

# Endophytic fungi in evergreen rhododendrons cultivated *in vitro* and *in vivo*

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## Abstract

The aim of the study was to survey fungal endophytes inhabiting healthy leaves and stems of evergreen rhododendrons and during *in vitro* tissue culture for their propagation. Fungi were identified using morphological traits. The main taxa observed in rhododendron leaves *in vivo* were *Phomopsis*, *Phoma*, *Alternaria* spp., *Colletotrichum*, *Cladosporium*, *Seimatosporium*, *Diplodina*, *Coleophoma*, *Cryptocline*, *Truncatella* and *Guignardia* spp. Contamination in tissue culture were caused by *Penicillium*, *Cladosporium*, and *Cephalosporium* spp. The study supported the view that endophytes can infect medium of *in vitro* cultivated rhododendrons. Fungal diversity increased after acclimatization of rhododendron plantlets *ex vitro*. The presence of microorganisms in rhododendron cultivated *in vitro* depended both on age of tissue and host genotype.

**Key words:** endophytes, fungi, *in vitro* culture, rhododendrons.

## Introduction

Rhododendrons (*Ericaceae*) are widely used as ornamental woody plants in horticulture. For this purpose rhododendrons are propagated by conventional and *in vitro* methods (Anderson 1984; Norton, Norton 1986; Cantos et al. 2007). Microorganisms, especially those involved in interactions with plants, are one of the main problems in tissue culture (Habiba et al. 2002). So far, no information is available on the taxonomic diversity of fungi causing contamination of rhododendron tissue cultures.

Plants are hosts for microorganisms and the associations can be classified either as epiphytic or endophytic (Samtamaría, Bayman 2005). Endophytic microorganisms are those that can be isolated from previously surface-sterilized plant material (Okane et al. 1998; Samtamaría, Bayman 2005; Hata, Sone 2008). It is known that endophytic microorganisms can be beneficial for plant by increasing biotic and abiotic stress tolerance and by synthesizing beneficial bioactive compounds (Shimizu et al. 2000; Surette et al. 2003; Rodriguez, Redman 2008; Quilliam, Jones 2010) etc.

Fungi from genera *Guignardia*, *Phomopsis*, *Discostroma*, *Colletotrichum*, *Phoma*, *Alternaria*, *Pestalotiopsis*, *Cladosporium*, *Coleophoma* are considered to be rhododendron endophytes (Farr et al. 1996; Okane et al. 1998). Several genera of fungi, such as *Phomopsis* (Petrini 1984; Petrini

1985; Udayanga et al. 2011), *Colletotrichum* (Vinnere et al. 2002; Thongsandee et al. 2011) and *Coleophoma* (Wu et al. 1996) have been identified as saprophytes, plant endophytes and pathogens.

The majority of endophytic fungi associations are likely localized infections by either latent pathogens or dormant saprophytes (Saikkonen et al. 2004). This may explain why plant pathogens (Vinnere 2002; Kowalik 2008) and endophytes (Okane et al. 1998) can be isolated with similar methods. Some species of *Colletotrichum* are pathogens that cause symptoms only on a particular host plant taxon. On other plants, these fungi may occur as asymptomatic endophytes (Feerman et al. 2001; Rodriguez, Redman 2008). In *Colletotrichum magna*, only a single gene confers the switch from a parasitic to mutualistic relationship, which results in the expansion of host plant range (Kogel et al. 2006). Some *Colletotrichum* species can express either a parasitic or mutualistic lifestyle depending on the host genotype (Rodriguez, Redman 2008), and thus the distinction is very labile.

The aim of the present study was to describe fungi present in tissue of rhododendron cultivated *in vitro* and *in vivo* and to determine whether fungi present in plant tissue can cause infection during cultivation *in vitro*. Also the effect of tissue culture age and genotype on richness of endophytic fungi was tested.

## Materials and methods

### Experimental design

The experiment was designed in three main parts: (i) *in vitro* shoot culture establishment and micropropagation of rhododendron with observation of possible infection; (ii) survey of endophytic fungi in asymptomatic two-year-old leaves ( $n = 105$ ) of various evergreen rhododendron taxa (*R. brachycarpum*, *R. catawbiense*, *R. smirnowii*, *R. maximum* and their hybrids) *in vivo*; (iii) survey of fungi in leaves and stems *in vivo* of two rhododendron cultivars propagated by tissue culture methods. In the latter part, 'Catawbiense Grandiflorum' and 'Emils' (*R. catawbiense* Michx.  $\times$  'Purple Splendour') were chosen as cultivars and three ages of tissue samples were tested.

### Establishment of rhododendron *in vitro* shoot culture

In February and March generative buds were collected from open-air-growing rhododendron cultivars 'Lee's Dark Purple', 'Cunningham's White' and 'Catawbiense Grandiflorum'. The buds were washed with antibacterial soap „Safeguard” (*Nelissa Lander Company*), rinsed under running tap water and sterile distilled water and surface sterilized in a laminar air flow box (*Kojair, BW-130 Standard*) with 96% ethanol and flame. Ovary, receptacle and peduncle were isolated as explants (Tomsone, Gertner 2003). These explants were placed in test tubes containing Anderson's medium (Anderson 1984) with additional sucrose 20 g L<sup>-1</sup>, glucose 10 g L<sup>-1</sup>, inositol 0.1 g L<sup>-1</sup>, adenine 0.8 g L<sup>-1</sup>, glycine 2 mg L<sup>-1</sup>, casein hydrolysate 5 g L<sup>-1</sup>, thiamine 0.4 mg L<sup>-1</sup>, pyridoxine 0.1 mg L<sup>-1</sup>, nicotinic acid 0.1 mg L<sup>-1</sup> and agar 8 g L<sup>-1</sup>. For stimulation of shoot formation, indole-3-acetic acid 3 mg L<sup>-1</sup>, indole-3-butyric acid 1 mg L<sup>-1</sup>, N<sup>6</sup>- $\Delta^2$ -isopentenyladenine 15 mg L<sup>-1</sup>, and thidiazuron 0.4 mg L<sup>-1</sup> were used. Medium pH was adjusted to 5.5.

Explants were incubated in 23  $\pm$  2 °C, with a photoperiod of 16 h and photon flux density 35 to 59  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . After each of five periods of nine to ten weeks explants were transferred to fresh nutrient medium with the same base composition adding N<sup>6</sup>- $\Delta^2$ -isopentenyladenine 3 mg L<sup>-1</sup>.

### Isolation of microorganisms

Microorganisms from *in vitro* cultivation tubes were isolated when infection was observed. In addition, microorganisms were isolated directly from 'Lee's Dark Purple', 'Cunningham's White' explants before the beginning of the experiment (three samples from each cultivar) and from apparently sterile tissue (three samples from each cultivar) after 20 weeks of cultivation *in vitro*. Plant material was crushed in an eppendorf tube by means of a glass rod and 0.5 mL sterile distilled water was added. Petri dishes containing malt extract agar ( $\rho = 1.028$ , agar 2%) as growth medium were inoculated with 0.1 mL of obtained suspension and incubated at room temperature for about 7 days.

In April and September 2009 two-year-old leaves of evergreen rhododendrons were collected in the Botanical Garden and Nursery of Rhododendrons "Babite", University of Latvia. In total 105 leaf samples of evergreen rhododendron (*R. brachycarpum*, *R. catawbiense*, *R. smirnowii*, *R. maximum* and their hybrids) were collected. Leaf and stem samples from 12-year-old 'Catawbiense Grandiflorum' and 'Emils' were collected in the Nursery of Rhododendrons "Babite" in October 2009. From these, one to three year old leaves and stems were collected in three replications.

Leaf and stem samples were washed with antibacterial soap „Safeguard” (*Nelissa Lander Company*) under running tap water. Leaves were sterilized in 70% ethanol for 2 min and 2% sodium hypochlorite solution for 3 min. Then samples were washed three times with sterile distilled water. Eight segments (5  $\times$  5 mm) were cut with a sterile scalpel from each leaf and plated in a Petri dish.

Stem samples were washed as above and immersed in 96% ethanol and sterilized in flame. Stem samples were sliced in thin segments and plated in Petri dish (eight segments per dish). Stems were peeled back to obtain bark segments (3  $\times$  6 mm) which were plated in Petri dishes (eight per Petri dish). Xylem left after peeling of the bark was sterilized in flame. After sterilization, xylem was sliced in thin segments and plated in Petri dishes (eight segments per dish).

Growth media consisted of malt extract agar (Biolife, Italy) and potato dextrose agar [boiled potatoes, filtered through a sieve (200 g L<sup>-1</sup>), dextrose (15 g L<sup>-1</sup>), and agar (15 g L<sup>-1</sup>)]. Plates were incubated at room temperature for approximately two months. Daily observations were made. When a microorganism colony was observed it was placed on fresh growth medium for further identification.

Colonization frequency (%) was calculated as the number of leaves from which a fungus was detected divided by total number of leaves examined.

### Identification of fungi

Filamentous fungi (Sutton 1980; Kiffer, Morelet 2000; Domsch et al. 2007) and yeasts (Boekhout et al. 2002) were identified by micromorphological and macromorphological traits in the University of Latvia and in the Centraalbureau voor Schimmelcultures.

## Results

### Microorganisms in tissue culture

No fungi were isolated from fresh rhododendron explants and from plant tissue after 20 weeks of cultivation. However, the rhododendron tissue was not sterile, and some bacteria from genus *Bacillus* was isolated.

However, fungi from the genera *Cephalosporium*, *Cladosporium*, *Paecilomyces*, *Penicillium*, *Tritirachium* as infection causing agents were isolated from rhododendron

**Table 1.** Fungi isolated from two-year-old leaves (n = 105) of evergreen rhododendron (*R. brachycarpum*, *R. catawbiense*, *R. smirnowii*, *R. maximum* and their hybrids), their frequency and culture collection accession number (CBS, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; MSCL, Microbial Strain Collection of Latvia, University of Latvia). Colonization frequency (%) = (number of leaves from which the fungus was detected / total number of leaves examined) × 100

Fungi	Colonization frequency (%)	Culture collection accession number
<i>Phomopsis</i>	46.67	CBSa 129167, CBS 129168, MSCLb 849, MSCL 1032
<i>Phoma</i>	36.19	MSCL 1031
<i>Alternaria</i>	23.81	–
<i>Colletotrichum</i>	17.14	MSCL 1033
<i>Cladosporium</i>	16.19	–
<i>Seimatosporium rhododendri</i>	14.29	CBS 129166, MSCL 860
<i>Diplodina</i>	12.38	MSCL 857
<i>Coleophoma empetri</i>	11.43	CBS 129169, MSCL 1028
<i>Cryptocline</i>	11.43	CBS 129163, MSCL 1029
<i>Truncatella</i>	10.48	CBS 129165, MSCL 902
<i>Guignardia rhodoraе</i> = <i>Botryosphaeria rhodoraе</i>	10.48	CBS 129172, MSCL 1027
<i>Sphaceloma</i>	6.67	–
<i>Fusarium</i>	6.67	–
<i>Pestalotiopsis baarnensis</i>	6.67	CBS 129164, MSCL 900
<i>Aureobasidium</i>	5.71	–
<i>Glomerella</i>	5.71	MSCL 1030
<i>Scytalidium</i>	4.76	–
<i>Sphaeropsis</i>	3.81	–
<i>Helicosporium</i>	3.81	–
<i>Acremonium</i>	2.86	–
<i>Monochaetia</i>	2.86	CBS 129171, MSCL 1034
<i>Aspergillus</i>	2.86	–
<i>Penicillium</i>	2.86	–
<i>Chaetomium</i>	1.90	–
<i>Hormiactis</i>	1.90	–
<i>Phyllosticta</i>	1.90	–
<i>Sordaria</i>	1.90	–
<i>Chaetosphaeronema</i>	0.95	–
<i>Gilmaniella</i>	0.95	–
<i>Kabatina</i>	0.95	–
<i>Trichoderma</i>	0.95	–

**Table 1. continued**

Fungi	Colonization frequency (%)	Culture collection accession number
<i>Pyrenochaeta</i>	0.95	–
<i>Pyricularia</i>	0.95	–
<i>Rhizosphaera</i>	0.95	–
<i>Cylindrosporium</i>	0.95	–
<i>Gliocladium</i>	0.95	–
<i>Leptostromella</i>	0.95	–
<i>Septoria</i>	0.95	–
<i>Stachylidium</i>	0.95	–
grey sterile I	40.95	–
grey sterile II	25.71	–
white unidentified	24.76	–

cv. 'Lee's Dark Purple', 'Catawbiense grandiflorum' and 'Cunningham's White' tissue cultures (Table 1). *Gliocladium* and *Verticillium* were isolated from the cultures of both 'Lee's Dark Purple' and 'Catawbiense grandiflorum'. Fungi from the genera *Aspergillus*, *Aureobasidium*, *Mucor* and *Rhodosporidium*, which are infection causing agents were isolated only from 'Catawbiense grandiflorum' *in vitro* culture. Also, *Pichia* from cultures of 'Lee's Dark Purple' and *Saccharomyces* and *Saccharomycopsis* from 'Cunningham's White' were isolated.

#### Endophytic fungi of evergreen rhododendrons in open-air conditions

In total 144 fungal isolates were obtained from two-year-old leaves of evergreen rhododendrons. Four isolated fungi were identified to species level, while 37 fungi were determined only to genera (Table 2). However, 103 fungal isolates from leaves could not be identified. All identified fungi belonged to the *Ascomycota*. Among them, *Phomopsis*, *Phoma*, *Alternaria* and *Monochaetia* (Fig. 1 A) were found at frequencies higher than 20%. Other important genera were *Colletotrichum* (Fig. 1 B), *Cladosporium*, *Seimatosporium* (Fig. 1 C), *Diplodina*, *Coleophoma*, *Cryptocline*, *Truncatella* and *Guignardia*, detected at frequencies higher than 10%. Genus *Pestalotiopsis* had a frequency of 6.67% (Fig. 1 D). Several genera were detected only once or twice (with frequencies 1.90% and 0.95%). Some of the unidentified fungi (Table 1) were found at high frequencies (24 to 41%).

Microorganism isolates from tissues of different age from rhododendron cv. 'Emils' and 'Catawbiense Grandiflorum' were studied. The samples were collected from visually healthy 12-year-old bushes. Leaves, stem together with bark, and separately bark and xylem were analyzed. Xylem appeared to be sterile as no fungi could be isolated from the tested samples. Similar levels of contamination were found in the other wood samples and therefore they were combined (Table 2). For cv. 'Emils', third year leaves had

**Table 2.** Fungi detected in leaves, entire stem with bark, and bark (1 to 3 years) of rhododendrons (each sample n = 3). Data obtained from entire stem and bark samples were combined as similar results were obtained. Xylem was sterile. For cv. 'Emils' three-year-old leaves have been abscised. y.o., year-old

Fungi	'Catawbiense Grandiflorum'						'Emils'					
	Leaves			Stem & bark			Leaves		Stem & bark			
	1 y.o.	2 y.o.	3 y.o.	1 y.o.	2 y.o.	3 y.o.	1 y.o.	2 y.o.	1 y.o.	2 y.o.	3 y.o.	
<i>Alternaria</i>			×				×	×			×	
<i>Acremonium</i>								×				
<i>Chaetomium</i>								×				
<i>Chaetosphaeronema</i>			×									
<i>Cladosporium</i>								×		×		
<i>Coleophoma empetri</i>								×				
<i>Colletotrichum</i>							×					
<i>Cryptocline</i>								×				
<i>Fusarium</i>					×	×					×	
<i>Glomerella</i>		×									×	
<i>Guignardia rhodorae</i> = <i>Botryosphaeria rhodorae</i>						×					×	
<i>Hormiactis</i>		×					×					
<i>Monochaetia</i>											×	
<i>Penicillium</i>				×						×		
<i>Pestalotiopsis baarnensis</i>						×						
<i>Phoma</i>	×	×	×	×	×	×	×	×	×	×	×	
<i>Phomopsis</i>	×	×	×	×	×	×				×	×	
<i>Pyrenochaeta</i>					×							
<i>Seimatosporium rhododendri</i>										×	×	
grey sterile I			×			×			×		×	
grey sterile II							×	×		×		
white unidentified		×	×		×	×				×		

already abscised and could not be collected.

The most frequent fungi observed in leaves and stems were *Phoma* and *Phomopsis* (Table 2). In addition, *Phoma* was found in all samples analyzed. Other dominant fungi were *Alternaria*, detected only in foliar samples, *Fusarium*, detected only in bark samples, and several unidentified fungi. Endophyte species composition differed with sample age and between cultivars. Considerably more fungi were observed in older leaves and stem samples than in one-year-old tissue. The one- and two-year-old leaves of cv. 'Emils' contained approximately double the number of species compared to that in cv. 'Catawbiense Grandiflorum'. No differences in diversity of endophytes between cultivars were found for stem samples.

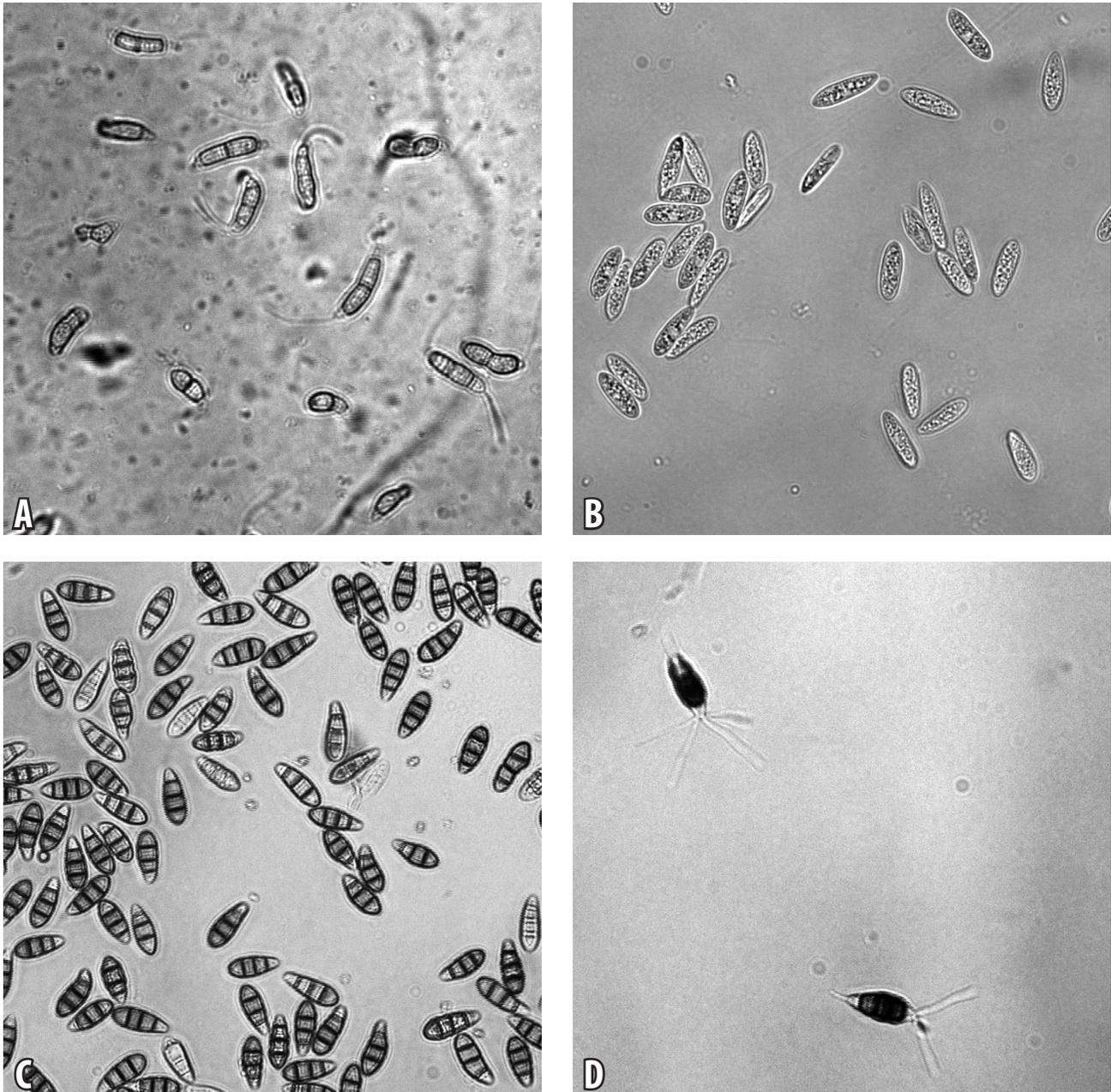
## Discussion

The tested rhododendron explant samples and tissue samples were sterile of fungi 20 weeks after cultivation *in vitro*. As the explants were taken from generative buds during the rest period, it is possible that flower

tissues forming inside the scales are well-protected from microorganisms (Schneider 1972).

However, fungi were found in plant tissue cultures as infection causing agents. It is not known if this is due to insufficient sterility or due to apparently insufficient samples tested for fungi, as microorganisms can be in a latent form within tissue (Saikkonen et al. 2004). Known endophytes in rhododendron are *Cladosporium* (Kowalik 2008), *Aspergillus* (Kowalik 2008), *Penicillium* (Kowalik 2008), *Gliocladium* (Farr et al. 1996), *Aureobasidium* (Okane et al. 1998) which were found also in surface-sterilized leaves (Table 1). Also fungi from *Mucor* (Kowalik 2008) and *Verticillium* (Farr et al. 1996) have been recorded from rhododendron. However, the origin of contamination from *Paecilomyces*, *Pichia*, *Rhodosporidium*, *Saccharomyces*, *Saccharomycopsis*, *Tritirachium* and *Cephalosporium* is unclear, as all of the species can be endophytes, saprophytes, pathogens and soil fungi (Wiese, Ravencroft 1975; Reddy et al. 1996; Vega et al. 2008; Zhao et al. 2010; Botha 2011).

In the present study, the most frequently detected genera in the leaves of evergreen rhododendrons *in vivo*



**Fig. 1.** Fungal isolates from rhododendron tissue samples. A, *Monochaetia* sp.; B, *Colletotrichum* sp.; C, *Seimatosporium* sp.; D, *Pestalotiopsis* sp.

were *Phomopsis*, *Phoma*, *Alternaria*, *Colletotrichum* and *Cladosporium* (Table 1). These genera are described as cosmopolites and endophytes in a wide range of unrelated host plants (Liu et al. 2010; Aly et al. 2011). These fungi have been found in *Rhododendron* in Japan (Okane et al. 1998) and Poland (Kowalik 2008). Previously in Latvia, *Colletotrichum acutatum* was isolated as a microorganism causing anthracnose on rhododendron (Vinnere et al. 2002). In Poland *Aspergillus* sp., *Coleophoma empetri*, *Septoria* sp., *Sordaria* sp. have been also mentioned as common microorganisms found in rhododendron (Kowalik 2008). *Guignardia* and *Pestalotiopsis*, that in our study were isolated

at quite high frequencies (respectively, 10.48 and 6.67%), have been described as common endophytes in leaves of rhododendron in Japan (Okane et al. 1998). Dominant taxa in the current research *Seimatosporium rhododendri*, *Diplodina*, *Truncatella*, *Fusarium* and *Aureobasidium* spp. have all been mentioned as fungi in the host plant rhododendron (Farr et al. 1996).

As the studied rhododendron cultivars ‘Catawbiense Grandiflorum’ and ‘Emils’ had been propagated via *in vitro* techniques, it can be supposed that the original plantlets were sterile of fungi. Thus, all fungi detected in plant tissue had probably been horizontally transmitted. It has

been observed that diversity and colonization frequency of endophytic fungi in plants increased with the host age (Okane et al. 1998; Suryanarayanan, Tennarasan 2004; Thongsandee et al. 2011), as was found in our study.

From field observations it is known that cv. 'Catawbiense Grandiflorum' is more resistant to infectious diseases than 'Emils' (Apine et al., unpublished data). Leaves of cv. 'Emils' hosted approximately twice more fungal genera than 'Catawbiense Grandiflorum' (Table 2). Furthermore, plants of cv. 'Emils' lacked three-year-old leaves. Therefore, it is obvious, that differences in plant genotypes can cause differences in host affinity to various fungi. It has been suggested that endophytic microorganisms can have effect on the physiological status of host leaves (Suryanarayanan, Tennarasan 2004) and induce senescence, or, conversely, microorganism invasion could be due to leaf senescence induced by the host itself (Promputtha et al. 2007). Probably, different mechanisms are used for endophyte recruitment, as leaves can recruit specific species of fungi as endophytes (Suryanarayanan, Tennarasan 2004).

In conclusion, the present study showed that infection in the medium during rhododendron cultivation *in vitro* may be caused by endophytes. In the case of rhododendron propagation *in vitro*, endophytes are transmitted mainly horizontally and infection occurs after acclimatization *ex vitro*. After acclimatization the number of fungal taxa present increased with tissue age. Microorganism presence depends on age of tissue and host genotype. In further research more emphasis should be placed on fungi identification to species level. The mechanism how fungi invade rhododendron plantlets is also important to elucidate in future study.

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