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Fungal endophytes in woody roots of Douglas-fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*)

By J. A. Hoff[1,2,4], N. B. Klopfenstein[1], G. I. McDonald[1], J. R. Tonn[1], M.-S. Kim[1], P. J. Zambino[1], P. F. Hessburg[3], J. D. Rogers[2], T. L. Peever[2] and L. M. Carris[2]

[1]USDA-FS, RMRS, Forestry Sciences Laboratory, 1221 S. Main St, Moscow, ID 83843, USA; [2]Department of Plant Pathology, Washington State University, PO Box 646430, Pullman, WA 99164-6430, USA; [3]USDA-FS, PNW, 1133 N. Western Ave., Wenatchee, WA 98801, USA; [4]Present address: J. A. Hoff, Plant Industries Division, West Virginia Department of Agriculture, 1900 Kanawha Blvd., E, Charleston, WV, 25305, USA. E-mail: nklopfenstein@fs.fed.us (for correspondence)

Summary

The fungal community inhabiting large woody roots of healthy conifers has not been well documented. To provide more information about such communities, a survey was conducted using increment cores from the woody roots of symptomless Douglas-fir (*Pseudotsuga menziesii*) and ponderosa pine (*Pinus ponderosa*) growing in dry forests on the eastern slope of the Cascade Mountains in Washington state, USA. Fungal isolates were cultured on standard media, and then were identified using a combination of molecular and morphological methods. Fungal genera and species identified in this study will provide baseline data for future surveys of fungal endophytes. Examination of internal transcribed spacer (ITS1 and ITS2) and 5.8S rDNA sequences and morphology of cultured fungi identified 27 fungal genera. Two groups predominated: *Byssochlamys nivea* Westling (20.4% of isolations) and *Umbelopsis* species (10.4% of isolations). This is the first report of *B. nivea* within large woody roots of conifers. Both taxa have been previously identified as potential biological control agents. Although some trends were noted, this study found no significant evidence of host species or plant association effects on total recovery of fungal endophytes or recovery of specific fungal taxa.

1 Introduction

The occurrence of fungal microorganisms within a forest ecosystem is influenced by disturbances (e.g., fires, herbivory, diseases, and insect attacks) that vary in frequency, intensity, duration, and extent. For example, ectomycorrhizal biomass may be reduced eightfold following a prescribed burn (Stendell et al. 1999). Ecosystem instability caused by such transitory phenomena is an evolutionary force in natural systems. Abiotic (e.g. fire, wind, landslides, and mudflows) and biotic disturbances (e.g. bark beetles and root diseases) have long been recognized for their critical influences on vegetation patterns within a forest ecosystem (Hessburg et al. 1994, 2000; Hagle et al. 1995; Harvey et al. 1995). In addition, pathogens, mutualists, and saprophytes are increasingly recognized for their roles in structuring natural forest ecosystems (Dickman and Cook 1989; Miller and Lodge 1997; McDonald et al. 2000). Although the roles of forest pathogens are well recognized, increased awareness of the roles of non-pathogenic species is needed.

Molecular techniques provide a powerful tool for exploring the unique trophic niches occupied by non-sporulating and unculturable fungi. For example, a recent study, using fungal-specific polymerase chain reaction (PCR) primers to amplify small subunit (SSU) ribosomal DNA (rDNA), found 49 fungal phylotypes in the roots of a single grass species, *Arrhenatherum elatius* (Vandenkoornhuyse et al. 2002). The phylotypes

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represented the four currently recognized fungal phyla, *Ascomycota*, *Basidiomycota*, *Chytridiomycota*, and *Zygomycota*, and suggested the existence of two previously unrecognized fungal phyla. In contrast, the authors noted that related culture-based studies yielded primarily ascomycetes (Vandenkoornhuyse et al. 2002). Kernaghan et al. (2003) assessed the fungi colonizing roots of containerized *Picea glauca* seedlings by direct amplification of fungal rDNA internal transcribed spacer (ITS) from samples of fine feeder roots. They found that the ectomycorrhizal basidiomycetes *Thelephora americana* and *Amphinema byssoides* were the predominant root-colonizing fungi, with ascomycetes less common. The results of these studies suggest that culture-based methods may select for specific phyla, thereby biasing perceptions regarding extant fungal communities.

The impact of endophytic fungi in forest ecosystems is relatively unknown, compared with that of fungal pathogens and mycorrhizal symbionts. Endophytic fungi occupy positions somewhere between the trophic niches of pathogen and mutualist, but their position within hosts enables them to potentially influence fungi (or other organisms) that occupy the same host. This influence can be expressed directly through inhibition or stimulation of fungal growth, or indirectly via effects on host physiology and morphology. Thus, endophytes can contribute to variation and unpredictability of pathogen interactions with host plants (Saikkonen et al. 1998).

Our objective in this study was to establish baseline information on latent fungal pathogens and endophytes associated with large woody roots of apparently healthy Douglas-fir and ponderosa pine occurring in the relatively dry forest ecosystems on the Wenatchee National Forest of the eastern Washington Cascades, USA. The study sites will be subjected to future prescribed burn treatments, and a subsequent re-survey in 5 years to determine the effects of burning on the occurrence of plant, insect, and fungal communities (Agee et al. 2001).

2 Materials and methods

2.1 Study design

Our study was conducted on all aspects, except north, in the Mission Creek watershed, on the Wenatchee National Forest in north-central Washington state, USA (Agee et al. 2001). On these aspects, ponderosa pine (*Pinus ponderosa* Douglas ex Lawson & C. Lawson) and Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] are dominant tree species within the dry, mixed-conifer forests, where annual precipitation seldom exceeds 762 mm. The mean presettlement fire-free interval in the Mission Creek drainage was about 17 years, with the last fire, a surface fire, occurring in 1929. The study consisted of 12 sampling units (Crow 1, Crow 3, Crow 6, L. Camas, Pendleton 30, Poison 6, Ruby, Sand 19, Sand 2, Slawson, Spromberg 4, and Tripp 9), 10–20 ha in size (Fig. 1). Units were placed on slopes <80%, with no more than 10% rock cover. Areas where rare plant species occurred were excluded. Units were placed in dry-forest environments that included Douglas-fir and ponderosa pine potential vegetation types. Sampling units typically contained the following plant associations (Williams and Smith 1991; Lillybridge et al. 1995): PSME/SPBEL (*P. menziesii/Spiraea betulifolia* var. *lucida* (Dougl.) C.L. Hitchc.), PSME/PUTR (*P. menziesii/Purshia tridentata* (Pursh) DC.), PSME/CARU (*P. menziesii/Calamagrostis rubescens* Buckley), PSME/SYAL (*P. menziesii/Symphoricarpos albus* (L.) S.F. Blake), PSME/AGSP [*P. menziesii/Agropyron spicatum* (Pursh.) Scribn and Smith], PIPO/PUTR (*P. ponderosa/P. tridentata*), PIPO/CARU (*P. ponderosa/C. rubescens*), and PIPO/AGSP, (*P. ponderosa/A. spicatum*).

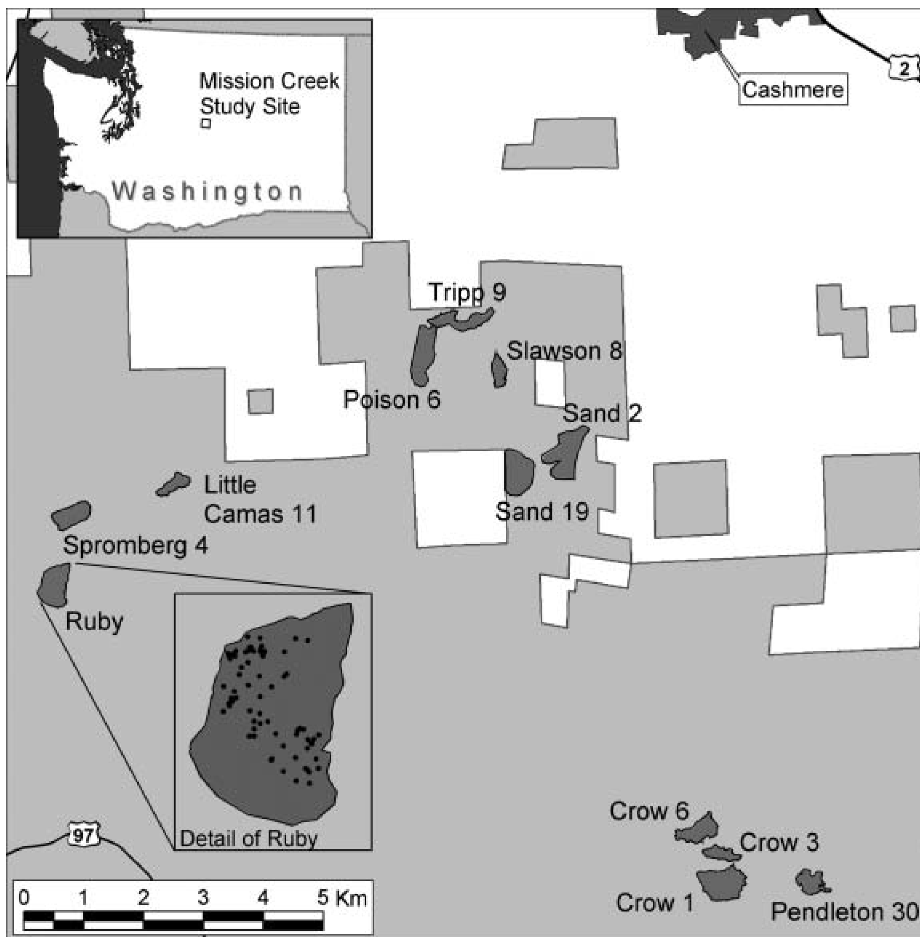

Fig. 1. Locations of the 12 sampling units at the Wenatchee National Forest. Inset: individual trees sampled within the sampling unit 'Ruby'; Shaded areas: Wenatchee National Forest ownership; Top of map is north

2.2 Sampling the units

Within each sampling unit, comprehensive surveys were conducted to determine the status (e.g. healthy, dead, crown thinning, dieback, topkill) of every tree. During the 2000 field season (May to September), root samples were collected from randomly selected, symptomless Douglas-fir and ponderosa pine. All sampled trees were mapped to a 0.25-m orthophoto, and site/tree attribute data were recorded (Agee et al. 2001). For each sampled tree, one major lateral root was excavated and examined for symptoms or signs of root disease out to a distance of 1 m prior to collecting root core samples. An increment borer, which was surface sterilized with ethanol after each use, was used to extract increment cores (ca. 5 mm diameter and 12 cm long) from the tree roots that were at least 15 cm in diameter. Root cores were placed in a plastic soda straw that was flame-sealed at both ends, stored at 4°C, and shipped to the Forestry Sciences Laboratory (USDA Forest Service, RMRS, Moscow, ID, USA) for fungal isolation, identification, and archiving.

2.3 Processing laboratory samples

The samples were maintained at ca. 4°C until processing (usually within 1–4 week) at the Forestry Sciences Laboratory in Moscow, ID, USA. Any remaining periderm and phloem tissues were excised from the root cores and discarded, so that only xylem tissue was used for fungal isolation. Depending on its length, each of the lateral root increment cores was cut into several pieces, ca. 2.5 cm in length. Each piece was submerged in 70% v/v ethanol, and flame-sterilized. To facilitate the isolation of basidiomycetous fungi, cores were initially placed into a 60×15 mm Petri dish containing a medium selective for basidiomycetes, then placed in an incubator in the dark at 21°C for 1–2 months. The selective medium was a benomyl-dichloran-streptomycin (BDS) agar that contained 15 g malt extract, 15 g agar, 40 mg benomyl, 20 mg dichloran (2,6-dichloro-4-nitroaniline), and 100 mg streptomycin sulphate per L (Worrall and Harrington 1993). Root core samples on which fungal growth did not visibly extend into the BDS medium within 8 weeks were transferred to a non-selective, 2% w/v Bacto™ (Becton, Dickinson and Co., Sparks, MD, USA) malt-extract agar (MEA) medium to facilitate growth and isolation of the primarily non-basidiomycetous fungi that are sensitive to BDS medium.

After incubation, fungal isolates were subjected to morphological examination. To facilitate the formation of specific morphological characteristics necessary for taxonomic identification, fungal isolates were subcultured on the following media: (i) BDS, (ii) a nutrient-rich medium containing 3% w/v dextrose, 1% w/v peptone, 1.5% w/v agar, and 3% w/v malt extract (Kim et al. 2000), and (iii) 2% w/v MEA medium. Representative fungal isolates are maintained as archival cultures at the Forestry Sciences Laboratory in Moscow.

2.4 Characterizing fungal morphology

To characterize the morphology of fungal isolates, slides prepared from cultures were stained with Melzer's Reagent (Melzer 1924) and examined with bright-field and phase-contrast microscopy (Olympus BX 60; Olympus America, Melville, NY, USA). Occurrence and dimensions of morphological features, such as hyphae, basidia/basidiospores, asci/ascospores, sporangiophores/sporangiospores, zygospores, and chlamydospores, were noted for different isolates. Photomicrographs were taken with a Nikon Cool Pix 990® digital camera (Nikon, Melville, NY, USA). Permanent slides were prepared using Shurmount® aqueous mounting medium (Triangle Biomedical Sciences, Durham, NC, USA).

2.5 Polymerase chain reaction of fungal nuclear ribosomal DNA

A pipette tip was lightly scraped across actively growing hyphae to obtain the DNA template for a 50-μl PCR amplification of the ITS1, 5.8S, and ITS2 regions of the ribosomal repeat. When direct mycelial scraping did not provide a suitable DNA template for the PCR, mycelial samples were treated with Lyse-N-Go™ PCR reagent (Pierce, Rockford, IL, USA) following the protocol of the manufacturer, which consisted of repeated cycles of incubation at 8, 65, and 97°C. Each 50-μl PCR amplification mixture contained 1.2 units AmpliTaq® (Applied Biosystems, Foster City, CA, USA), 5 μl 10X PCR buffer (Applied Biosystems), 200 μM dNTPs, 4.0 μM $MgCl_2$, 0.5 μM ITS-1 primer, and 0.5 μM ITS-4 primer (White et al. 1990). The cycling parameters were modified from Pimentel et al. (1998) as follows: DNA was denatured for 5 min at 94°C, followed by 30 cycles of denaturation at 94°C for 1 min 30 s, annealing at 50°C for 1 min 30 s, and extension at 72°C for 2 min 30 s, with a final 5-min extension at 72°C. Samples (4 μl) of amplified products were subjected to agarose-gel electrophoresis at 9 V/cm for 2 h, then viewed with UV light after staining with ethidium bromide.

The DNA products were prepared for sequencing using ExoSap-IT™ (USB, Cleveland, OH, USA) PCR product cleanup following the manufacturer's protocol. The reaction conditions were 37°C for 15 min followed by 80°C for 15 min. DNA products were sequenced by Amplicon Express (Pullman, WA, USA) using an ABI Prism 377 (Applied Biosystem). BioEdit (Hall 1999; http://www.mbio.ncsu.edu/BioEdit/bioedit.html) was used to delete ambiguous terminal regions of the single stranded sequence data, and to generate double-stranded contigs (ca. 500–600 bp) containing the full sequence of the 5.8S coding region and the trimmed partial sequences of the flanking ITS1 and ITS2 regions. Species identification was facilitated by comparing nucleotide similarity between ca. 500–600 bp sequences from our isolates with those of known fungi, using the National Center for Biotechnology Information's GenBank nucleotide BLAST search. To better identify and characterize the group of *Umbelopsis*-like fungi in this study, cultures and/or DNA sequences were also obtained from independent researchers for rDNA and morphological comparisons. *Umbelopsis isabellina* (Oudem.) W. Gams isolates were obtained from K. O'Donnell (USDA-ARS, Peoria, IL, USA; isolate 1757), E. Vandegrift (Oregon State University, Corvallis, OR, USA), and R.Vilgalys (Duke University, Durham, NC, USA; isolate DFMO JSNH 13); isolate 5844 of *Umbelopsis ramanniana* (A. Möller) W. Gams (as *Micromucor ramannianus*) was also obtained from K. O'Donnell. Representative ITS sequences from fungal isolates of this study were deposited into GenBank.

2.6 Identification of fungal genera

Isolates showing similar morphology were grouped together. In general, fungal isolates were considered as belonging to a single genus if (i) their morphology in culture was within ranges previously reported, and/or (ii) sequences of representative isolates used in a BLAST search of GenBank (and other sources) resulted in sequence identity that was greater than the lowest similarity (i.e. 92%) among recognizable species within other genera examined in this study (Hoff 2002).

2.7 Statistical analysis

Significant difference in recovery of fungal genera across host species and plant associations was tested using Z-tests of significance for correlated proportions using the 'Vassar Stats' package (http://faculty.vassar.edu/lowry/VassarStats.html). The statistical significance of host species and plant association on fungal recovery was tested using chi-square tests for independence, using 'Web Chi Square Calculator' (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html).

3 Results

3.1 Identified fungal taxa

A summary of fungal taxa isolated from experimental units is presented in Table 1. A total of 289 root cores were evaluated, and fungi were isolated from woody roots of 138 (47.8%) symptomless ponderosa pine and Douglas-fir (Table 2). Averaged across all experimental plots, fungal isolates were obtained from 50% of the ponderosa pine roots and 45% of the Douglas-fir roots (Table 2). Twenty-seven cores (9.3%) yielded multiple fungal genera, 111 (38.4%) yielded growth of one fungal genus, and 151 (52.2%) yielded no fungi. A wide diversity of fungi was recovered from root cores in this study, comprising three phyla, 11 orders, 13 families, and 17 genera. Fourteen genera were isolated from ponderosa pine, and nine genera were isolated from Douglas-fir. Although a medium selective for basidiomycetous fungi was used, the only basidiomycetes isolated were *Tremella* sp. (two isolations)

Table 1. **Summary of fungal species isolated from roots of symptomless trees within each sampling unit**

Sampling unit	Number of trees	Plant association[1]	No growth	*Byssochlamys nivea*	*Umbelopsis* spp.	*Mucor* sp.	*Penicillium* sp.	Other species
Crow 1	31	PIPO & PSME	11	6	12	1	0	1 *Cladosporium* sp. 4 Unidentified
Crow 3	18	PIPO & PSME	5	8	6	0	0	1 *Cladosporium* sp. 1 *Epicoccum* sp.
Crow 6	35	PIPO & PSME	17	1	4	0	3	1 *Alternaria* sp. 1 *Phialophora* sp. 1 *Rhinocladiella* sp. 1 *Ulocladium* sp. 7 Unidentified
L. Camas	10	PIPO & PSME	5	2	0	0	0	3 Unidentified
Pendleton 30	28	PIPO	15	7	0	0	0	1 *Verticillium* sp. 2 *Tremella* sp. 1 *Heterobasidion* sp. 3 Unidentified
Poison 6	21	PSME & PIPO	14	2	1	2	1	2 *Verticillium* sp. 1 *Eupenicillium* sp.
Ruby	24	PSME & PIPO	10	4	0	5	2	1 *Verticillium* sp. 1 *Pesotum* sp. 2 Unidentified
Sand 19	28	PSME	23	3	0	0	2	1 Unidentified
Sand 2	25	PSME	14	9	1	0	1	1 *Hormonema* sp. 2 Unidentified
Slawson	22	PSME & PIPO	12	4	0	2	1	1 *Cladosporium* sp. 1 *Merimbla* sp. 2 Unidentified
Spromberg 4	28	PSME & PIPO	13	6	6	8	0	3 Unidentified
Tripp 9	19	PSME & PIPO	12	7	0	0	0	0

Total	289	151	59	30	18	10	4 *Verticillium* sp. 3 *Cladosporium* sp. 2 *Tremella* sp. 1 *Epicoccum* sp. 1 *Phialophora* sp. 1 *Alternaria* sp. 1 *Ulocladium* sp. 1 *Rhinocladiella* sp. 1 *Heterobasidion* sp. 1 *Eupenicillium* sp. 1 *Merimbla* sp. 1 *Pesotum* sp. 1 *Hormonema* sp. 27 Unidentified

[1]For this study, PIPO typically included PIPO/PUTR, PIPO/CARU, and PIPO/AGSP plant associations; PSME included PSME/SPBEL, PSME/PUTR, PSME/CARU, PSME/SYAL, and PSME/AGSP plant associations (see text; WILLIAMS and SMITH 1991; LILLYBRIDGE et al. 1995). AGSP, *Agropyron spicatum*; CARU, *Calamagrostis rubescens*; PIPO, *Pinus ponderosa*; PSME, *Pseudotsuga menziesii*; PUTR, *Purshia tridentata*; SPBEL, *Spiraea betulifolia*; SYAL, *Symphoricarpos albus*.

Table 2. **Summary of fungal species isolated from roots of symptomless ponderosa pine and Douglas-fir hosts growing within PIPO and PSME plant associations**[1]

	Ponderosa pine host		Douglas-fir host		
	PIPO plant association [no. (%)]	PSME plant association [no. (%)]	PIPO plant association [no. (%)]	PSME plant association [no. (%)]	Total [no. (%)]
Total number of trees	48	112	1	128	289
Trees with isolated fungal endophyte	22 (45.8)	58 (51.8)	0 (0.0)	58 (45.3)	138 (47.8)
Trees with no isolated fungal endophytes	26 (54.2)	54 (48.2)	1 (100)	70 (54.7)	151 (52.2)
Trees with multiple fungal endophytes	6 (12.5)	8 (7.1)	0 (0.0)	13 (10.2)	27 (9.3)
Byssochlamys sp.	5 (10.4)	22 (19.6)	0	32 (25.0)	59 (20.4)
Umbelopsis spp.	4 (8.3)	16 (14.3)	0	10 (7.8)	30 (10.4)
Mucor sp.	1 (2.1)	8 (7.1)	0	9 (7.0)	18 (6.2)
Penicillium sp.	1 (2.1)	3 (2.7)	0	6 (4.7)	10 (3.5)
Verticillium sp.	2 (4.2)	0 (0.0)	0	2 (1.6)	4 (1.4)
Cladosporium sp.	1 (2.1)	1 (0.9)	0	1 (0.8)	3 (1.0)
Tremella sp.	1 (2.1)	1 (0.9)	0	0 (0.0)	2 (0.7)
Epicoccum sp.	1 (2.1)	0 (0.0)	0	0 (0.0)	1 (0.3)
Phialophora sp.	1 (2.1)	0 (0.0)	0	0 (0.0)	1 (0.3)
Alternaria sp.	0 (0.0)	1 (0.9)	0	0 (0.0)	1 (0.3)
Ulocladium sp.	1 (2.1)	0 (0.0)	0	0 (0.0)	1 (0.3)
Rhinocladiella sp.	0 (0.0)	1 (0.9)	0	0 (0.0)	1 (0.3)
Heterobasidion sp.	0 (0.0)	1 (0.9)	0	0 (0.0)	1 (0.3)
Eupenicillium sp.	1 (2.1)	0 (0.0)	0	0 (0.0)	1 (0.3)
Merimbla sp.	0 (0.0)	0 (0.0)	0	1 (0.8)	1 (0.3)
Hormonema sp.	0 (0.0)	0 (0.0)	0	1 (0.8)	1 (0.3)
Pesotum sp.	0 (0.0)	0 (0.0)	0	1 (0.8)	1 (0.3)
Unidentified	7 (14.6)	11 (9.8)	0	9 (7.0)	27 (9.3)

[1]For this study, PIPO typically included PIPO/PUTR, PIPO/CARU, and PIPO/AGSP plant associations; PSME included PSME/SPBEL, PSME/PUTR, PSME/CARU, PSME/SYAL, and PSME/AGSP plant associations (see text; Williams and Smith 1991; Lillybridge et al. 1995). AGSP, *Agropyron spicatum*; CARU, *Calamagrostis rubescens*; PIPO, *Pinus ponderosa*; PSME, *Pseudotsuga menziesii*; PUTR, *Purshia tridentata*; SPBEL, *Spiraea betulifolia*; SYAL, *Symphoricarpos albus*.

and *Heterobasidion* sp. (one isolation). Most of the fungi isolated were ascomycetes and zygomycetes (Table 1). *Byssochlamys nivea* Westling, *Umbelopsis* spp., and *Mucor* sp. were the most frequently recovered fungi from ponderosa pine (16.9, 12.5 and 5.6% of cores, respectively) and Douglas-fir (24.8, 7.8 and 7.0% of cores, respectively). Averaged across hosts, the recovery of *B. nivea* was significantly greater than that of *Umbelopsis* spp. (z-ratio = 3.442; $p < 0.001$; d.f. = 1) or any other fungus. Recovery of *Umbelopsis* spp. was significantly greater than that of *Mucor* spp. (z-ratio = 1.947; $p < 0.03$; d.f. = 1), *Penicillium* spp. (z-ratio = 3.162; $p < 0.001$; d.f. = 1), and other less frequently recovered fungi.

3.2 Influence of host species and plant association

Host species did not significantly influence the proportion of cores from which fungi were recovered ($\chi^2 = 0.727$; $p = 0.394$; d.f. = 1), nor did host species influence recovery of the individual fungal species *B. nivea* ($\chi^2 = 2.765$; $p = 0.096$; d.f. = 1), *Umbelopsis* spp. ($\chi^2 = 1.731$; $p = 0.188$; d.f. = 1), *Mucor* spp. ($\chi^2 = 0.223$; $p = 0.636$; d.f. = 1), or

Penicillium spp. ($\chi^2 = 0.989$; $p = 0.320$; d.f. = 1). Similarly, plant association (PIPO and PSME) did not influence the overall recovery of fungi from cores ($\chi^2 = 0.192$; $p = 0.661$; d.f. = 1), nor recovery of *B. nivea* ($\chi^2 = 3.787$; $p = 0.052$; d.f. = 1), *Umbelopsis* spp. ($\chi^2 = 0.312$; $p = 0.577$; d.f. = 1), *Mucor* spp. ($\chi^2 = 1.772$; $p = 0.183$; d.f. = 1), or *Penicillium* spp. ($\chi^2 = 0.355$; $p = 0.551$; d.f. = 1). Within the PSME plant association, host species (ponderosa pine or Douglas-fir) did not influence overall recovery of fungi ($\chi^2 = 1.002$; $p = 0.317$; d.f. = 1), nor recovery of the individual species *B. nivea* ($\chi^2 = 0.983$; $p = 0.321$; d.f. = 1), *Umbelopsis* spp. ($\chi^2 = 2.591$; $p = 0.107$; d.f. = 1), *Mucor* spp. ($\chi^2 = 0.001$; $p = 0.973$; d.f. = 1), or *Penicillium* spp. ($\chi^2 = 0.668$; $p = 0.414$; d.f. = 1) (Table 2).

3.3 Identification of *B. nivea*

The ITS sequences from representative isolates (e.g. AY376406, Genbank) showed 97% sequence identity with *B. nivea* (stat. anam. *Paecilomyces*) (U18361, GenBank). However, *B. nivea* is the only one of the five accepted *Byssochlamys* species for which ITS sequence information was available in GenBank. Thus, identification to the species level could not be determined by sequence data alone. Morphological data were compared with morphological characterizations of the *Byssochlamys* genus (Brown and Smith 1957; Stolk and Samson 1971). When root cores were placed on BDS medium, growth of *B. nivea* isolates was restricted to the portion of the core farthest from the medium, and did not extend into the selective medium. Normal fungal growth was obtained after root cores were transferred to MEA. Morphologically, the *Byssochlamys* isolates agreed with published descriptions of *B. nivea* (Brown and Smith 1957; Stolk and Samson 1971; Hanlin 1998). Ascospores of our isolates were smooth-walled, ellipsoid, ca. 5 × 3 μm, and asci were ca. 11 μm diam, compatible with described *B. nivea* ascospores of 4–5.5 × 2.5–3.5 μm, and asci of 8.5–11 μm diam (Brown and Smith 1957; Stolk and Samson 1971). Coiled ascogonial initials similar to those illustrated for *B. nivea* by Stolk and Samson (1971) were observed twice in culture. Chlamydospores (5–7 μm diam) and conidia [(3.0)–4.0 μm × (1.5)–2.5 μm] were frequently observed (Fig. 2). Chlamydospores of *B. nivea* are given as 4–7 × 2.5–5 μm, and conidia as 3–5.7 × 2.2–4 μm (Stolk and Samson 1971). The conidiogenous apparatus of the *Paecilomyces* anamorph consisted of long (18–45 × 2.7–5.0 μm), tapering phialides with conidia borne in chains; hyphae were irregularly branched and septate. The colour of the mycelia in culture on 2% MEA was highly variable, ranging from olive to brown, with tufts of white mycelia dispersed across most cultures (Fig. 2). Brown and Smith (1957) described the colony colour in *B. nivea* cultures as white, but Stolk and Samson (1971) described a range of colony colours, including olive-buff to deep olive buff, among the isolates they examined. The morphological features of *B. nivea* differ from those of other described species in the genus. *Byssochlamys fulva* Olliver & Smith has larger ascospores (mostly 6.5 μm long), phialides borne in groups of two to three on short metulae, conidia 4–8.7 × 1.5–5 μm, and lacks chlamydospores (Stolk and Samson 1971). *Byssochlamys zollerniae* Ram produces black, echinulate chlamydospores (Ram 1968), and *Byssochlamys verrucosa* Samson & Tansey and *B. striata* (Raper & Fennell) Arx have conspicuously ornamented ascospores (Stolk and Samson 1971; Samson and Tansey 1975).

3.4 Identification of *Umbelopsis* spp.

Thirty *Umbelopsis*-like isolates obtained from root samples of both Douglas-fir and ponderosa pine (Table 2) had morphological and/or rDNA sequence similarity (ca. 92–98%) with reference isolates of *U. isabellina* (provided by R. Vilgalys, E. Vandegrift, and K. O'Donnell) and *U. ramanniana* (provided by K. O'Donnell, as *M. ramannianus*). A subset of *Umbelopsis* isolates was sent to A. Schüßler (Institute of Botany, TU,

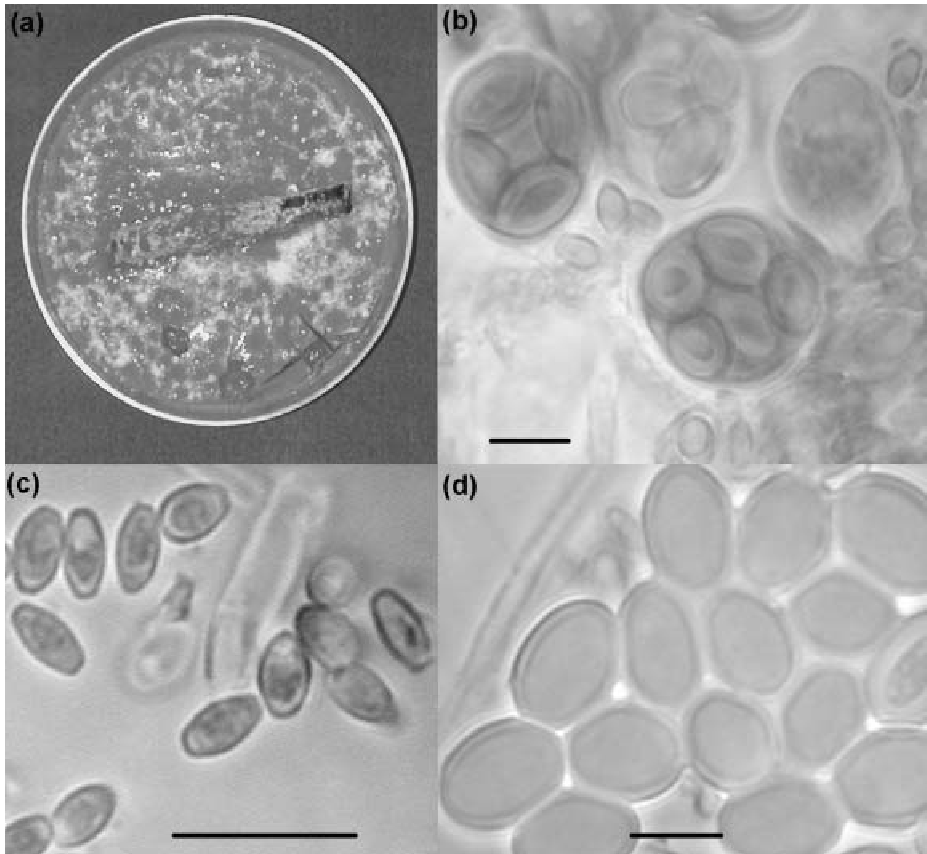

Fig. 2. *Byssochlamys nivea* (a) culture on nutrient-rich medium; (b) asci containing ascospores; (c) conidia; (d) chlamydospores. Scale bars: B, C, D = 5 μm

Darmstadt, Germany) for small subunit (SSU) rDNA sequence analyses. Two of the cultures had a 99% SSU sequence identity to *U. isabellina*. However, ITS sequences from other isolates of *Umbelopsis* (e.g. AY376407, AY376408, AY376409, AY376410, and AY376411, GenBank) also showed a 92–99% identity with additional *Umbelopsis* species in the GenBank database (e.g. *U. vinacea*, AJ495425; *U. versiformis*, AJ495434).

O'Donnell et al. (2001) determined that *U. isabellina* and *M. ramannianus* formed a well-supported basal clade to all other *Mucorales*. Meyer and Gams (2003) placed all species formerly classified as *Micromucor* in *Umbelopsis* and identified two subclades within the *M. isabellina* group based on RFLP and ITS sequence analyses. These authors noted that additional work is needed to establish reliable species limits within *Umbelopsis* (Meyer and Gams 2003). Thus, our related group of isolates belongs in *Umbelopsis*, but species identification is not possible at this time (Hoff 2002). Isolates are referred to herein as *Umbelopsis* spp.

Umbelopsis spp. isolates produced abundant growth on BDS, the nutrient-rich medium, and 2% w/v MEA. Colonies ranged from cream to tan in colour, and some exhibited a grayish colour. Three isolates had colonies that were pink (Fig. 3). Variations in colour did not correspond to specific sequence variants. Mycelia and chlamydospores were filled with

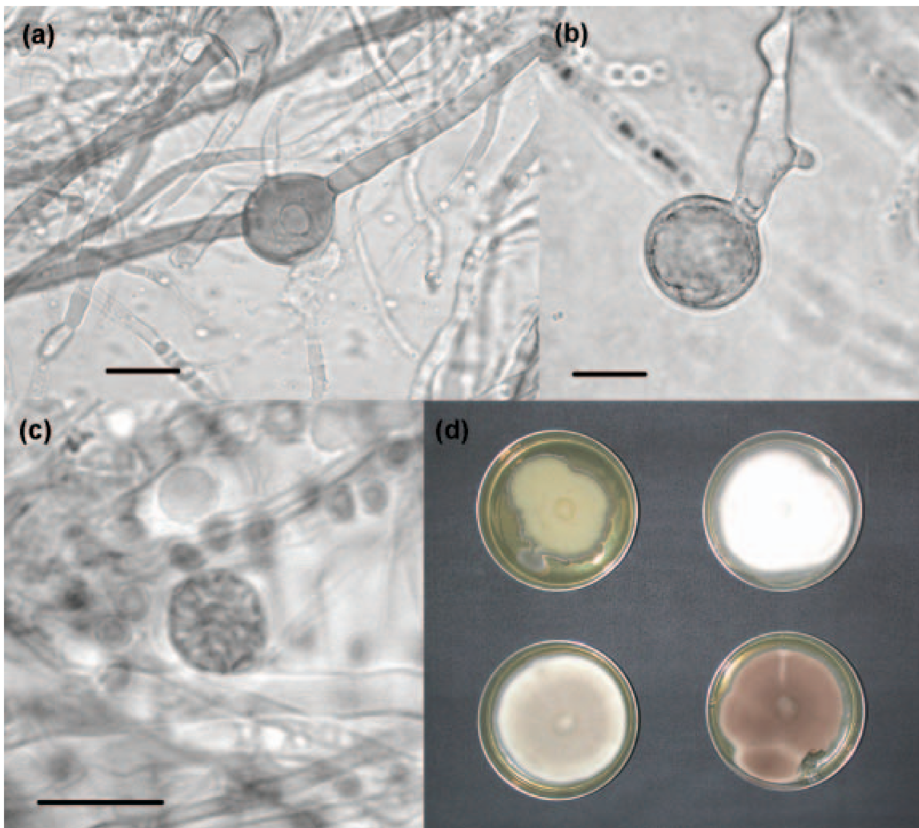

Fig. 3. Morphology of *Umbelopsis* sp. fungal isolates (a) intercalary chlamydospores; (b) terminal chlamydospores; (c) sporangia; (d) colour variations in colonies. Scale bars: A, B, C = 10 μm

what appeared to be lipid droplets (Fig. 3). Hyphae were irregularly septate, with a random branching pattern. Intercalary and terminal chlamydospores were 20–(49) × 22–(49) μm diam (Fig. 3). Dull reddish-coloured, globose sporangia (7–12.5 μm diam) and angular sporangiospores (3.5–4.5 × 2.5–3.5 μm diam) were occasionally observed in culture of some isolates.

4 Discussion

Byssochlamys nivea and *Umbelopsis* spp. predominated among fungi isolated from the study sites. *Byssochlamys* is a genus of ascomycetes characterized by rudimentary cleistothecia (Beuchat and Pitt 2002). *B. nivea* has been isolated from soil and a range of organic substrates, including wood, barley and oat grains, apple juice, and mummified plums (Stolk and Samson 1971). *B. nivea* is a potential biological control species, and is known to inhibit *in vitro* growth of such plant pathogenic fungi as *Fusarium oxysporum* Schlecht, *Gaeumannomyces graminis* (Sacc.) von Arx and Olivier var. *tritici* J. Walker, *Phytophthora cinnamomi* Rands, *Pythium irregulare* Buisman, and *Rhizoctonia solani* Kuhn (Park et al. 2001). *Byssochlamys nivea* also inhibits egg hatching, and causes cuticle disruption and death in certain nematodes (Park et al. 2001). *Byssochlamys nivea* and

B. fulva are heat-resistant fungi; ascospores can survive temperature exposure up to 99°C (Beuchat and Pitt 2002). Piirto et al. (1998) isolated *B. fulva* from 38% of fire scars of giant sequoia. In their study, recovery of *B. fulva* was greatest (85%) from fire scars of trees 5 years after fires, moderate (29%) from unburned trees, and poorest (8%) from trees 1 year after fire. Considering its heat tolerance, biological control potential, and widespread occurrence, *B. nivea* may have significant interactions with forest fungal pathogens. The frequency of *B. nivea* will be assessed again after the 2004 prescribed burn treatment to compare its recovery relative to that of other fungal species, and evaluate the influence of burning on the fungal flora of these dry forests. Further research is needed to evaluate potential biological control implications of *B. nivea*.

Umbelopsis is a genus of zygomycetes that has velvety to leather-like mycelia of gray to tan colour and sporangia of ochre to red colour. *Umbelopsis* colonies produce dense, cymosely or verticillately branched sporangiophores and lipid-filled chlamydospores/vesicles, but apparently lack zygospores (Kendrick et al. 1994; Meyer and Gams 2003). *Umbelopsis* spp., including many species formerly included in *Mortierella*, are very common soil fungi that are found in forest ecosystems around the world (Domsch et al. 1980; Meyer and Gams 2003). In previous studies, members of this genus have been also obtained from the ascocarp of a *Peziza* species (Kendrick et al. 1994). Although *Umbelopis* spp. do not grow at high temperatures, they have been characterized as heat resistant (Bollen and van der Pol-Luiten 1975). *U. isabellina* has been isolated as *Mortierella isabellina* from surface-sterilized, 2–3-year-old roots of healthy, mature trees of *Pinus sylvestris* L. in Exeter, U.K. (Fisher et al. 1991). *U. isabellina* (as *M. isabellina*) and *U. ramanniana* (as *M. ramanniana*) have each been isolated from serially washed, fine (3–4 mm diam) roots of healthy, 50- to 100-year-old trees of *Picea abies* (L.) Karst. in Southern Bavaria, Germany (Holdenrieder and Sieber 1992). Holdenrieder et al. (1994) also isolated these two species from healthy and discoloured cores taken from main lateral roots of *P. abies*. In addition, Amos and Barnett (1966) isolated an *Umbelopsis* sp. while isolating *Ceratocystis fagacearum* (Bretz) Hunt in the roots of naturally infected oaks. Thus, *Umbelopsis* spp. are common associates of woody roots. The frequent recovery of *Umbellopsis* spp. in this study contrasts sharply with the absence of other common soil- and wood decay-associated fungi (e.g. *Trichoderma*) that can also grow on the selective medium (BDS) used in this study. This finding indicates that *Umbelopsis* spp. recovered in this study were present as endophytes in root xylem tissue, in support of earlier findings by Holdenrieder et al. (1994).

The ecological roles of *Umbelopsis* spp. in forest ecosystems of northwestern USA are potentially quite important, but largely undetermined. In a recent study covering three geographic regions in the western USA (Oregon coast, Washington Cascades, and high desert in Oregon), Vandegrift (2002) isolated fungi similar (e.g. 98% ITS sequence identity) to our *Umbelopsis* spp., from woody roots of Douglas-fir, western hemlock [(*Tsuga heterophylla* (Raf.) Sarg.], Sitka spruce [(*Picea sitchensis* (Bong.) Carriére], ponderosa pine, and lodgepole pine (*Pinus contorta* Louden). *Umbelopsis* spp. with similar ITS sequences to the endophytic isolates have been isolated from wood of Douglas-fir located in a laminated root-rot (caused by *Phellinus* sp.) mortality center near Chewelah, WA (J. W. Hanna, USDA Forest Service, Moscow, ID, USA, pers. comm.) and from decomposing wood stakes in contact with forest soil at multiple sites in eastern Washington and northern Idaho (R. C. Rippy, USDA Forest Service, Moscow, ID, USA, pers. comm.). More studies are needed to determine the potentially diverse ecological roles of *Umbelopsis* spp. in forests of northwestern USA.

Unanswered questions concerning *Umbelopsis* spp. involve their mode of root ingress, interactions with other fungi and forest organisms, the nature of interactions with their hosts, and the effects of these ecological interactions on the surrounding plant community. Because *Umbelopsis* spp. may impact ecosystem processes indirectly,

ecological interactions should be considered at multiple levels to determine the potential ecological significance of these fungi.

Surveys of fungi inhabiting older sections of living woody roots are uncommon, and we cannot yet confirm whether the fungi found in this study represent the breadth of fungi that favour dry forest environments, including those disturbed by fire. For such studies, sampling biases are necessarily interconnected with the choice of culture media used for fungal isolation. The use of selective medium is important to assure representation of slower growing fungi, while non-selective medium is important to assure representation of fungi that are sensitive to the selective medium (Clubbe 1978). In this study, a benomyl-containing medium was used for initial isolation, with subsequent transfer to a less-restrictive medium. This approach was intended to avoid discrimination against slower growing basidiomycetes, while also encouraging recovery of other diverse fungi. Nevertheless, the fungi most frequently recovered in this study were zygomycetous, ascomycetous, and imperfect fungi, not basidiomycetous fungi that are typically favoured by the benomyl-containing medium used for initial isolation. In a previous study, Varese et al. (2003) found that MEA and benomyl-amended medium both allowed recovery of diverse fungi from inoculated stumps. In our study, *Umbelopsis* spp. grew well on the BDS medium, whereas *B. nivea* exhibited strong growth only after transfer to non-selective MEA. Benomyl tolerance of *Umbelopsis* species, formerly included in *Mortierella sensu lato*, has been observed previously (Strauss et al. 2000). As with many media selective for basidiomycetes, media containing benomyl allow preferential colonization of basidiomycetes and other select fungal groups (e.g. *Mucor* spp.), but may be only fungistatic to other fungi (Edgington et al. 1971; Hunt and Cobb 1971; Hale and Savory 1976). Benomyl and its breakdown product carbendazim both have low solubility in water (ARS Pesticide Properties Database; http://www.ars usda.gov/acsl/services/ppdb/listall.html) and are tightly bound by soil and organic compounds (Kerle et al. 1996). Thus, survival and/or growth of benomyl-sensitive fungi can occur on portions of woody material that do not directly contact the selective medium, a phenomenon previously noted by Hunt and Cobb (1971). The wide diversity of fungi recovered in this study indicates that the transfer of root cores from selective to non-selective medium appears to be an effective approach for surveying fungal endophytes. The infrequent recovery of basidiomycetous fungal endophytes in this study is also typical of fungal endophyte isolations among woody and non-woody hosts (Redlin and Carris 1996).

The fungal community that develops in large, living woody roots after a burn treatment, such as is planned for the study sites represented here, would be expected to exhibit ruderal characteristics, including rapid dispersal, efficient uptake of nutrients, rapid capture of resources, and stress tolerance. These characteristics should be shared by fungi that are generally favoured by disturbance (Pugh and Boddy 1988). Fungal taxa may be differentially affected by prescribed burns, depending upon their prevalence in the different litter and/or soil layers that are most impacted by fire (Stendell et al. 1999). For this reason, Stendell et al. (1999) suggested that prevalence of ectomycorrhizal fungi prior to a prescribed burn is a poor indicator of persistence after the fire.

Although the two hosts and two plant associations examined here showed no significant interaction with the fungal species recovered within the PSME series, *Umbelopsis* spp. were nonetheless isolated more frequently from ponderosa pine than from Douglas-fir. This weak trend may indicate that this endophyte could show a host preference under certain environmental or disturbance conditions. This relationship warrants further investigation. In addition, *B. nivea* was somewhat more abundant in the PSME series, although the influence of plant association on *B. nivea* occurrence was not significant. This latter trend could be because of the fact that the PSME plant associations occur on sites where soil

moisture is slightly less limiting during the growing season than the PIPO plant associations.

The overall lack of correlation between host, plant association, and fungal occurrence is likely because of the high degree of similarity in the PSME and PIPO plant associations of the sampling units and/or the lack of strong host specialization by these fungi. Had our study incorporated more extreme variation in environmental conditions, such as wet habitats *vs.* dry habitats, or a larger number of samples, a more significant influence among the potential vegetation types and number of fungal species isolated might have been observed. In 5 years, a re-survey is planned to observe how burn treatments impact fungal communities inhabiting these sites, and determine whether fungal communities change in response to treatments. Host vigour, the associated fungal community, and protection offered by niches within the interior of roots might all influence the distribution of fungal endophytes within woody roots. It will be of further interest to study how fungi occupying this recently discovered niche might also influence the dynamics of fungi in the rhizosphere of forest tree roots.

Results revealed that only a small number of cores from woody roots of Douglas-fir and ponderosa pine were colonized by various other fungal genera (e.g. *Mucor* sp., *Penicillium* spp., *Verticillium* sp., *Cladosporium* sp., *Tremella* sp., etc.). Many of the taxa isolated infrequently in this study were also isolated in studies of root-colonizing fungi of *P. abies* (Holdenrieder and Sieber 1992) and *P. sylvestris* (Fisher et al. 1991). Fisher et al. (1991) speculated that the presence of common soil and organic debris-colonizing fungi, such as *Cladosporium*, *Penicillium*, *Mucor*, and *Umbelopsis* (as *Mortierella*), in woody roots may indicate that many different saprotrophic fungi have some limited ability to live in living root tissue.

The diversity of fungi obtained from healthy, large, woody roots of two conifer species growing in a relatively narrow range of dry-forest settings suggests that an even broader flora of fungal endophytes might be found across diverse forest environments. The use of molecular tools aided in rapid identification of our cultured fungi to the genus level. However, the use of molecular databanks, such as GenBank, for species identification was limited in several regards. Until molecular databanks accurately reflect species collected from a broad range of geographic areas/environments and the taxonomic identification of the fungi from which submitted sequences were obtained can be accepted as reliable, molecular techniques must remain coupled with cultural/microscopic techniques to help ensure accurate identification. This combined approach will enable researchers to tap into expanding databanks representing previous surveys of extant fungal diversity, while capturing important local information on population structure and genecology through cultural assays. Aside from providing morphological features, cultural techniques provide strong information on vegetative and sexual incompatibility that, when coupled with available molecular evidence, may allow differentiation of closely related species and interpretation of biological implications related to DNA sequence divergence.

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Résumé

Champignons endophytes des grosses racines de Douglas (Pseudotsuga menziesii) et Pin ponderosa (Pinus ponderosa)

La communauté fongique des grosses racines de conifères sains est mal connue. Pour apporter des informations dans ce domaine, une prospection a été réalisée en utilisant des carottes de sondage dans les racines de Douglas (*Pseudotsuga menziesii*) et de Pin ponderosa (*Pinus ponderosa*) asymptomatiques poussant dans les forêts sèches du versant oriental des Cascade Mountains dans l'Etat de Washington, USA. Les isolats fongiques ont été cultivés sur des milieux classiques et identifiés en utilisant une combinaison de méthodes morphologiques et moléculaires. Les genres et espèces fongiques identifiés dans cette étude pourront servir de données de référence pour de futures études sur les champignons endophytes. 27 genres ont été identifiés à partir de la séquence des ITS1, ITS2 et 5.8S rDNA et de la morphologie en culture. Deux groupes sont prédominants: *Byssochlamys nivea* Westling (20.4% des isolements) et *Umbelopsis* species (10.4% des isolements). Cette étude constitue la première mention de ces champignons dans des grosses racines de conifères. Les deux taxons ont été précédemment identifiés comme des agents potentiels de lutte biologique. Bien que certaines tendances soient observées, aucun effet significatif de l'espèce hôte ou des associations végétales sur le résultat total des isolements de champignons endophytes ou sur l'isolement de certains taxons spécifiques n'a été démontré.

Zusammenfassung

Pilzliche Endophyten in verholzten Wurzeln von Douglasie (Pseudotsuga menziesii) und Gelb-Kiefer (Pinus ponderosa)

Die Pilzgemeinschaften, die grosse, verholzte Wurzeln von gesunden Koniferen besiedeln, wurden bisher kaum untersucht. Aus diesem Grunde wurden im Rahmen einer Inventur in trockenen Wäldern am Ostabhang der Kaskaden in Washington, USA aus verholzten Wurzeln symptomloser Douglasien (*Pseudotsuga menziesii*) und Gelb-Kiefern (*Pinus ponderosa*) Bohrkerne entnommen. Die daraus isolierten Pilze wurden auf Standardmedien kultiviert und mit Hilfe molekularer und morphologischer Methoden bestimmt. Die in dieser Untersuchung identifizierten Taxa liefern die Basisdaten für zukünftige Aufnahmen der pilzlichen Endophyten. Mit Hilfe der ITS 1, ITS 2 und 5,8 S rDNA Sequenzen und der Kulturmorphologie wurden insgesamt 27 Pilzgattungen nachgewiesen. Dabei dominierten zwei Taxa: *Byssochlamys nivea* (20,4% der Isolierungen) und *Umbelopsis* spp. (10,4%). Dies ist der Erstnachweis von *B. nivea* in verholzten Koniferenwurzeln. Beide Pilze wurden zuvor als mögliche biologische Kontrollorganismen identifiziert. Trotz einiger Tendenzen ergaben sich in dieser Studie keine signifikanten Hinweise auf Auswirkungen von Wirtsart oder Pflanzengesellschaft auf das Vorkommen der pilzlichen Endophyten insgesamt oder von einzelnen Pilzarten.

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