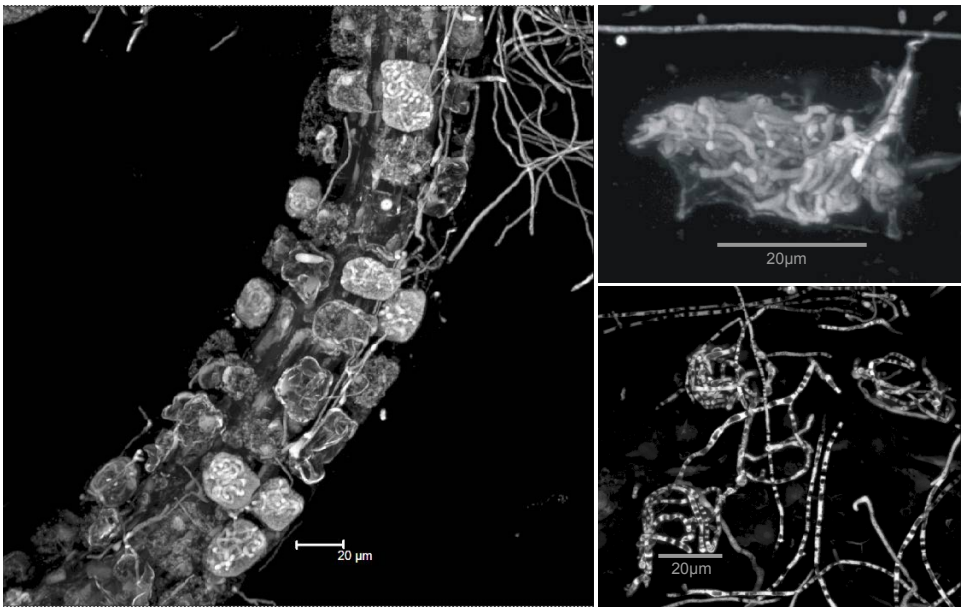

Fig. 6. Rhododendron hair root-infection with ERMF isolate E3-24a; CLSM-technique, maximum projection, acid fuchsine-lactophenol stain, transformation to black-white colour; colonisation pattern (left), coil formation (above, right), vacuolated mycelium (bottom, right).

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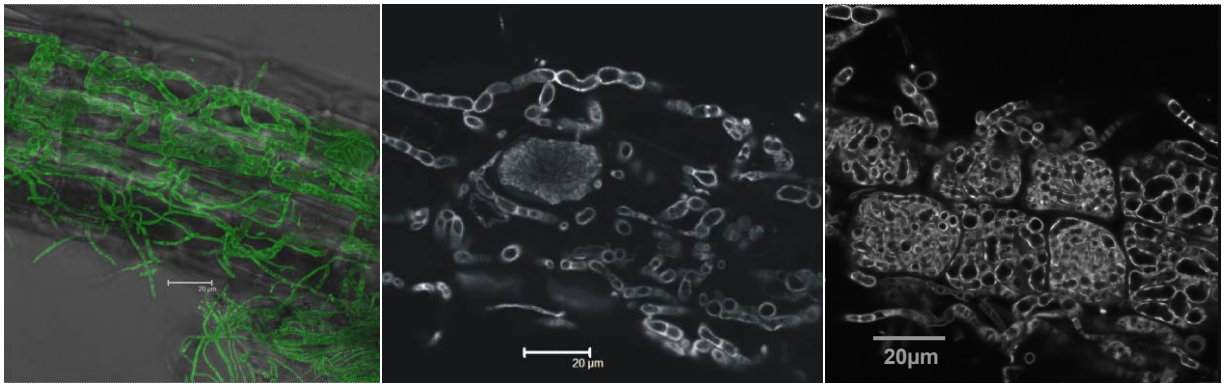

Fig. 7. Rhododendron hair root-infection with ERMF isolate E3-8; CLSM-technique, maximum projection, acid fuchsine-lactophenol stain, transformation to black-white colour; extramatrical hyphae and internal colonisation pattern (overlay of fluorescence-images and transmission-images, left), coil formation (centre), intracellular dense structures 10 weeks after application (right).

stained primary infection sites could be detected as early as one and five days after application in *Pythium* spec. and *P. cinnamomi* trials, respectively. Pathogens developed a vigorous branched mycelium and directly penetrated the epidermal cells or invaded the intercellular space to spread to the cortex parenchyma. The root tips and basal regions of the emerging lateral roots were the primary infection sites for *Pythium* spec. and *P. cinnamomi*. As the infection progressed, the *Pythium* spec. developed numerous oogonia and vesicles and destroyed root apices. *P. cinnamomi* tended to enter the stele for swift spread to the shoot (Fig. 9).

Assessment of the root colonisation of ERM: With detailed class ratings, the patchy distribution of the internal root colonisation could be assessed and displayed. Based on these scoring data the calculated Myc-Indices gave information about the quantity of potentially effective biocontrol fungi (Table 1). *O. maius* and the Karwendel isolate E3-8 were the candidates with the best colonisation in all trials, despite some failure in single segments leading to raised standard deviations in trial Py I. In the first experiments, Pc I and Py I, the Karwendel isolate E3-24a did not establish mycorrhiza at a sufficient

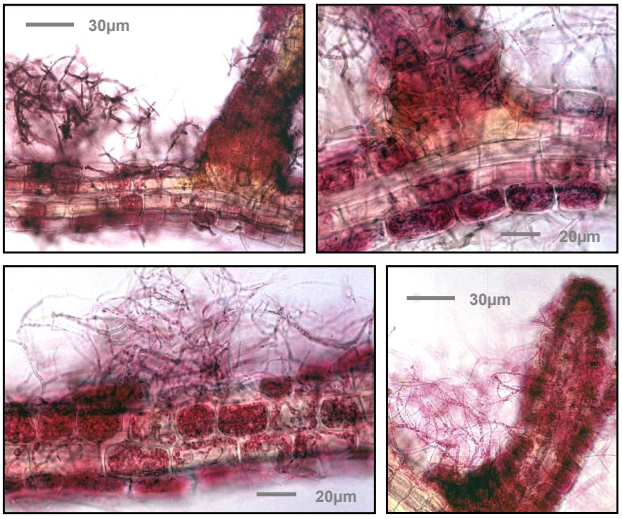

Fig. 8. Infections of *O. maius* (above) and ERMF isolate E3-8 (bottom) in Rhododendron hair roots in liquid culture with melanised extramatrical hyphae (5 weeks after application). Acid fuchsine-lactophenol staining, bright field DIC microscopy.

level or with steady frequency. The Karwendel isolate E3-5 performed inconsistently with a total failure of external and internal root colonisation in trial Py I and minor internal establishment in trial Pc I. Therefore, these isolates were omitted in the repetition trials Py II and Pc II. Isolate Rh11-4, which was highly efficient in the dual culture tests, showed only moderate internal colonisation, but had a constant high frequency on the root surface as compared to the other ERMF isolates.

The scoring data in class frequencies for the mycorrhizal establishment in trials Pc II and Py II (Fig. 10 A, B) affirmed the significant better colonisation of *O. maius* in comparison to the Karwendel isolates E3-8 and Rh11-4, which both showed less intense colonisation in the epidermal cell layer and on the root surface.

Effects of ERMF on pathogen infection: The biocontrol effect of the ERMF was measured with an assessment of the number of infection sites that the added pathogens were able to initiate (Table 1). All ERMF could protect the host plants from pathogen infections when they were sufficiently present in or on the roots. In comparison to the abundant pathogen infections in non-mycorrhizal control plants, the suppressive effects of ERMF on both pathogens were obvious and significant. Even in cases of a low mycorrhizal level with < 2.0 Myc-Index (trials Pc I, Py I), the Karwendel isolates E3-24a, E3-5 and Rh11-4 could significantly reduce the number of infection sites of Pythium and Phytophthora. The total failures of mycorrhizal establishment with isolate E3-24a in trial Py I resulted in the unhampered development of Pythium.

In particular, the relation of colonisation efficiency and biocontrol effect became evident with the details of the results of Karwendel isolate E3-5 and isolate E3-24a (Table 1): With nearly no colonisation by E3-5 in and on the root segments of four samples in trial Py I, Pythium could infect the roots like those of control plants (Fig. 11, right). However, in trial Pc I (Fig. 11, left), the antagonistic effect was detectable and coincided with medium mycorrhiza formation and root surface colonisation in all four tested samples. Equally, uneven mycorrhiza formation of isolate E3-24a in plants within one experiment (Pc I) led to heavy infestation with *P. cinnamomi* in six plantlets free from mycorrhiza, whereas the roots of two plants showed mycorrhizal formation and were protected from pathogen attack. These data prove that a sufficient symbiosis development is a prerequisite

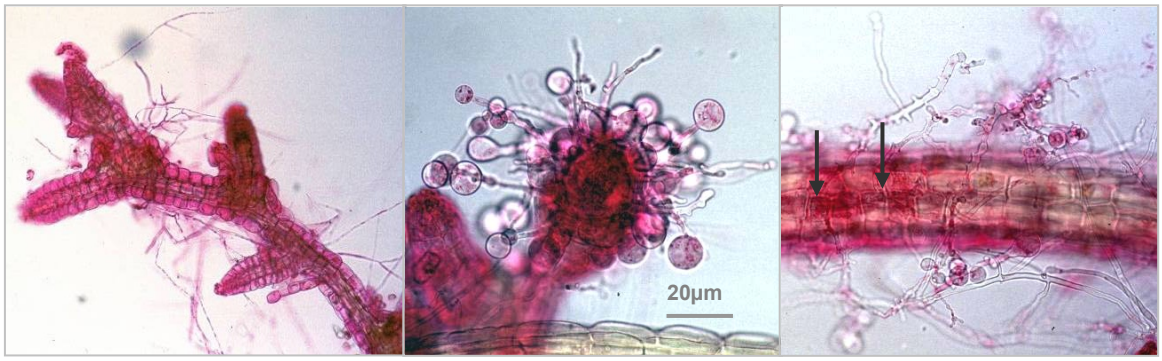

Fig. 9. Rhododendron hair root tissue of control plantlets in liquid culture with *Pythium* spec., three days after inoculation (left); root tip infection with outgrowing hyphae and vesicles of *P. cinnamomi*, 7 days after inoculation (centre); two primary infection sites (arrows) and extramatrical mycelium with vesicles of *P. cinnamomi*, 10 days after inoculation (right). Acid fuchsine-lactophenol stain, bright field DIC microscopy.

Table 1. Effects of ERMF colonisation in hair root segments of Rhododendron shown as mycorrhizal structures (Myc-Index ± SD) and of ERMF root surface colonisation shown as extramatrical hyphae (Extr- Index ± SD) on the number of pathogen infection sites of *Phytophthora cinnamomi* (Pc) and *Pythium* spec (Py), respectively, in four independent assays; Pc I: n=240; Pc II: n=240; Py I: n=240; Py II: n=300. In comparisons against controls with a one-sided Wilcoxon permutation test the level of significance was $\alpha = 0.05$, $^{n.s.}$ = non significant, *significant differences to the respective pathogen control with $p_{adj.} < 0.0152$.

Treatment in assay	Myc-Index ± SD	Extr-Index ± SD	No of Pathogen Infection Sites
Pc I			
E3-5+Pc	1.60 ±0.25	2.08 ±0.33	40*
E3-8+Pc	2.59 ±0.49	2.68 ±0.16	0*
E3-24a+Pc	0.62 ±1.24	0.85 ±1.28	1865*
Rh11-4+Pc	1.35 ±0.78	2.85 ±0.14	0*
O.m.+Pc	3.45 ±0.25	3.50 ±0.17	12*
Contr.+Pc	0.00 ±0.00	0.00 ±0.00	5627
Pc II			
E3-8+ Pc	3.15 ±0.16	2.54 ±0.26	0*
Rh11-4+Pc	2.51 ±0.41	2.60 ±0.24	0*
O.m.+Pc	3.58 ±0.23	3.43 ±0.22	10*
Contr.+Pc	0.00 ±0.00	0.00 ±0.00	3255
Py I			
E3-5+Py	0.00 ±0.01	0.00 ±0.02	1145 $^{n.s.}$
E3-8+Py	2.46 ±2.55	0.13 ±0.15	1*
E3-24a+Py	0.08 ±0.55	0.13 ±0.36	763*
Rh11-4+Py	0.47 ±0.83	0.52 ±0.38	305*
O.m.+Py	3.36 ±3.81	0.13 ±0.09	2*
Contr.+Py	0.00 ±0.00	0.00 ±0.00	1981
Py II			
E3-8 +Py	2.64 ±0.50	2.46 ±0.20	3*
Rh11-4 +Py	1.82 ±0.30	2.45 ±0.25	9*
O.m. +Py	3.40 ±0.32	3.15 ±0.23	19*
Contr +Py	0.00 ±0.00	0.00 ±0.00	2805

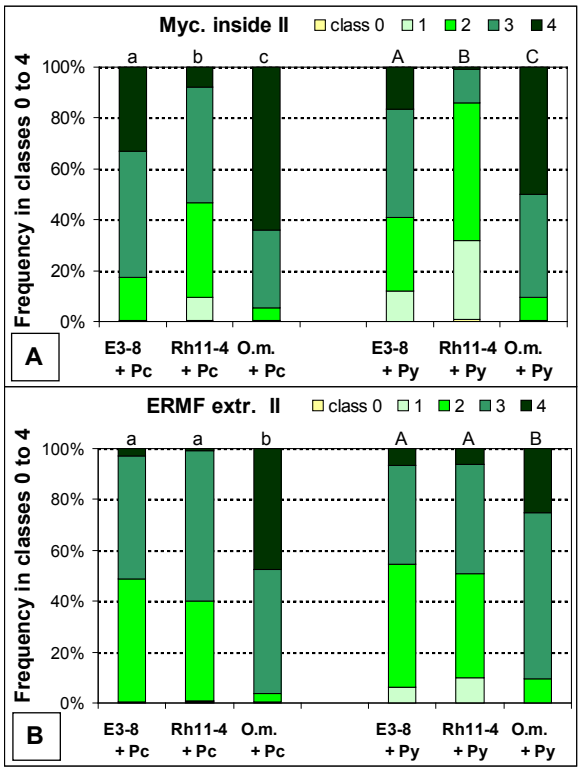

Fig. 10. Colonisation of Rhododendron hair roots in liquid culture by ERMF isolates E3-8 and Rh11-4 and *O. maius* (O.m.) in trials Pc II and Py II. **A:** Frequencies in scoring classes 0 to 4 in trials with *Pythium* spec. (Py) and with *P. cinnamomi* (Pc) showing the internal mycorrhizal status (Myc inside), **B:** Frequencies in scoring classes 0 to 4 in trials with *Pythium* spec. (Py) and with *P. cinnamomi* (Pc) showing the root surface colonisation (ERMF extr.,), n=300 (Py) root segments, n=240 (Pc) root segments, harvested 5 weeks (Py) and 6 weeks (Pc) after ERMF application; significant differences calculated in pairwise permutation tests with $p < 0.05$ are marked with different letters.

for biocontrol success.

Pathogen infections occurred in such root segments that were only sparsely colonised by *O. maius* or the Karwendel isolates E3-8 and E3-24a. If pathogens were able to infect epidermal cells, they were also capable of immediate spread into the cortex tissue, even in mycorrhizal areas. Pathogens and mycorrhizal fungi could indeed infect the same cell; however, such double infections (Fig. 12 and Fig. 13) were extremely rare. In the root tissue no cellular structures that would contribute to protection mechanisms (lignotubers, papilla, hypersensitive collapses) were detected.

Further, microscopic observations revealed a distinct reduction in the quantity of hyphae and uncovered several disorders as hyphal collapse, lysis, malformation, distortion and stunting in pathogen mycelium around the root surface of mycorrhizal plants. In contrast, in the liquid of non-mycorrhizal controls the structures of pathogens grew undisturbed and with vigorous appearance.

On account of technical reasons (harvested roots) the relationship of reduced infection sites and disease severity could not be assessed in identical plants. Plantlets in trials Pc II and Py II that were additionally kept back for the evaluation of disease symptoms clearly mirrored the preceding results on root infections: Visual assessment of non harvested control plantlets inoculated with the pathogens alone were all heavily diseased with wilted or brown shoots after four weeks of liquid culture, whereas all mycorrhizal plantlets appeared healthy with green turgescent leaves over the test period despite the inoculation with a pathogen.

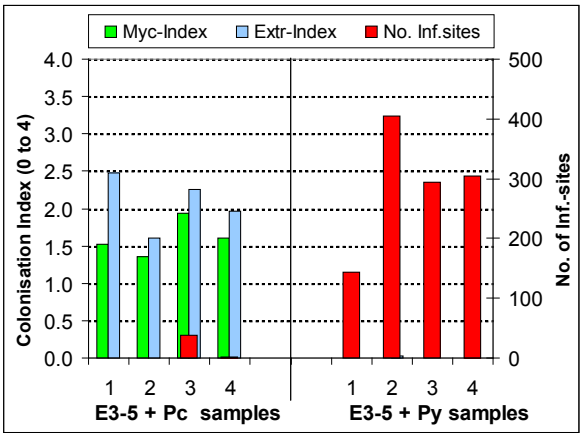

Fig. 11. Mycorrhiza formation and pathogen infection in hair roots of Rhododendron plants in sterile liquid culture with ERMF isolate E3-5 plus *P. cinnamomi* and *Pythium* spec., respectively. Single scoring data of ERMF colonisation from trial I shown as Myc-Index, Extr-Index, and absolute numbers of pathogen infections sites in four samples, each of two plants with n=60 root segments.

Discussion

Ericoid mycorrhizal fungi are not standardly used in the commercial propagation and production of ericaceous plants, because convincing advantages of ERMF applications have not been shown yet. Jansa and Vosatka (2000) followed first reports of Lemoin et al. (1992) to improve Rhododendron micro-cutting establishment in the nursery. In a screening of autochthonous ERMF isolates, they identified several beneficial ones grouped within the genus Oidiodendron or as sterile dark septate fungi (DSE) which induced positive growth effects in micro-cuttings when grown in peat-based media and inoculated *post vitro*. Other isolates, however, showed negative effects on plant growth. Similarly, Scagel et al. (2005) referred to the dependence of ERMF isolates on host plant variety and growth conditions to effectively colonise and to increase the root biomass during the raising phase of *Vaccinium corymbosum* under nursery conditions. In studies to expand the blueberry cultivation in Chile to edaphic and climatic zones outside of regions with natural favourable growth conditions, Vega et al. (2009) introduced *Rhizoscyphus ericae* as well as native ERMF inoculum, and gained rather variable, inconsistent results. Thus, the commercial production of ERMF inoculum and its practical use are not widely developed and need more stable information on the selection of appropriate strains for distinct targets.

Mycorrhiza and root disease relationships regarding arbuscular mycorrhiza (AM) were the subject of many earlier studies (e.g. Dehne 1982, Linderman 1994, Azcón-Aguilar and Barea 1996) up to recent reports (Borowicz 2001, Whipps 2004, Fritz et al. 2006, Sjöberg et al. 2007, Steinkellner et al. 2012). Also, in experiments with ectomycorrhizal fungi direct or indirect interactions with soil-borne root pathogens were demonstrated to be based on antibiosis, competition, or mechanisms of induced resistance leading to inhibition (reviewed in Marx 1972, Schönbeck et al. 1994, Whipps 2004).

Our approach was chosen due to the need of plant protection alternatives for the production of ericoid plants exposed to economic relevant root diseases caused by Oomycetes. The probability of Phytophthora root infections was increased through the cultivation of Rhododendron plants at higher pH-levels, after breeding and selection of lime-tolerant root stock varieties. The five different ERMF isolates used here showed suppressive effects against Pythium and Phytophthora in axenic culture

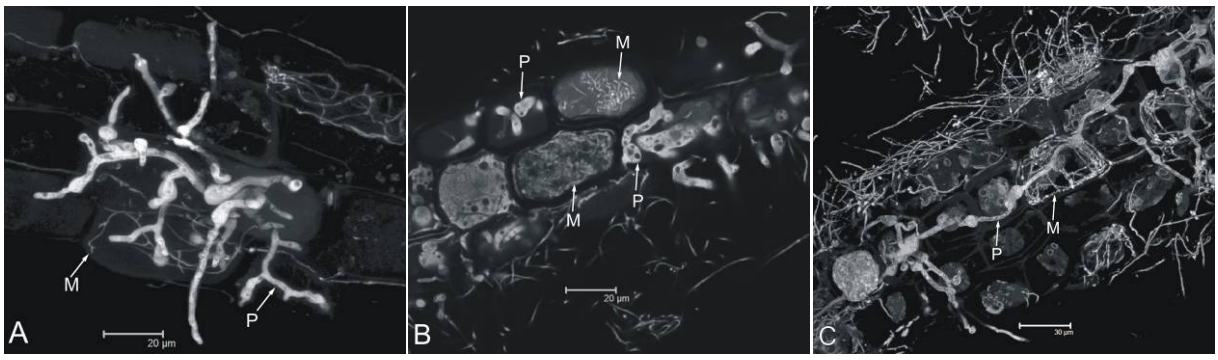

Fig. 12. Infections of Rhododendron hair root cells by *Pythium* spec. (P) and *O. maius* (M); **A**: infections in adjacent, but separate cells as revealed by 3D-animation, three days after pathogen inoculation, **B**: dual infections, five days after pathogen inoculation, **C**: mixed infections, 9 days after pathogen inoculation. CLSM-technique, fluorescence images transformed to black-white modus, A and C maximum projection of serial sections.

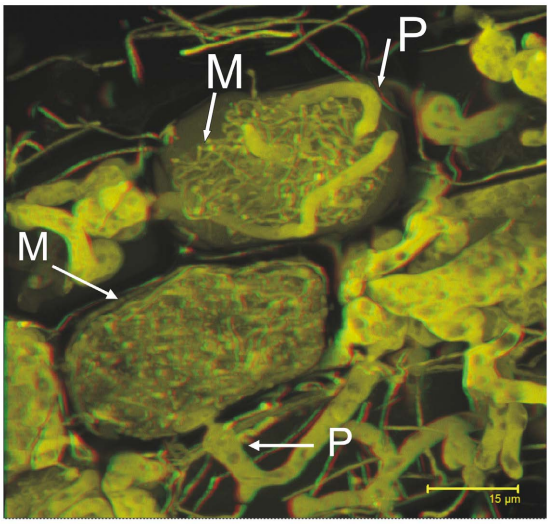

Fig. 13. *Pythium* spec. (P) penetrates and infects mycorrhizal hair root cells (M) filled with coiled hyphae of *O. maius*. CLSM-technique, maximum stereo projection of 47 serial sections with a total depth of 18.8 µm, (use anaglyph 3D glasses red/green).

and *in planta*, supporting our hypothesis that ERMF can promote health in the host plants. As the Karwendel isolates are adapted to soil reactions higher than pH 5.5, they are attractive candidates for further pot and field assays. The clear-cut results of symptomless and healthy appearing mycorrhizal plantlets in liquid tests after pathogen inoculation give a first hint for the relationship between pathogen suppression by ERMF and protection from disease. Further experiments *in planta* under greenhouse conditions have to confirm the actual biocontrol effects.

The four Karwendel isolates used in this study are verified mycorrhizal in Rhododendron (and *Calluna vulgaris*; data not shown) based on morphological evidence in roots. They differ from *Oidiodendron* and *Rhizoscyphus* species in mycelium morphology and cultural characteristics. As DNA sequences suggest, these sterile isolates are most likely members of the genus Leohumicola. Several species of Leohumicola have previously been described (Hambleton et al. 2005) and may show features of ERMF (intracellular hyphal coil formation) as well as of DSE (sterility, microsclerotia, melanisation of cell walls, distribution in soils of alpine and arctic meadows, ericaceous hosts; Jumpponen and Trappe 1998, Girlanda et al. 2006).

Ericoid mycorrhizae and DSE commonly occur in many ericoid host plants. Recently, Vohník and Albrechtová (2011) observed simultaneous ERM and DSE associations in the hair roots of six European native Rhododendron species. Brundrett (2006) set up morphological and functional criteria for fungal root associations (like specialised hyphae in plant root, synchronised development, dependence on host plant, mutual physiological benefits) to separate mycorrhizal symbioses from endophytic or parasitic associations. However, details in ERMF show that members of this group can share features fitting both endophytic and mycorrhizal fungi and are therefore difficult to categorise (Brundrett 2006, Rice and Currah 2006). Also Newsham (2011) showed in a meta-analysis of plant responses to DSE that, at least under some conditions, the DSE symbiosis was functionally similar to a mycorrhizal association.

Previous studies addressed the possibility of ERMF (e.g. *O. maius*, Rice and Currah 2006) and DSE (Mandyam and Jumpponen 2005) to protect from biotic stress, but this assumed function was rarely investigated. Few examples for biocontrol effects have been reported for DSE. Isolates of the endophytic DSE *Heteroconium chaetospira* forming ericoid mycorrhizal structures in *Rhododendron obtusum* roots (Usuki et al. 2003, Usuki and Narisawa 2005) revealed an antagonistic feature towards soil-borne clubroot disease and Verticillium yellows in Chinese cabbage (Ohki et al. 2002, Narisawa et al. 2005). Andrade-Linares et al. (2011) showed a weak biocontrol effect of an unknown ascomycete DSE towards *Verticillium dahliae* in tomato. It was suggested that, similar to mycorrhizal symbioses, DSE associations are multifunctional and exceed nutritional acquisition and plant growth support. They were also assumed to be deterrents against plant pathogens based on competition for nutrients, colonisation sites or production of inhibitory compounds (Mandyam and Jumpponen 2005). In our studies with five different ERMF in the sterile test system, we found inhibitory effects against Oomycetes, supporting the latter attribute.

The sterile liquid culture system we used with Rhododendron allowed continuous sampling for microscopy and easily exceeded Dalpé´s (1986) *in vitro* system with *Vaccinium angustifolium* in solid media regarding mycorrhizal development and also the liquid culture system of Douglas et al. (1989) regarding the duration of experiments. Factors not being controlled in greenhouse pot cultures (such as substrate or rhizosphere organisms) were excluded in our experimental design. However, the inhibitory effects observed in the liquid test system must not appear in naturally growing plants, as metabolites may accumulate in the liquid and are not bound to substrate components or drained by water.

In our studies on ERMF-pathogen interactions in liquid culture, the mycorrhizal fungi colonised even the apical region of roots which in nature remains non-colonised (Peterson et al. 1980, Berta and

Bonfante-Fasolo 1983, Harley and Smith 1983, Jackson and Mason 1984, Smith and Read 2008). Obviously, the liquid culture favours mycorrhizal development. In most AM associations, fungal colonisation influences the root morphology of the host, mainly increasing the number of root tips and lateral branching (Fusconi et al. 2000). This might influence the impact of biocontrol, as has been shown in mycorrhizal tomato plants (Berta et al. 2005). Also in *Calluna vulgaris* under simulated soil conditions, a modified root morphogenesis was attributed to the ERMF *R. ericae* (former *Pezizella ericae*, Berta et al. 1988). In our Rhododendron assays, especially the root tips and basic cells of lateral hair roots were preferred infection sites for *Pythium* spec. and *P. cinnamomi* in non-mycorrhizal control plants. However, there was no significant evidence of an alteration in lateral hair root formation in mycorrhizal plants (data not shown) that could have modified the biocontrol effect.

For the microscopic evaluation of ERM development several modifications of the original AM quantification procedures (Giovannetti and Mosse 1980, McGonigle et al. 1990, Rajapakse and Miller 1992) have been used in the last decades. Similarly, the magnified inter-grid section method was varied to gain more precise data for estimating the root length colonisation of ERMF (Urcelay et al. 2003, Villarreal-Ruiz et al. 2004) and, recently, of DSE (Mandyam et al. 2012). However, with a magnified inter-grid section method we did not adequately reflect the quantity of ERMF (Grunewaldt-Stoecker, unpublished data). Our methodology of ERMF colonisation assessment used a detailed scoring system of different classes and the Myc-Index, whereby the entire length of each root piece was rated. This method considers the patchy distribution of mycorrhizal infections better than other methods using too small or only few root pieces (Dalpé 1986, McLean et al. 1998). Thus, larger deviations in intensity and frequency within one root sample and between isolates could be detected. Additional information on the outer root surface colonisation (Extr-Index) provided data to estimate the part of extramatrical hyphae in the biocontrol potential. This separated examination of ERMF together with the pathogen assessment in identical root segments is essential for an advanced evaluation of the ERMF performance. All these advantages were facilitated by the liquid tests system used.

In our microscopic assessments we observed that pathogenic infections mainly started in non-mycorrhizal areas of the mycorrhizal plantlets. Ross and Marx (1972) previously described identical infection patterns on ectomycorrhizal Pinus seedlings infected with *P. cinnamomi*. This supports our assumption that defence reactions are more present in mycorrhizal areas than in non-mycorrhizal areas. But, as visible structures of cellular defence were missing in the epidermal root cells, the suppression of pathogen infections was not caused by morphological barriers. Such morphological defence reactions were described for AM-induced resistance to fungal root pathogens (AMF *Glomus etunicatum* towards *Olpidium brassicae*, Schönbeck and Dehne 1979; *Cochliobolus sativus*, Dehn and Dehne 1986; and *Thielaviopsis basicola*, Dehne 1987). We cannot exclude a plant mediated resistance induced by ERMF in general, because our experiments were not designed to detect further criteria of an induced resistance against disease (Steiner and Schönbeck 1995). But, the impairment of even the few pathogen hyphae associated with mycorrhizal Rhododendron roots as well as the localised protection of colonised root tips, colonised mature epidermal tissues and nearby surrounding cells do not approve the criteria of a systemic induced resistance. More likely, our results suggest a direct antibiotic activity of the ERMF against the pathogens: i.e., the production and release of anti-Oomycetes substances into the intra- and intercellular space and to the root surface which could disturb the hyphal integrity and infectivity of the pathogens and thereby prevent pathogen attack. At least one component of ERMF biocontrol was active outside of the mycorrhizal roots.

Notably, because the culture liquid was renewed prior to pathogen inoculation, we only considered the effects of substances available at the same time as pathogen infections could occur. We used Oomycetes (Kingdom Chromista) as pathogens, which have cell walls mainly composed of cellulose. Possibly, the antagonistic activity of ERMF is based upon the production of cell wall-degrading hydrolases. It remains unknown whether increased melanin levels, such as those found in other ERMF and DSE (Mandyam and Jumpponen 2005), could potentially be involved in the production of secondary metabolites that are toxic for Oomycetes.

The results obtained from experiments in liquid culture are consistent with the pathogen damage and growth inhibition in dual agar plate tests. As all of the tested Karwendel isolates turned the PDA plates to brown colour we assume a production of extracellular polyphenol oxidases (Rice and Currah 2001, Lin et al. 2011). Additionally proteolytic enzymes of ERMF (Baywa et al. 1985, Schulz et al. 2002) may play an antagonistic role. The inhibitory effects of single metabolic substances on Pythium or Phytophthora have not been tested yet. Thus, further investigations on the biochemical aspect of the ERMF mode of action are required.

As in any effective biocontrol system the quantity

and efficiency of the symbionts must be suitable for the inoculum quantity and aggressiveness of the pathogen. From our results, we cannot specify any mycorrhizal threshold that has to be accomplished for a reduction of pathogen infection sites. More likely, a homogenous distribution of the ERMF in the root system is important to offer enough of its antibiotic potential. *O. maius* with its high colonisation potential gained biocontrol effects similar to those of the best Karwendel isolates E3-8 and Rh11-4 which had moderate colonisation levels. Further investigations *in planta* must clarify the dose-response issue. At the moment, many features of the Karwendel isolates are still unknown. But with the aim to exploit beneficial fungi for practical use, the evidence of their biocontrol ability encourages for screenings in an expanded group of root-inhabiting fungi including DSE and ERMF.

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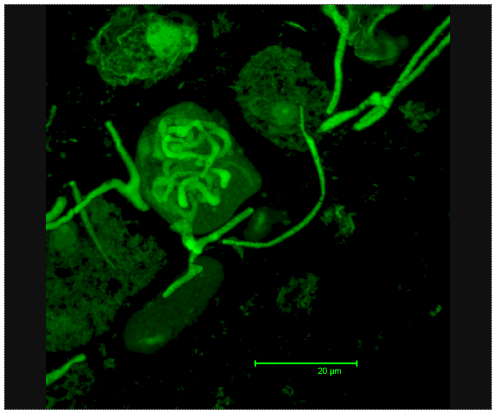
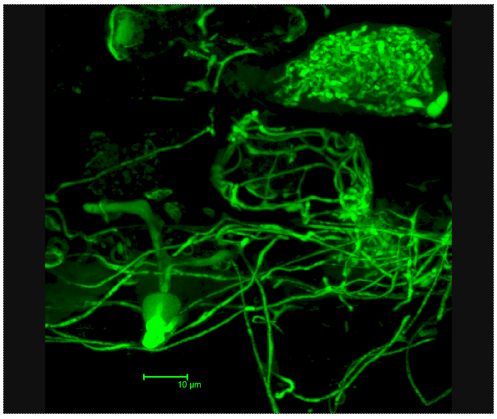
Supplemental Data



Animation 1: Mycorrhizal coil structures of Karwendel-isolate E3-24a established in Rhododendron hair root epidermis. CLSM-technique after acid fuchsine-lactophenol staining, 30 serial sections of 0.407 µm, total depth 11.8 µm; 3D-animation with rotation.

[Download] Animation 1 (19.2 MB)



Animation 2: *Pythium spec.* infection (bottom left, thick hypha) in Rhododendron hair root in liquid culture, 3 days after pathogen application; penetration of the epidermal cell layer in a mycorrhizal root segment colonised by *Oidiodendron maius* (thin hyphae). CLSM-technique after acid fuchsine-lactophenol staining, maximum projection of 40 serial fluorescence images with a total depth of 19.05 µm; 3D-animation with rotation.

[Download] Animation 2 (39.0 MB)

Dr. Gisela Grunewaldt-Stöcker is a plant pathologist at Leibniz University Hanover with main interests in impacts of fungal root endophytes on plant health, and she specialized in microscopy.

Biologist Dr. Christiane von den Berg joined the Mycorrhiza working group at Leibniz University Hanover during her Diploma Thesis about antibiosis of ericoid mycorrhizal fungi against root pathogens.

M.Sc. Johanna Knopp contributed to ericoid mycorrhiza research during her master course. Now as a Ph.D. student she is investigating the effects of arbuscular mycorrhiza on chilling stressed ornamentals.

Dr. Henning von Alten is leader of the group working about aspects of the arbuscular and the ericoid mycorrhizal symbiosis at the Dept. of Phytomedicine, Leibniz University Hanover.