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High-Throughput Sequencing Shows High Fungal Diversity and Community Segregation in the Rhizospheres of Container-Grown Conifer Seedlings

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Abstract: Forest nurseries in Sweden produce *ca.* 360 million seedlings of *Pinus sylvestris* L. and *Picea abies* (L.) Karst. annually. Fungi represent the largest microbial component in rhizospheres and may significantly affect health and, consequently, quality of the seedlings. The aim of this study was to assess fungi focusing on pathogens in roots and the sphagnum peat growth substrate of healthy-looking *P. sylvestris* and *P. abies* seedlings from nine forest nurseries situated in northern, central and southern regions of Sweden. We hypothesized that nursery stock and the growth substrate can provide a venue for dissemination of fungal diseases. In each nursery and for each tree species, 100 seedlings with the growth substrate were collected during the dormant period. DNA was isolated from parts of root systems and from samples of the growth substrate, amplified using internal transcribed spacer of rDNA as a marker and 454-sequenced. Clustering at 98.5% similarity of 169,844 high-quality sequences resulted in 619 non-singleton fungal taxa. Although results showed that management practices in forest nurseries generally give a healthy stock, latent establishment of pathogenic fungi in both roots and the growth substrate supported the hypothesis. Furthermore, seedling roots and the growth substrate were inhabited by distinct communities of fungi, and lifestyles of these fungi largely determined community segregation into particular ecological niche.

Keywords: forest nursery; mycorrhiza; pathogens; sequencing; seedlings; tree health

1. Introduction

New and invasive diseases of forest trees have been reported at an increasing rate and such incidents are predicted to be even greater in the future [1,2]. Changing environment and different anthropogenic factors (e.g., international trade, lack of legislation, *etc.*) are generally thought to be responsible for their arrival and spread [2]. One of the recent examples of such diseases is dieback of European ash (*Fraxinus excelsior* L.), which is caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz, Hosoya [3], and which is invasive in Europe [4]. Its origin is in the Asian Far East, where it colonizes petioles of the native Manchurian ash (*Fraxinus mandshurica* Rupr.) without causing severe damage to living trees [5,6]. In contrast, the fungus causes severe damage to *F. excelsior*, often resulting in mortality [7]. It appears that the pathogen invaded Europe together with saplings of *F. mandshurica* imported from China to forest nursery in eastern Poland, and via airborne spores spread from there in all geographic directions [8]. Another example is *Phytophthora ramorum* Werres, de Cock & Man in 't Veld, which is the causal agent of sudden oak death as well as ramorum blight to numerous species of

woody ornamental and forest understorey plants [9]. It was introduced from Asia independently to both Europe and US [10] and then spread through the trade with nursery plants [11].

The latter examples demonstrate that forest nurseries are frequent sources of tree seedling imports and trade, and can provide dissemination routes for any diseased plants [12]. As symptoms of new diseases are normally not well described, and latent or cryptic disease phases may be visually undetected for prolonged periods, plants may be disseminated without any disease problems being recognized [1]. Furthermore, due to the intensive nature of both management and production, forest nurseries are among the most vulnerable environments affected by tree pathogens. Intensive management practices (e.g., large monocultures of densely grown seedlings, intensive fertilization, irrigation, chemical weed and pest control) may often stress plants and create conditions favorable for establishment and rapid spread of fungal infections, resulting in tremendous economical losses [13]. Early detection of such diseases is critical as this can prevent further establishment and spread to new environments. In addition to detrimental pathogenic fungi, roots of tree seedlings harbor beneficial communities of fungi [14,15], which may promote seedling growth both in forest nurseries and after outplanting [16]. Mycorrhizal fungi are known to provide nutritional benefits to their hosts and may increase tolerance to abiotic and biotic stress factors [17]. Mycorrhizal fungi forms a compact sheath around the rootlet, from which hyphae grow inward to the cortex, forming a continuous network between the cortical cells, and outward to the surrounding soil. A fine network of fungal hyphae explores and extracts nutrients from a volume of soil far beyond that directly influenced by the roots themselves. A proportion of these nutrients are translocated through the hyphal network to the short roots. The mycorrhizal short roots are the functional units of the symbiosis where exchange of nutrients, carbon and water between the symbiotic partners take place [17]. Another group of fungi are the endophytes which live asymptomatically within plant tissues for at least part of their life cycle [18]. Endophytes have been found in all woody plants studied to date and represent numerous fungal species [19]. Many taxa found in roots have darkly pigmented hyphae. Endophytes live in a habitat, which involves continual metabolic interactions between fungus and host. Substances produced by endophytes may be toxic to plant pathogens or act as repellents against insects or herbivores [20]. Some endophytes of woody roots may be selectively antagonistic to plant pathogens and/or pest insects and may be an important source of new biologically active secondary metabolites [21].

In a recent study, which deployed fungal culturing and direct Sanger sequencing from segments of lateral roots of healthy-looking *P. sylvestris* and *P. abies* seedlings, rarefaction analysis showed that significantly higher diversity of fungal taxa could be detected with increased sequencing effort [22]. More importantly, the study showed the predominant establishment in roots of potentially new and invasive fungal species such as *Atradidymella muscivora* Davey & Currah (anamorph *Phoma muscivora*) [22]. The latter demonstrated the need for more detailed study encompassing larger portions of root systems and the growth substrate, which can both act as potential vectors for diseases. The aim of this study was to assess fungi focusing on pathogens in roots and the sphagnum peat growth substrate of healthy-looking conifer seedlings from nine containerized forest nurseries situated in northern, central and southern regions of Sweden through the use of high-throughput sequencing methods. Previously initiated studies with similar aims were largely based on culturing methods and mainly detected fast-growing fungi [23,24], whereas high-throughput sequencing allows exploration of complex fungal communities and individual community components even at low abundance [25]. We hypothesized that nursery stock and the growth substrate can provide a venue for dissemination of fungal diseases whose symptoms can be undetected due to the new nature of the disease and/or be hidden due to the intensive nature of both management and production in forest nurseries.

2. Experimental Section

2.1. Seedling Materials and Sampling

Scots pine (*Pinus sylvestris* L.) and Norway spruce (*Picea abies* (L.) Karst.) seedlings were collected at dormancy in winter 2011 from nine major containerized forest nurseries in Sweden (Table 1). Three of these nurseries were situated in northern, three in central and three in southern regions of Sweden (Figure 1). Seedling cultivation in forest nurseries is highly mechanized and each nursery produces between 10 and 70 million seedlings annually [26]. The main focus is to produce high quality seedlings, that is, healthy seedlings showing high survival and growth rates after outplanting. Cultivation scheme includes production of one-year-old, 1.5- and two-year-old seedlings (Figure 2). Recommended fertilization with macro- and micronutrients as proportional to N is: N 100%, P 13%, K 65%, Ca 7%, Mg 8%, S 9%, Fe 0.7%, Mn 0.4%, B 0.2%, Zn 0.06%, Cu 0.03%, Cl 0.03% and Mb 0.007% [26]. Fertilizers are often applied dissolved in water through the automated watering system. The content of water is monitored to compose between 70% and 85% of the total containerized system content. Pesticides are applied as preventive measures against fungal diseases and to control weeds. The most common fungal diseases in Swedish forest nurseries include grey mould, *Gremmeniella* shoot dieback, Lophodermium needlecast, pine twisting rust, Sirococcus shoot blight and different root diseases [23,27].

Table 1. Sampled root systems (*roots*) and the sphagnum *peat* growth substrate of healthy-looking *Pinus sylvestris* and *Picea abies* seedlings from nine forest nurseries in Sweden, generated high-quality fungal ITS rDNA *sequences* using high-throughput 454-sequencing and detected diversity of fungal *taxa*.

No.	Nursery	Location	*Pinus sylvestris* [a]						*Picea abies* [a]					
			Roots	Sequences	Taxa	Peat	Sequences	Taxa	Roots	Sequences	Taxa	Peat	Sequences	Taxa
1	Gideå	N63°29′, E18°58′	100	6528	59	100	7246	70	100	2291	46	100	5221	76
2	Kilåmon	N63°28′, E16°40′	100	2242	72	100	6988	100	100	2599	35	100	3390	83
3	Bogrundet	N62°31′, E17°18′	100	1548	52	100	2122	96	100	664	41	100	4028	126
4	Nässja	N60°16′, E16°42′	100	637	30	100	3813	48	100	1201	36	100	5363	92
5	Lugnet	N59°41′, E16°36′	100	1171	50	100	7261	127	100	1339	46	100	7941	124
6	Stakheden	N60°16′, E14°57′	100	712	23	100	8005	120	100	1964	39	100	9836	110
7	Trekanten	N56°41′, E16°06′	100	8113	100	100	10392	149	100	9209	75	100	8351	139
8	Flåboda	N56°34′, E15°08′	100	1330	40	100	5611	92	100	686	53	100	3131	90
9	Kolleberga	N56°03′, E13°15′	100	2763	92	100	9357	164	100	438	33	100	7668	197
	Total		900	25044	237	900	60795	406	900	20391	159	900	54929	442

[a] Number of seedlings used in this study corresponds to number of "Roots".

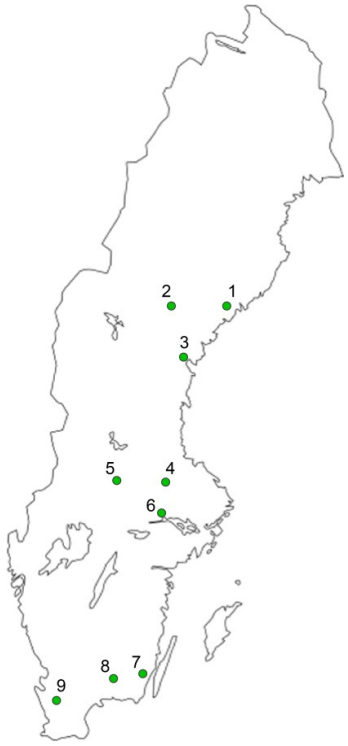

Figure 1. Map of Sweden showing geographical distribution of forest nurseries of the present study. Forest nurseries are numbered as in Table 1.

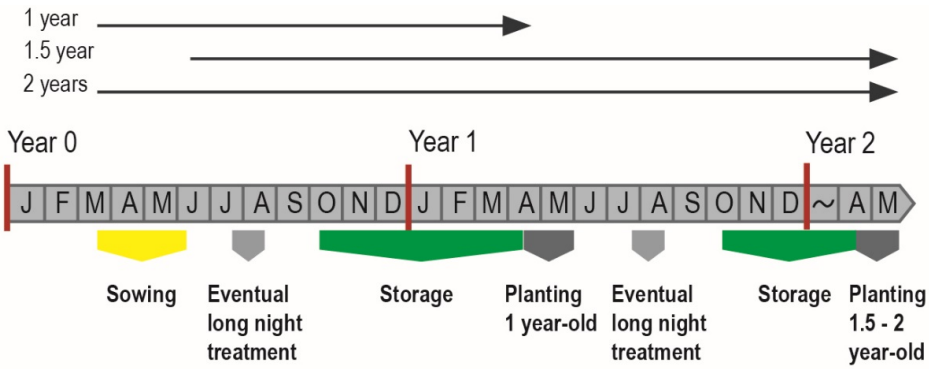

Figure 2. Cultivation scheme of containerized conifer seedlings in Swedish forest nurseries (adapted from [26]).

In each nursery, 100 seedlings of *P. sylvestris* and 100 seedlings of *P. abies* were randomly collected resulting in 900 seedlings for each tree species in total (Table 1). In the laboratory, *ca.* 1/5 of the root system (including fine and lateral roots) of each seedling was excised together with the growth substrate, which was carefully separated from roots. Roots were then washed in tap water to remove the remaining growth substrate and cleaned roots were individually placed in 2 mL centrifugation tubes. The growth substrate, which was mainly composed of a sphagnum peat, was sieved (mesh size 2 × 2 mm) to remove larger particles and any of remaining roots, and individually placed in 50 mL centrifugation tubes. Half of the growth substrate was used to determine pH and concentration of macro- and micronutrients, and the other half was used for DNA isolation and sequencing of fungal communities. Chemical analysis of the growth substrate was carried out at the Agrochemistry laboratory, Lithuanian Research Centre for Agriculture and Forestry, Kaunas, Lithuania. All chemical analyses were done in two replicates.

2.2. DNA Work and Bioinformatics

Prior to isolation of DNA, roots and the growth substrate were freeze-dried at −60 °C for two days. Isolation of DNA, amplification and sequencing were carried out as described by Menkis *et al.* [25]. Briefly, within each tree species and forest nursery, collected roots and the growth substrate were separately pooled together resulting in nine root samples and in nine growth substrate samples for *P. sylvestris*, and in corresponding number of root and the growth substrate samples for *P. abies*. A total of 18 root and 18 growth substrate samples were each homogenized and 1 g dry-weight of roots and 1 g dry-weight of the growth substrate were used for isolation of genomic DNA. Following DNA isolation, concentration of genomic DNA was determined using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Diluted (1–10 ng/μL) genomic DNA samples were amplified separately using the primer pair ITS9 (5′-GAACGCAGCRAAIIGYGA-3′) [28] and ITS4 (5′-xxxxxxxxTCCTCCGCTTATTGATATGC-3′) [29] containing 8-bp sample identification barcodes denoted by x. The concentration of purified PCR products was determined using Quant-iTTM dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA), and an equimolar mix of all PCR products was used for pyrosequencing. Construction of FLX library and pyrosequencing in a 1/4 run was carried out by Macrogen Inc. (Seoul, Korea), utilizing a GS-FLX Titanium 454 system (454 Life Sciences, Branford, CT, USA).

The sequences generated were subjected to quality control and clustering in the SCATA NGS sequencing pipeline (http://scata.mykopat.slu.se). Quality filtering of the sequences included the removal of short sequences (<200 bp), sequences with low read quality, primer dimers and homopolymers, which were collapsed to 3 bp before clustering. Sequences that were missing a tag or primer were excluded. The primer and sample tags were then removed from the sequence, but information on the sequence association with the sample was stored as meta-data. The sequences were then clustered into different taxa using single-linkage clustering based on 98.5% similarity. The most common genotype (real read) for clusters was used to represent each taxon. For clusters containing two sequences, a consensus sequence was produced. The fungal taxa were taxonomically identified using the ribosomal database project RDP pipeline classifier [30]. Sequences showing similarity < 80% to phylum level were considered to be of non-fungal origin and were excluded from further analysis. In addition, 33 most common fungal taxa were identified using GenBank (NCBI) database and the Blastn algorithm [31]. The criteria used for identification at GenBank were: sequence coverage > 80%; similarity to taxon level 98%–100%, similarity to genus level 94%–97%. Sequences not matching these criteria were considered unidentified and were given unique names as shown in Table 2. All non-singleton fungal sequences are available from GenBank under accession numbers KU188523-KU189141.

Table 2. Relative abundance of the 33 most common fungal taxa 454-sequenced from root systems (*roots*) and the sphagnum peat growth substrate (*peat*) of healthy-looking *Pinus sylvestris* and *Picea abies* seedlings from nine forest nurseries in Sweden. The data from different forest nurseries is pooled within each tree species.

Fungal taxa	Phylum	NCBIreference	Similarity, %*	*Pinus sylvestris*		*Picea abies*		All
				Roots	Peat	Roots	Peat	
Unidentified sp. 2588_2	Ascomycota	JX317435	216/216 (100)	41.6	9.1	43.1	16.5	21.0
Thelephora terrestris	Basidiomycota	KP814380	329/331 (99)	15.6	19.5	22.0	13.3	17.1
Cryptococcus terricola	Basidiomycota	HF558655	342/347 (99)	0.7	15.6	0.3	20.4	13.0
Leucosporidium drummii	Basidiomycota	FN908919	323/324 (99)	0.9	15.4	0.5	13.3	10.6
Atradidymella muscivora	Ascomycota	EU817828	266/267 (99)	8.1	7.2	7.5	9.4	8.1
Phialophora sp. 2588_10	Ascomycota	DQ069046	246/247 (99)	5.3	2.1	1.6	2.8	2.8
Umbelopsis isabellina	Mucoromycotina	EU484210	308/309 (99)	0.1	2.4	0.2	3.7	2.2
Trichoderma viride	Ascomycota	KT876533	199/200 (99)	4.0	1.1	4.7	1.3	2.1
Mortierella turficola	Mucoromycotina	JX976040	348/349 (99)	0.4	4.4	0.0	0.6	1.9
Laccaria proxima	Basidiomycota	KM067833	199/199 (100)	0.8	2.2	0.9	2.1	1.8
Rhodotorula fujisanensis	Basidiomycota	HF558662	327/328 (99)	0.3	3.7	0.0	0.1	1.5
Mortierella parvispora	Mortierellomycotina	JX976005	345/347 (99)	0.2	1.8	0.3	1.9	1.4
Oidiodendron rhodogenum	Ascomycota	NR_119425	252/252 (100)	0.3	2.0	0.1	1.4	1.3
Botrytis cinerea	Ascomycota	KR055052	206/206 (100)	2.3	0.4	1.7	0.2	0.8
Cladosporium cladosporioides	Ascomycota	KT151593	260/262 (99)	2.3	0.6	0.6	0.4	0.8
Meliniomyces variabilis	Ascomycota	KP753330	258/259 (99)	0.8	0.0	4.5	0.2	0.7
Tylospora fibrillosa	Basidiomycota	KR019831	306/306 (100)	-	-	2.3	1.0	0.6
Inocybe jacobi	Basidiomycota	HQ604812	290/292 (99)	0.2	0.4	0.0	0.6	0.4
Tylospora asterophora	Basidiomycota	HM190017	305/306 (99)	0.0	0.0	2.1	0.3	0.4
Cryptococcus sp. 2588_18	Basidiomycota	KT372800	337/338 (99)	0.1	0.6	0.0	0.2	0.3
Suillus variegatus	Basidiomycota	JQ753773	276/277 (99)	2.1	0.0	-	0.0	0.3
Rhodotorula colostri	Basidiomycota	JX188225	317/319 (99)	0.1	0.7	0.0	0.0	0.3
Cystofilobasidium capitatum	Basidiomycota	HF558659	337/339 (99)	0.0	0.8	-	0.0	0.3
Fusarium avenaceum	Ascomycota	KT823772	275/276 (99)	0.5	0.3	0.4	0.1	0.3
Wilcoxina mikolae	Ascomycota	LC029799	273/274 (99)	1.6	0.0	0.0	0.0	0.3
Cryptococcus victoriae	Basidiomycota	KP299253	251/252 (99)	0.3	0.4	0.0	0.1	0.3
Unidentified sp. 2588_22	Ascomycota	AM292201	274/274 (100)	0.0	0.2	0.0	0.5	0.2
Pezoloma ericae	Ascomycota	DQ069026	247/249 (99)	0.5	0.2	0.5	0.1	0.2
Amphinema sp. 2588_23	Basidiomycota	JN943911	285/289 (99)	-	0.0	0.6	0.5	0.2
Lecythophora sp.	Ascomycota	KJ542236	263/267 (99)	0.0	0.3	0.0	0.4	0.2
Mortierella elongata	Mortierellomycotina	KP772767	361/364 (99)	0.1	0.4	0.0	0.2	0.2
Hebeloma cavipes	Basidiomycota	EU887517	320/321 (99)	1.3	0.0	-	-	0.2
Trichosporon porosum	Basidiomycota	HG737348	275/276 (99)	0.6	0.1	0.1	0.2	0.2
Total of 33 taxa				90.9	92.1	94.3	91.9	92.1

* Sequence similarity column shows base pairs compared between the query sequence and the reference sequence at NCBI databases, and the percentage of sequence similarity in the parenthesis.

2.3. Statistical Analyses

Differences in relative abundance (estimated as a number of sequences) of dominant fungal taxa detected in roots and in the growth substrate of each *P. sylvestris* and *P. abies* was compared by non-parametric chi-square test [32]. In cases where the datasets were subjected to multiple comparisons, confidence limits for *p*-values of the chi-square tests were reduced a corresponding number of times, as required by the Bonferroni correction [33]. Soil chemical parameters were tested for normality using the Ryan–Joiner test in Minitab v.16.2.4. [34]. Differences in chemical parameters of the growth substrate were compared by one-way analysis of variance (ANOVA) and Tukey's test which provides confidence intervals for all pairwise differences between means [35,36]. Shannon diversity index, quantitative Sorensen similarity index and correspondence analysis (CA) in Canoco 4.5 were used to characterize diversity and composition of fungal communities in different datasets: ecological niches (roots *vs.* the growth substrate), tree species (*P. sylvestris* vs. *P. abies*) and different forest nurseries [32,37,38]. MANOVA was used to evaluate degree of separation (along CA Axes 1 and 2) between the fungal communities in different ecological niches, that is, roots *vs.* the growth substrate.

3. Results

A total of 628,198 sequences was generated by 454-sequencing from 18 root and 18 growth substrate samples representing two tree species and nine forest nurseries (Table 1). However, 458,354 (72.9%) of sequences were of low quality and were excluded from further analysis. Clustering of remaining 169,844 high-quality sequences (279 bp on average) resulted in 839 non-singleton contigs (at 98.5% similarity representing different taxa) and in 2005 singleton contigs, which were excluded from further analysis. Taxonomic identification using an RDP pipeline classifier showed that 619 taxa were of fungal origin and 220 of non-fungal origin, which were excluded. The detected fungi were 49.9% Ascomycota, 37.5% Basidiomycota, 12.3% Zygomycota and 0.3% Chytridiomycota. In *P. sylvestris* (data pooled from all forest nurseries), the richness of fungal taxa was higher in the growth substrate (406 taxa out of 60,795 sequences) than in roots (237 out of 25,044), and the chi-squared test showed that the richness of taxa between these datasets differed significantly ($p < 0.0001$). In *P. abies*, by contrast, the richness of fungal taxa between roots and the growth substrate did not differ significantly ($p > 0.05$). Information on the 33 most common fungal taxa representing 92.1% of all fungal sequences is shown in Table 2. The most common fungi were Unidentified sp. 2588_2 (21.0% of all fungal sequences), *Thelephora terrestris* Ehrh. (17.1%), *Cryptococcus terricola* Pedersen (13.0%), *Leucosporidium drummii* Yurkov, Schäfer & Begerow (10.6%) and *A. muscivora* (8.1%), which at variable abundances occurred in all forest nurseries (Figure 3).

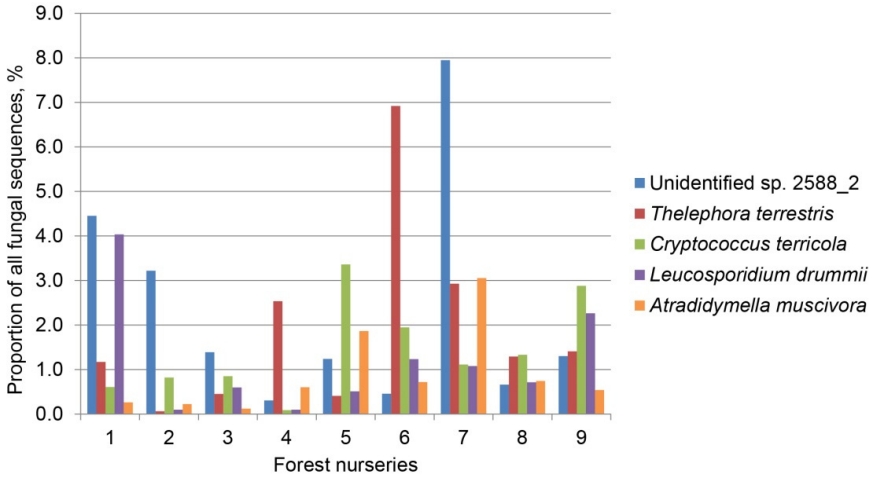

Figure 3. Relative abundance of the five most common fungal taxa (data is pooled within each nursery) in nine forest nurseries of a study. Forest nurseries are numbered as in Table 1.

Among these, in *P. sylvestris*, abundance of Unidentified sp. 2588_2 was significantly higher in roots than in the growth substrate ($p < 0.0001$), abundance of *C. terricola* and *L. drummii* was significantly higher in the growth substrate than in roots ($p < 0.0001$), while it was similar in *T. terrestris* and *A. muscivora*. In *P. abies*, abundance of Unidentified sp. 2588_2 and *T. terrestris* was significantly higher in roots than in the growth substrate ($p < 0.0001$), while in *C. terricola*, *L. drummiii* and *A. muscivora*, it was significantly higher in the growth substrate than in roots ($p < 0.001$). The plant pathogen *Botrytis cinerea* Pers. (0.8%) was the fourteenth most common fungus which at low abundance was detected in both tree species and in both ecological niches, that is, roots and the growth substrate (Table 2). The remaining 586 fungal taxa were rare, and their relative abundances varied between 0.001% and 0.2% (Table S1).

Chemical analysis showed that, among different forest nurseries, only pH differed significantly in the sphagnum peat growth substrate of each *P. sylvestris* and *P. abies* (Table 3). By contrast, concentrations of N, P and K macronutrients in the growth substrate of each tree species were similar among different forest nurseries. Among different forest nurseries, concentrations of Ca and Mg but not of Cl micronutrients varied significantly in the growth substrate of *P. sylvestris*, while concentrations of Cl but not of Ca and Mg varied significantly in the growth substrate of *P. abies.* Pooling values within each tree species from different nurseries showed that N was significantly higher in the sphagnum peat growth substrate of *P. abies* than of *P. sylvestris* ($p < 0.04$), while pH and the remaining macro- and micronutrients did not differ significantly between the tree species.

Table 3. Chemical characteristics of the sphagnum peat growth substrate of *Pinus sylvestris* and *Picea abies* seedlings from nine forest nurseries in Sweden. Values show the mean value ± standard error. Forest nurseries are numbered as in Table 1.

Forest nursery	1	2	3	4	5	6	7	8	9
Pinus sylvestris									
pH	3.5 ± 0.1 cd	3.2 ±0.1 d	3.7 ± 0.00 cd	4.0 ± 0.1 bc	3.9 ± 0.2 c	4.5 ± 0.0 ab	3.9 ± 0.1 c	3.8 ± 0.2 c	4.5 ± 0.1 a
N (mg/L)	0.7 ± 0.1 a	2.3 ± 0.9 a	1.1 ± 0.1 a	1.2 ± 0.1 a	0.7 ± 0.3 a	0.4 ± 0.1 a	0.6 ± 0.1 a	1.2 ± 0.5 a	0.5 ± 0.0 a
P (mg/L)	7.8 ± 2.9 a	5.6 ± 1.4 a	5.5 ± 0.4 a	7.9 ± 2.6 a	8.9 ± 1.1 a	12.1 ± 0.2 a	7.5 ± 0.1 a	7.5 ± 2.1 a	6.4 ± 0.1 a
K (mg/L)	114.5 ± 2.5 a	131.5 ± 10.5 a	115.0 ± 19.0 a	135.0 ± 18.0 a	105.0 ± 3.0 a	91.3 ± 6.6 a	94.6 ± 1.8 a	93.5 ± 12.5 a	88.2 ± 4.4 a
Ca (mg/L)	16.2 ± 1.1 ab	14.3 ± 0.3 b	17.0 ± 1.2 ab	18.4 ± 0.6 ab	23.5 ± 3.2 a	16.5 ± 0.4 ab	19.9 ± 0.4 ab	16.2 ± 0.1 ab	23.9 ± 3.2 a
Mg (mg/L)	5.6 ± 0.3 b	5.2 ± 1.1 b	5.9 ± 0.3 b	7.7 ± 1.2 ab	9.7 ± 0.3 a	5.9 ± 0.1 b	7.7 ± 0.0 ab	5.1 ± 0.0 b	8.6 ± 0.9 ab
Cl (mg/L)	30.8 ± 4.4 a	23.5 ± 2.9 a	17.6 ± 2.9 a	17.6 ± 11.7 a	17.6 ± 2.9 a	20.5 ± 2.9 a	41.1 ± 5.9 a	22.0 ± 4.4 a	29.3 ± 8.8 a
Picea abies									
pH	3.5 ± 0.0 b	3.6 ± 0.1 b	3.4 ± 0.2 b	3.5 ± 0.1 b	3.7 ± 0.1 ab	3.7 ± 0.1 ab	3.8 ± 0.2 ab	3.6 ± 0.1 b	4.5 ± 0.3 a
N (mg/L)	3.0 ± 0.9 a	6.1 ± 2.9 a	2.0 ± 0.9 a	2.4 ± 0.7 a	1.2 ± 0.1 a	0.5 ± 0.0 a	0.4 ± 0.0 a	6.8 ± 4.7 a	0.5 ± 0.0 a
P (mg/L)	9.8 ± 0.6 a	9.9 ± 3.6 a	5.4 ± 1.0 a	6.9 ± 1.5 a	4.7 ± 0.6 a	8.7 ± 1.9 a	6.9 ± 0.5 a	7.8 ± 0.0 a	12.6 ± 1.1 a
K (mg/L)	117.5 ± 2.5 a	129.5 ± 26.5 a	107.5 ± 7.5 a	152.5 ± 18.5 a	88.6 ± 6.0 a	124.5 ± 0.5 a	96.7 ± 2.1 a	99.5 ± 25.5 a	111.0 ± 4.0 a
Ca (mg/L)	14.7 ± 1.9 a	29.6 ± 9.1 a	18.1 ± 4.2 a	15.9 ± 0.4 a	19.9 ± 0.9 a	21.4 ± 5.2 a	20.2 ± 0.1 a	23.3 ± 0.8 a	24.7 ± 3.1 a
Mg (mg/L)	5.1 ± 0.5 a	12.5 ± 4.9 a	5.7 ± 1.3 a	5.1 ± 0.1 a	6.2 ± 0.2 a	7.5 ± 1.7 a	7.6 ± 0.2 a	7.6 ± 0.1 a	8.6 ± 0.2 a
Cl (mg/L)	23.5 ± 2.9 ab	23.5 ± 2.9 ab	13.2 ± 1.5 b	16.2 ± 1.5 ab	24.9 ± 1.5 ab	24.9 ± 4.4 ab	41.0 ± 11.7 a	17.6 ± 2.9 ab	29.3 ± 8.8 ab

Within rows of respective tree species, values followed by the same letter in ANOVA test are not significantly different.

Correspondence analysis of fungal communities explained 22.2% of the variation on Axis 1 and 21.2% on Axis 2. Correspondence analysis showed that fungal communities in roots and in the sphagnum peat growth substrate were separated from each other along a diagonal (Figure 4), thereby showing specificity of fungal communities in each ecological niche. MANOVA showed that separation of fungal communities was significant along Axis 1 ($p < 0.014$) but not along Axis 2.

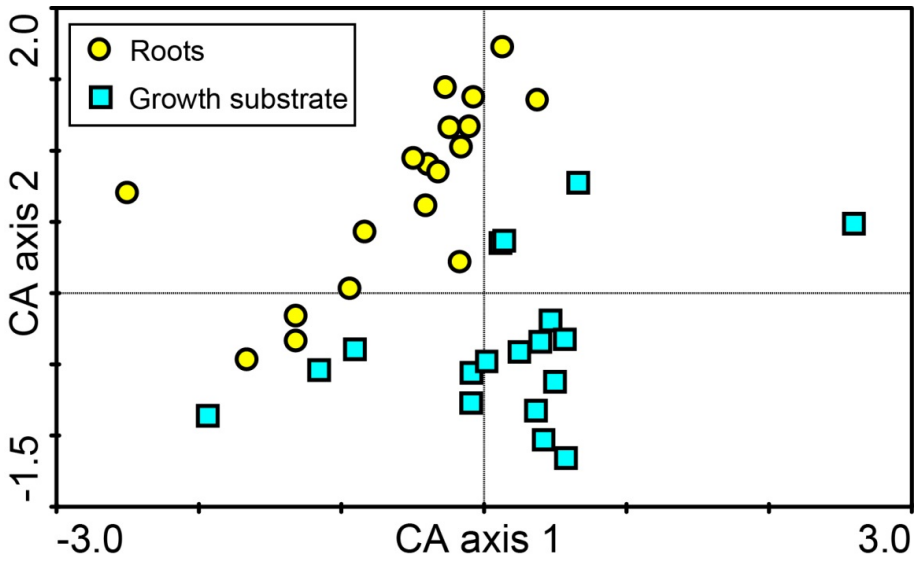

Figure 4. Ordination diagram based on correspondence analysis of fungal communities in roots and in the sphagnum peat growth substrate of *Pinus sylvestris* and *Picea abies* seedlings from nine forest nurseries in Sweden.

In comparison, CA showed that geographical position of forest nurseries (northern, central or southern), tree species (*P. sylvestris vs. P. abies*) and management practices in different forest nurseries had little effect on fungal community structure (data not shown). Sorensen similarity index of fungal communities was moderate (0.54) when compared between roots and the growth substrate, high (0.68) when compared between *P. sylvestris* and *P. abies* and moderate (0.36–0.59) when compared among different forest nurseries. Shannon diversity index of fungal communities was high and ranged between 1.8 and 3.0 in different forest nurseries.

4. Discussion

The study demonstrated that, despite intensive management practices and homogenous growth substrate (sphagnum peat), the rhizospheres in containerized forest nurseries are inhabited by a high diversity of fungal taxa (Table 2 and S1). Moreover, the observed fungal diversity was significantly higher than what was known from forest nurseries before [15,22,39], showing high efficiency of the 454-sequencing method. Combination of the taxonomic information and the abundance data provided important practical information on the relative importance of individual fungal taxa and their potential roles in forest nurseries. Consequently, the fungal community in roots and in the sphagnum peat growth substrate of *P. sylvestris* and *P. abies* seedlings from different forest nurseries were largely composed of saprotrophic, mycorrhizal and endophytic fungi, while pathogens were relatively rare (Table 2 and S1). The latter suggests that management practices in forest nurseries created suitable conditions for the predominant establishment of beneficial fungi but limited plant pathogens. Despite the relatively large geographical separation, fungal communities and the chemical composition of the growth substrate in different forest nurseries were similar (Table 2 and 3, Figure 3) suggesting that management practices in these were not only similar but also of high standard, generally resulting in healthy stock.

Nevertheless, latent establishment of several pathogenic fungi showed that nursery stock and the growth substrate can provide a venue for dissemination of fungal diseases, thereby supporting the hypothesis. Although the intensive control measures in forest nurseries may suppress the development of fungal diseases, these may develop rapidly after seedling replanting, which often predispose seedlings to infection due to replanting stress. Lilja and Rikala [40] showed that *P. sylvestris* and *P. abies* seedlings infected with root pathogenic fungi may exhibit up to 70% reduced survival rates the first year following outplanting, even when disease symptoms in the nursery are absent. Moreover, the symptoms of new diseases in forest nurseries can be undetected due to new nature of these diseases. The latter demonstrates that careful assessment of fungi in forest nurseries is critical even when disease symptoms are absent as this may prevent the spread of fungal diseases and contribute to production of high quality planting stock.

In the present study, fungal communities represented dormant periods, but abundance and composition of these may change throughout the growing season. For example, Voříšková *et al.* [41] demonstrated that, in a temperate oak forest soil saprotrophic genera reached their seasonal maxima in autumn, while mycorrhizal taxa dominated in summer, suggesting that decomposition of organic matter and phytosynthate allocation represent important factors contributing to the community variations during the growing season. Despite the high fungal diversity, fungal community was dominated by a limited number of fungal taxa (Table 2) many of which were reported from forest nurseries before [14,15,22,39,42]. Therefore, our results corroborate previous observations and demonstrate specificity of fungal communities in forest nurseries.

In the present study, Unidentified sp. 2588_2 dominated the fungal community, showing higher abundances in roots of both tree species than in corresponding samples of the growth substrate (Table 2). Although the taxonomic affiliation for this fungus could not be reliably established, the fungus appears to belong to order Helotiales which is one of the largest orders in kingdom fungi and mainly includes saprotrophs but also includes some pathogens, endophytes and mycorrhizal fungi [43]. Predominant occurrence of Unidentified sp. 2588_2 in healthy-looking roots may suggest that this fungus is most likely not pathogenic. *T. terrestris*, the second most common fungus of this study (Table 2), is known as one of the most common mycorrhizal fungi in forest nurseries worldwide [17]. While our results are in agreement with previous observations, they also revealed that *T. terrestris* occurred at similar abundances in both roots and the growth substrate, suggesting not only the presence but also high activity in the latter ecological niche. Despite *T. terrestris* being well adapted to environmental conditions present in forest nurseries [14], it often fails to support seedling growth after outplanting and is gradually replaced by indigenous mycorrhizal fungi [44]. Among other dominant fungi, both *C. terricola* and *L. drummii* were more abundant in the growth substrate than in roots (Table 2), which reflected on their saprotrophic lifestyle and reportedly common isolation from the soil [45,46].

A. muscivora was also among the most common fungi found in all nurseries, on both tree species, and in both ecological niches: roots and the growth substrate (Table 2). Although this fungus was recently described as a pathogen of bryophytes [47], its presence in roots of nursery-grown conifer seedlings was suggested to be latent [22]. However, pathogenicity tests using several *A. muscivora* cultures isolated from *P. sylvestris* and *P. abies* seedlings [22] demonstrated that different isolates can cause variable degree of pathogenic colonisation, necrosis and eventually dieback of stressed conifer seedlings (unpublished). The latter suggests that *A. muscivora* may potentially have a negative impact on seedling survival and establishment after outplanting as this often stresses the plants. Although the route of *A. muscivora* arrival in forest nurseries is not known, the possibility should not be excluded that it is disseminated with the sphagnum peat growth substrate. As *A. muscivora* is originally described as a pathogen of bryophytes [47], the sphagnum peat infected with the propagules of *A. muscivora* might provide a venue for dissemination of this fungal species. The other pathogenic fungi, including *B. cinerea* Pers. and *Fusarium avenaceum* Cook, are mainly known as facultative parasites, which usually cause the disease to predisposed plants [13,48]. Although *B. cinerea* can be

a major threat to aboveground parts and cause grey mould disease to containerized conifer seedlings in forest nurseries [48], its detection in roots is likely to be a latent form. While richness of pathogenic fungi such as *B. cinerea* and *F. avenaceum* was relatively low, a number of beneficial mycorrhizal taxa were detected (Table 2). In addition to *T. terrestris*, mycorrhizal fungi included *Laccaria proxima* (Boud.) Pat., *Tylospora fibrillosa* (Burt) Donk, *Inocybe jacobi* Kühner, *Tylospora asterophora* (Bonord.) Donk, *Suillus variegatus* (Sw.) Kuntze, *Wilcoxina mikolae* (Yang and Wilcox) Yang and Korf and *Hebeloma cavipes* Huijsman (Table 2), thereby repeatedly demonstrating the suitability of management practices for the establishment of diverse mycorrhizal fungi in forest nurseries.

Although the quantitative Sorensen similarity index showed that the number of fungal taxa was moderately similar in roots and in the growth substrate, CA analysis, which also incorporated the abundance data, revealed a clear separation of fungal communities between these two ecological niches (Figure 4). The latter separation appears to be largely determined by lifestyles of different fungi as mycorrhizal, endophytic and pathogenic fungi were often more abundant in roots while saprotrophs in the growth substrate (Table 2 and S1). However, the abilities of mycorrhizal fungi to utilize a range of different organic nutrient sources was reported previously [49], which may also explain the presence and possibly enzymatic activity of a number of mycorrhizal fungi in the growth substrate. Moreover, the capture and transfer of nutrients from saprotrophic mycelia to mycorrhizal fungi and their host plants [50] may further suggest active resource competition in rhizospheres, which likely contributes to improved nutrition and growth of seedlings in forest nurseries.

5. Conclusions

The study demonstrated that seedling production in forest nurseries generally give a healthy stock. Nevertheless, latent establishment of several pathogenic fungi supported the hypothesis that nursery stock and the growth substrate can provide a venue for dissemination of fungal diseases. Seedling roots and the growth substrates were inhabited by diverse communities of fungi, and different lifestyles of these fungi largely determined community segregation into particular ecological niches.

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