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The development of foliar fungal communities of nursery-grown *Pinus sylvestris* seedlings

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ABSTRACT

In forest nurseries, foliar fungi play a key role in the health of tree seedlings. The aim of this study was to study the diversity and the development of foliar fungal species associated with nursery-grown *Pinus sylvestris*, and to evaluate the effect of two biological control products and two growth-stimulating products on seedling growth and disease control, as well as seedling associated fungal community. The study was conducted at four Swedish forest nurseries. Fungal communities were assessed from non-symptomatic needles using high-throughput sequencing of the ITS2 rRNA region. The fungal pathogens *Cladosporium* sp. (15.1%), *Phoma herbarum* (14.5%), and *Alternaria alternata* (5.5%) were among the most abundant fungi. Results showed that the nurseries and the development of fungal communities influenced the occurrence of dominant fungal taxa. Disease prevalence was low and microbial treatments had no significant impact on seedling growth and survival, nor on the number of fungal operational taxonomic units (OTUs), species diversity, and species evenness ($p > 0.05$). In conclusion, the results showed a dynamic change in foliar fungal community structure over the growing season. With appropriate nursery management strategies and under suitable climatic conditions, nursery seedlings can remain healthy even in the presence of fungal pathogens.

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Botrytis cinerea; *Cladosporium* spp.; high-throughput sequencing; forest nursery; fungal pathogens; *Phoma herbarum*; scots pine needles

Introduction

In Sweden, the annual production of forest seedlings has reached 450 million seedlings, constituted primarily of *Pinus sylvestris* and *Picea abies* (Fürst 2022). Forest nursery production faces several disease challenges, e.g. the potential establishment of diseases and their rapid spread within nurseries, the spread of fungal pathogens between nurseries, and the spread of fungal pathogens into the forest system (Lilja et al. 2010). Several diseases are of economic importance as they cause significant losses in forest nurseries, for instance grey mould, caused by *Botrytis cinerea*, or damping-off and root dieback diseases among very young seedlings (Capieau et al. 2004; Lilja et al. 2010). Increased knowledge of foliar fungal communities could improve pest management in forest nurseries, for example by improving the prediction of disease outbreaks and early detection of fungal infections.

Foliar fungal communities are complex and diverse as they include a variety of epiphytic (on the surface of the host tissue) and endophytic (within the host tissue) fungal species. These species play a vital role in plant health as they can include both harmful plant pathogens and/or beneficial symbionts (Inacio et al. 2002; Sieber 2007; Cordier et al. 2012). The activity and function of these fungi are often influenced by both biotic and abiotic factors e.g. host species (Lebeis 2015), site characteristics (Wurth et al. 2019), or latitudinal gradients (Millberg et al. 2015). Moreover, the abundance and composition of fungal communities are influenced by the host characteristics, which can lead to a variation in fungal community structure within individual hosts, e.g. owing the variation in needle age within conifers (Hata et al. 1998; Terhonen et al. 2011; Wurth et al. 2019).

In the Nordic countries, forests are dominated by coniferous tree species, mainly *P. sylvestris* and *P. abies* (Ekström and Hannerz 2021). Coniferous tree species often host a high diversity of foliar fungal communities, which are dominated by a few host-specific species (Sieber 2007). Coniferous seedlings from natural systems have also been observed to host a high diversity of foliar fungi (Oono et al. 2015). However, the structure and composition of the foliar fungal community in forest nursery stocks are still poorly studied, whereas studies on root-associated fungal communities in forest nurseries are more common (Stenström et al. 2014; Menkis et al. 2016; Okorski et al. 2019).

The production of *P. sylvestris* seedlings in Sweden has increased progressively over the last decade (Fürst 2022). Seedlings are typically grown at high density using containerised cultivation system (multi-cell growing trays) and aseptic peat substrate (Lilja et al. 2010; Menkis et al. 2016). The intensive and highly advanced production procedure

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exposes the seedlings to conditions that may stress the seedlings and create favourable conditions for fungal pathogens, e.g. different environmental conditions (from greenhouses to outdoor cultivation), high moisture (automatic irrigation systems from above), rigorous fertilisation, repeated fungicide treatments and sparse herbicide treatments. Today, fungal infections are prevented by cultural control measures (good hygiene, clean equipment, removal of infected seedlings, reduced humidity by mechanical removal of water droplets from needles) as a part an integrated pest management (IPM) strategy. The use of biological control, as an alternative to chemical control, has not yet been fully implemented in Swedish forest nurseries, and fungicides are normally used to prevent or cure fungal diseases.

Biological control is commonly based on a living microorganism(s) that can suppress disease development (Stenberg et al. 2021). Several microorganisms (e.g. *Trichoderma* spp., *Clonostachys rosea*, and *Bacillus subtilis*) are used as biological control agents (BCAs) due to their complex abilities to control pathogens through different mechanisms, including competition, antagonism, and mycoparasitism, as well as their ability to modify the rhizosphere, increase plant growth, and stimulate plant defence mechanisms (Benitez et al. 2004; Fravel 2005). However, due to the complex role of BCAs, the result of disease control can be very unpredictable and include unknown negative effects on non-target species and beneficial species (Prospero et al. 2021). Several commercial microbial products are available on the market, but their performance and efficacy under forest nursery conditions is still largely unknown.

The aim was to study the diversity, composition, and dynamics of foliar fungal communities associated with *P. sylvestris* seedlings in four containerised Swedish forest nurseries with a focus on fungal pathogens. Four commercial microbial products were tested on seedling growth, survival, and disease incidence, as well as their effects on fungal communities. We hypothesised that *P. sylvestris* seedlings host a high diversity of foliar fungi, and that aging of seedlings during the growing season will impact the overall fungal community structure towards higher diversity at the end of the season. In addition, we hypothesised that seedlings subjected to microbial treatments will have a higher growth rate and lower disease incidence, and microbial additives will alter fungal community composition by inhibiting pathogenic species (e.g. *B. cinerea*) or promoting other fungal species.

Materials and methods

Study sites, treatments, and sampling

The study was conducted in four forest nurseries: Kilåmon, Stakheden, Lugnet and Trekanten, all belonging to the company Svenska Skogsplantor. The nurseries are located in geographically different regions of Sweden and are therefore exposed to different environmental conditions (Figure 1, Table 1) (Ahti et al. 1968). The surroundings of the nurseries vary from a combination of agricultural land and mixed deciduous forests (Trekanten), or mixed *P. abies*/*P.sylvestris* forests (Lugnet) to *P. sylvestris* dominated forests in the north (Kilåmon and Stakheden) (Table 1). Each forest nursery has its own production system in terms of design of cultivation trays, and the seedlings in the study were of different sizes and origins. The number of trays included in the study was chosen to provide a representative number of seedlings from each nursery and treatment (Table 1), and a total of 33,683 seedlings were included in the study. Containerised *P. sylvestris* seedlings were sown in March 2019 and transferred outdoors when they were approximately ten weeks old.

Four products with microbial additives were used in the study. Two biological control products: Prestop® (10^7–10^9 cfu/g of *C. roseae* strain J1446; Verdera 2019) and Serenade ASO® (13.96 g/l *Bacillus amyloliquefaciens* QST 713; Bayer 2021) and two growth stimulats: Binab® (>10^7 cfu/g of *T. polysporum* and *T. atroviride*; Binab Bio-Innovation 2020) and Mikroferm® (containing a mixture of *Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, *Lactobacillus plantarum*, *L. casei*, *Saccharomyecs cerevisiae*; Agriton Sverige 2018). Three of the products (Binab®, Prestop®, Serenade ASO®) have been developed and evaluated for forest nursery conditions and are marketed to producers of forest seedlings. For this study, the products were diluted to 1000 ml solutions with following proportions: Prestop® (0.25 g/m^2), Serenade ASO® (0.4 ml/m^2), Binab® (0.2 g/m^2), and Mikroferm® (10 ml/m^2), and sprayed manually. Prestop® and Serenade® solutions were supplemented with 0.5 ml of 0.025–0.05% Silwet Gold® to improve dispersion of the treatment according to the manufacturer's instructions. Seedlings used as negative controls were treated with tap water. In addition, a set of reference seedlings (positive control) followed the ordinary nursery production protocol, including both fungicidal treatments and microbial treatments (Table 1, Table S1). Seedlings subjected to the different microbial treatments and negative control seedlings were all removed from the field before each application according to the nursery protocol. Treated seedlings were placed in single blocks and separated by a buffer zone consisted of untreated seedlings to avoid any possible contamination between treatments (Figure 2). A sample of 13 ml of each product solution (including the negative control) was collected prior to treatment application and stored at −18°C until further analysis.

Sampling and treatment were carried out during one growing season, i.e. the outdoor period from late May to mid-November. Growth measurements were taken at the beginning and end of the experiment. The height of ten randomly selected seedlings from five randomly selected trays from each treatment was measured using a folding rule on both occasions ($n = 50$ per treatment and time point). In addition, the diameter at the base of the stem was measured using a calliper at the end of the experiment. Survival was estimated by counting the total number of seedlings at the start of the experiment and then subtracting the remaining number of seedlings at the end of the experiment, together with any seedlings that had died or showed symptoms of disease and were therefore removed during the season. For the analysis of foliar fungal communities, regular sampling of non-symptomatic needles was performed prior to treatment application, which occurred every third week (Figure 2). Sampled needles were fully elongated and collected from the middle part of the stem and up to the top of the shoot. Three randomly

Figure 1. Map of Sweden (indicated as the shaded area in Northern Europe) where sampling of non-symptomatic *Pinus sylvestris*-needles were carried out in four forest nurseries: Kilåmon, Stakheden, Lugnet and Trekanten.

selected trays were sampled from each treatment (i.e. three replicates per treatment). Each tray gave one sample of two needles from each of five randomly selected seedlings (i.e. ten needles per sample). Needles were sampled into 15 ml Falcon tubes using disposable tweezers and gloves, and these were changed between trays. Collected samples were stored frozen at −18°C prior to further analysis.

Biomass measurements

Five seedlings per treatment were collected at the end of the study and stored at −18°C for seedling biomass measurements. Seedlings were thawed and the root plug placed in water overnight to remove the growth substrate. The root system was then carefully cleaned from the growth substrate under tap water and separated from the shoot at the base of the stem. The shoot and root system were placed separately in paper bags and dried at 60°C for 7 days before dry weight was measured using a precision scale (Precisa Gravimetrics AG, Diektikon, Switzerland).

DNA extraction, amplification, and sequencing

The total DNA was extracted from 378 samples: 4 nurseries × 6 treatments × 5 time points × 3 replicates, plus an additional time point added to Kilåmon nursery: 6 treatments × 3 replicates. No surface sterilisation was carried out. All samples were freeze-dried at −85°C for 72 h using a ScanVac freeze drier (Labogene, Lillerød, Denmark). Lyophilised samples were crushed using 5.5 mm metal screw nuts in 15 ml Falcon tubes, and samples up to 73 mg dry weight were homogenised in a high-speed homogeniser machine (Bertin instruments, Montigny-le-Bretonneux, France). The extraction procedure followed the protocols of the NucleoSpin®Plant kit (Macherey-Nagel, Düren, Germany). DNA concentration in each sample was determined using a NanoDrop™ One spectrophotometer (Thermo Scientific, Rodchester, NY, USA). The ITS2 rRNA region was amplified using the fungal-specific forward primer fITS7 (Ihrmark et al. 2012) and the reverse universal primer ITS4 (White et al. 1990), both with 8 bp unique sample identification tags attached to each primer. PCR was performed in 50 µl reactions in duplicates, using DNA extracts diluted to 0.5 ng/µl. Each reaction included primers at 500 nM (fITS7) and 300 nM (ITS4), 1xRB-buffer, 0.2 mM dNTP, 2.75 mM $MgCl_2$, 0.025 unit/µl DreamTaq Green Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The PCR cycling programme was set as follows: 5 min at 95°C, followed by 26–35 cycles of 30s at 95°C, annealing at 57°C for 30s and 30s at 72°C, final extension at 72°C for 7 min. Final PCR products were analysed using gel electrophoresis on 1% agarose gels

Table 1. Four Swedish forest nurseries where foliar fungal community associated with non-symptomatic Pinus sylvestris-needles were investigated after treatment of microbial additives using direct sequencing methods.

				Experimental setup in present study						
Forest Nursery	Location and vegetation zones	Description of nursery location	Yearly production	Seedlings	Growing trays	Growing trays per treatment	Cell volume (cm^3)	Seedlings per m^2	Reference seedlings	Fungal sequences
Kilåmon	63°28.9906′N, 16°42.3242′E; middle boreal zone	Dominated by *Pinus sylvestris*-forests, occurrence *of Picea abies* and broadleaved tree species.	ca. 50 million	7981	48	6	30	1322	Binab® Serenade®	216,344
Stakheden	60°16.7138′N, 14°57.7879′E; southern boreal zone	Mix of *Pinus sylvestris*-forests and fields, occurrence of *Picea abies* and broadleaved tree species.	ca. 30 million	9195	48	6	48	840	Amistar® Cantus® Teldor® Tilt®	124,921
Lugnet	59°37.9045′N, 17°30.8673′E; hemiboreal zone	Mix of *Picea abies*/*Pinus sylvestris*-forests and fields, occurrence of broadleaved tree species.	15–20 million	8407	108	18	90	561	Amistar® Cantus® Frupica® Geoxe® Teldor® Signum® Switch® Teldor® Tilt®	143,052
Trekanten	56°42.0871′N, 16°7.4627′E; hemiboreal zone	Dominated by fields, occurrence of *Pinus sylvestris*, *Larix* spp., and broadleaved tree species.	15–17 million	8100	180	30	90	547	Amistar® Binab® Cantus® Signum® Switch® Tilt®	135,621

Note: Products applied on reference seedlings are listed in the table.

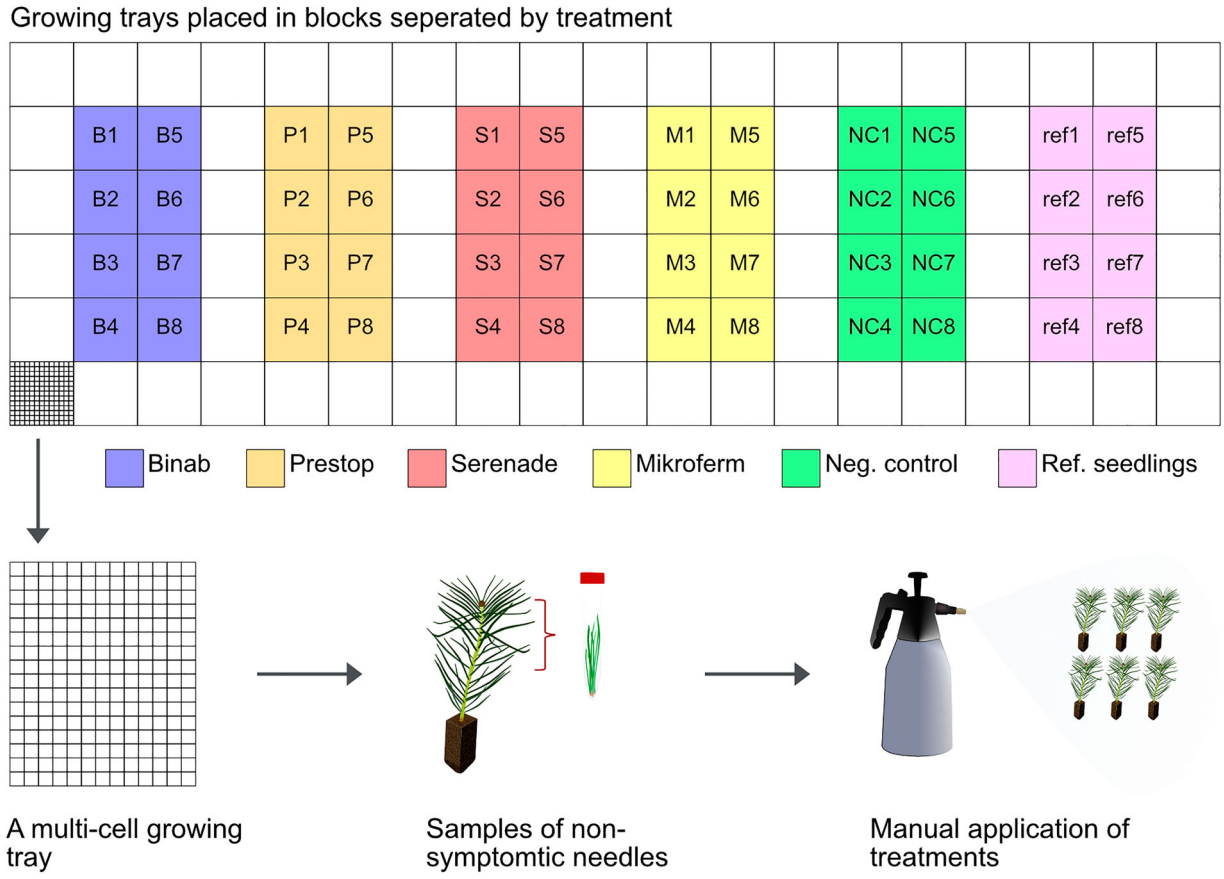

Figure 2. Experimental setup with example from Stakheden nursery. Growing trays of each treatment were placed in blocks with a fixed order and separated by a buffer of non-treated trays of seedlings. Randomised sampling of non-symptomatic needles were followed by manual application of treatments every third week.

stained with Nancy-520 (Sigma-Aldrich, Stockholm, Sweden). The number of PCR cycles was optimised to reduce bias by re-running PCR cycles until the minimum number of cycles required to obtain a visible band on the gel was achieved (Castano et al. 2020). PCR products were cleaned using the AMPure kit (Beckman Coulter, Indianapolis, IN, USA) and DNA concentration was quantified using a Quantus Fluorometer (Promega Biotech, Madison, WI, USA). An equivalent molar-mixture of purified PCR products was pooled into four pools and purified using the E.Z.N.A. Omega cycle pure kit (Omega Biotek, Norcross, GA, USA). Four samples did not contain sufficient PCR products and were excluded from the pools. Amplicon quality and size distribution were controlled using the BioAnalyser DNA 7500 (Alignment Technologies, Boulder, CO, USA), and pooled libraries were sequenced on the PacBio RSII platform using four SMRT cells from SciLifeLab NGI (Uppsala, Sweden).

The product solution samples (collected prior treatment application) were analysed to verify the included microorganisms. Twenty product solutions (4 nurseries × 4 product solutions + 4 negative controls) were collected in 15 ml Falcon tubes and DNA was extracted from a pellet obtained after centrifugation of the samples at 6,000 ppm for 30 min. DNA extraction, amplification, and sequencing followed the same procedure as described for needle samples. In addition, Prestop® product solutions were further verified using the CRnA (TTTCTCGGCCTTTGTCCACTAACG) and CRnB (CGCCCCGCCCCCATTCTA) primer-pair for amplification of a 124 bp region of the *C. rosea* IK726 genome (Alvarez Nordström 2014; J. Wang 2012). The PCR cycling programme followed the same settings as for needles, except that an annealing temperature of 60°C was used. The final PCR product was confirmed on a 1.5% electrophoresis gel and a positive control from *C. rosea* was used as a reference.

Bioinformatics

Sequences were filtered for quality and clustered using the SCATA pipeline (Brandstrom-Durling et al. 2011). Sequences with low read quality, sequences too short (<200 bp) and primer dimers were removed by filtering, as were sequences missing a primer or a sample tag. Homopolymers of the sequences were collapsed down to 3 base pairs (bp) before clustering into different operational taxonomic units (OTUs) by single linkage clustering with a minimum of 98.5% similarity to provide a good compromise between intraspecific variation, variance between closely related species, and sequencing errors (Tedersoo et al. 2022). Two needle samples and two microbial product samples did not contain high quality sequences and were lost after filtering. Fungal OTUs were taxonomically classified using a Ribosomal Database Project (RDP) pipeline classifier (Q. Wang et al. 2007), and sequences with less than 80% similarity at the phylum level were considered non-fungal and not included

in further analysis. The final dataset (1718 fungal OTUs) was taxonomically identified through the PROTAX-fungi and massBLASTer (UNITE/INSD fungi) databases using the PlutoF biodiversity platform (Abarenkov et al. 2010). Species hypotheses were assigned to each fungal OTU using SH Matching (v2.0.0). Fungal taxonomies were assigned by manual comparison of the output files for fungal OTUs represented by >5 sequences. Identification criteria were set at minimum >80% sequence coverage, with >94% similarity for genus level identification, and >98% similarity for taxon-level identification (Stenström et al. 2014; Menkis et al. 2016). Fungal OTUs with high similarity to multiple species were assigned to a shared genus. In addition, the 80 most common fungal OTUs were manually taxonomically identified through the GenBank (NCBI) database using the Blastn algorithm. Fungal OTUs containing sequences that did not meet these criteria or fungal OTUs represented by <5 sequences were considered as unidentified and were given unique names. Fungal OTUs are available from the GenBank database under accession numbers ON749862–ON751563.

Statistical analysis

All statistical analyses were performed using R v 4.2.0 and RStudio (Posit team 2022; R Core Team 2022). No statistical comparisons were made between nurseries due to differences in production systems, seedling size, and seed origin. A linear mixed-effects model was built to assess the effects of treatments on seedling growth using the R package lme4 (Bates et al. 2015). Growth rate ($height_{end}$ − $mean\ height_{start}$) and diameter were used as response variables, respectively, whereas treatment was used as a fixed effect and cultivation tray as a random effect. General linear models were used to assess the effect of treatments on seedling biomass (root and shoot dry weight, data adjusted with sample rank transformation to improve the distribution of residuals within the models) and generalised linear models to assess the effect of treatments on seedling survival (data adjusted for overdispersion with a binomial distribution).

Rarefaction curves were constructed for each nursery to estimate the amplification depth of samples using the R package vegan (Oksanen et al. 2022). Rarefied datasets were constructed by taking random subsamples from each sample, where each subsample was of the same size as the smallest sample in the original dataset (≥ 90 reads) of each nursery. Shannon diversity index, Simpson's evenness index, and the number of fungal OTUs observed between treatments and time points were analysed using rarefied datasets because of uneven amplification depth between samples. General linear models were used to assess the effect of treatments, time points, and their interactions (data adjusted using a sample rank transformation to improve the distribution of residuals within the models). Differences were analysed using pairwise comparisons of estimated marginal means in the R package emmeans v 1.7.4.1 (Lenth 2022).

An ordination diagram of the fungal communities was constructed using a nonmetric multidimensional scaling (NMDS) analysis based on the Bray–Curtis dissimilarity matrix and relative fungal OTU abundances. The number of dimensions was selected to be k = 3, which gave a stress value of 0.149, but the stress value was no longer improved by adding more dimensions. A permutational multivariate analysis of variance (PERMANOVA) on the Bray–Curtis dissimilarity matrix with 999 permutations was used to determine fungal community dissimilarities. The effect of treatments, time points, and any interactions between them was tested on non-rarefied datasets, adjusted using a Hellinger transformation. Differences were analysed using pairwise comparisons in the R package pairwise Adonis v 0.4 (Martinez Arbizu 2017), and *p*-values were adjusted using the Bonferroni correction. Variability in species composition between treatments and sampling time points was assessed through the analyses of multivariate homogeneity of group dispersion using a permutational analysis of multivariate dispersion (Anderson et al. 2006). Differences in distance to the centroid between groups were analysed using a permutation test and compared using TukeyHSD.

Primary lifestyles were assigned to fungal OTUs identified at the genus level and represented by >5 sequences (742 OTUs) using the FungalTraits database (Polme et al. 2020). The output of FungalTraits assigned 17 different primary lifestyles to the whole dataset, and the lifestyles were further grouped into the following categories: Ectomycorrhizal, Endophyte (foliar/root), Epiphyte, Lichenised, Mycoparasite, Parasite (animal/lichen), Plant pathogen, Saprotroph (dung/litter/nectar/soil/sooty mould/wood/unspecified), and Unidentified. Primary lifestyle categories with less than 3% representation each (Endophyte, Epiphyte, Lichenized, Parasite) were further categorised into "Others". Relative abundances were calculated for primary lifestyle categories based on the non-rarefied datasets and visualised per time point using the R packages reshape2 and ggplot2 for each nursery, respectively (Wickham 2007, 2016). Relative abundances were also calculated for the 19 most abundant fungal OTUs, based on the non-rarefied datasets, and visualised per treatment and time point for each nursery. The remaining number of fungal OTUs after the 19 most abundant fungal OTUs (i.e. 1,699 OTUs) were grouped as "Others".

Results

Survival and growth of P. sylvestris seedlings

Almost all *P. sylvestris* seedlings from all four forest nurseries in the study, including the negative control, showed no symptoms of fungal infection throughout the study period. Seedling survival was generally high in all nurseries (>97.9 ± 3.1%), and microbial treatments were not found to have a clear effect on the number of dying seedlings ($p > 0.05$, Figure S2), which was generally low (<1.9 ± 2.8%). Of a total of 67 diseased seedlings collected during the study, 57 seedlings were infected by *B. cinerea*, four seedlings by *Sydowia polyspora*, two seedlings by *P. herbarum*, and one seedling was found to be infected by *Diplodia sapinea* (Larsson et al. 2021). The seedlings grew well during the study period and the microbial treatments had no significant ($p > 0.05$) effect (neither positive nor negative) on seedling growth or shoot and root biomass (Figures S3-S4).

Characteristics of the foliar fungal community composition

In total, 687,824 (56.0%) high-quality sequences were generated from 372 needle samples using the PacBio platform, while 540,366 (44.0%) low-quality sequences were excluded. The high-quality sequences were clustered into 3764 global clusters (OTUs) and 4,629 singletons, and the singletons were excluded from further analysis. The final dataset included 1718 fungal OTUs (after excluding 2046 "non-fungal" OTUs), represented by 619,938 sequences across 372 samples (Table S2). Rarefaction curves showing the relationship between the cumulative number of fungal OTUs and the number of sequences for each sample were constructed for each nursery, respectively (Figure S1). The total number of fungal OTUs (1718) belonged to four different phyla: 54.2% Ascomycota, 44.7% Basidiomycota, 1.0% Mucoromycota and 0.1% Chytridiomycota. The fungal OTUs represented by >5 reads (1073) were identified at different taxonomic levels; 29.5% species, 39.7% genus, 6.9% family, 12.2% order, 2.0% class and 9.8% phylum. Stakheden nursery had the highest total number of fungal OTUs (923), followed by Kilåmon nursery (816), Lugnet nursery (725) and Trekanten nursery (583) (Figure 3). A total of 178 fungal OTUs were found in all four nurseries, with Kilåmon nursery having the highest number of unique OTUs (317), and Trekanten nursery having the lowest (130) (Figure 3). Among the fungal OTUs, active fungal BCAs were identified and verified in the applied product solutions (Table S2). As two samples were lost after clustering, an additional test for Prestop® product solutions further verified the active BCA.

A nonmetric multidimensional scaling (NMDS) of all samples showed an overall large overlap between samples (Figure 4). A clear but overlapping separation of fungal communities on *P. sylvestris* needles over time was found in each nursery (Figure 4). Communities were separated between different time points, which was reflected as a gradient along the NMDS axis 1 in Stakheden ($R2 = 0.619$, $p < 0.001$) or along the NMDS axis 2 in Kilåmon ($R2 = 0.614$, $p < 0.001$), Lugnet ($R2 = 0.635$, $p < 0.001$) and Trekanten ($R2 = 0.632$, $p < 0.001$). An exception to this was between the fourth and fifth time point in Stakheden ($R2 = 0.091$, $p = 0.028$, p (adjusted) = 0.28) and Lugnet nurseries ($R2 = 0.079$, $p = 0.024$, p (adj) = 0.24). However, microbial treatments were not found to impact the separation of fungal communities in any of the nurseries ($p > 0.05$). In addition, the fungal community composition within treatments changed over time, which was reflected as an altered distance to the centroid (Figure 5(a). At Kilåmon nursery, the distance to the centroid decreased at the beginning of September ($p < 0.001$)), while at Stakheden nursery ($p < 0.001$) and Lugnet nursery ($p < 0.001$) the distance decreased at the end of August and increased again in the end of the season. At Trekanten nursery, the decrease in the distance to the centroid occurred in August ($p < 0.01$). The treatments had no effect on the distance to the centroid (Figure 5(b), $p > 0.05$).

Foliar fungal OTU richness and species diversity

The richness, diversity, and evenness of fungal OTUs changed over time (Figure 6, Table 2). At Kilåmon nursery, the number of fungal OTUs fluctuated over the season and significantly increased and decreased between each time point ($p < 0.05$), except between the last two observations ($p = 0.287$). Stakheden nursery showed a similar fluctuating trend as Kilåmon nursery, with the highest number of fungal OTUs in July and October ($p < 0.001$). Lugnet nursery had the highest number of fungal OTUs in July ($p < 0.05$), while Trekanten nursery had the highest number in May ($p < 0.001$). Shannon diversity index and Simpson's evenness index showed similar patterns to fungal OTU richness, but with less differences between time points (Figure 6). Microbial treatments were not found to significantly impact fungal OTU richness, diversity, or evenness ($p > 0.05$) (Table 2).

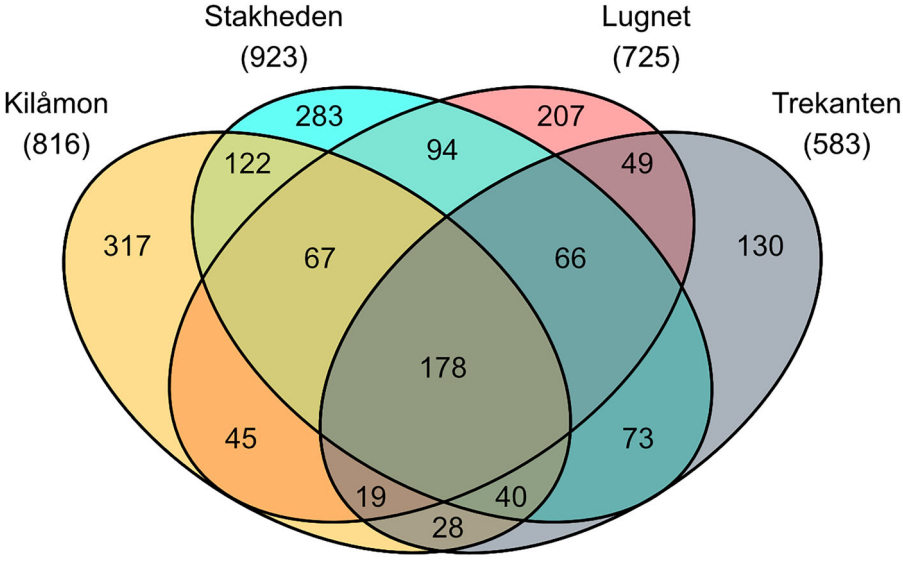

Figure 3. Venndiagram over number of observed fungal OTUs at each nursery (non-rarefied data set). The number of shared and unique fungal OTUs are presented within the ellipses and the total number of fungal OTU from each forest nursery (Kilåmon, Stakheden, Lugnet, Trekanten) is presented within parentheses. The total number of unique fungal OTU found in the study was 1718.

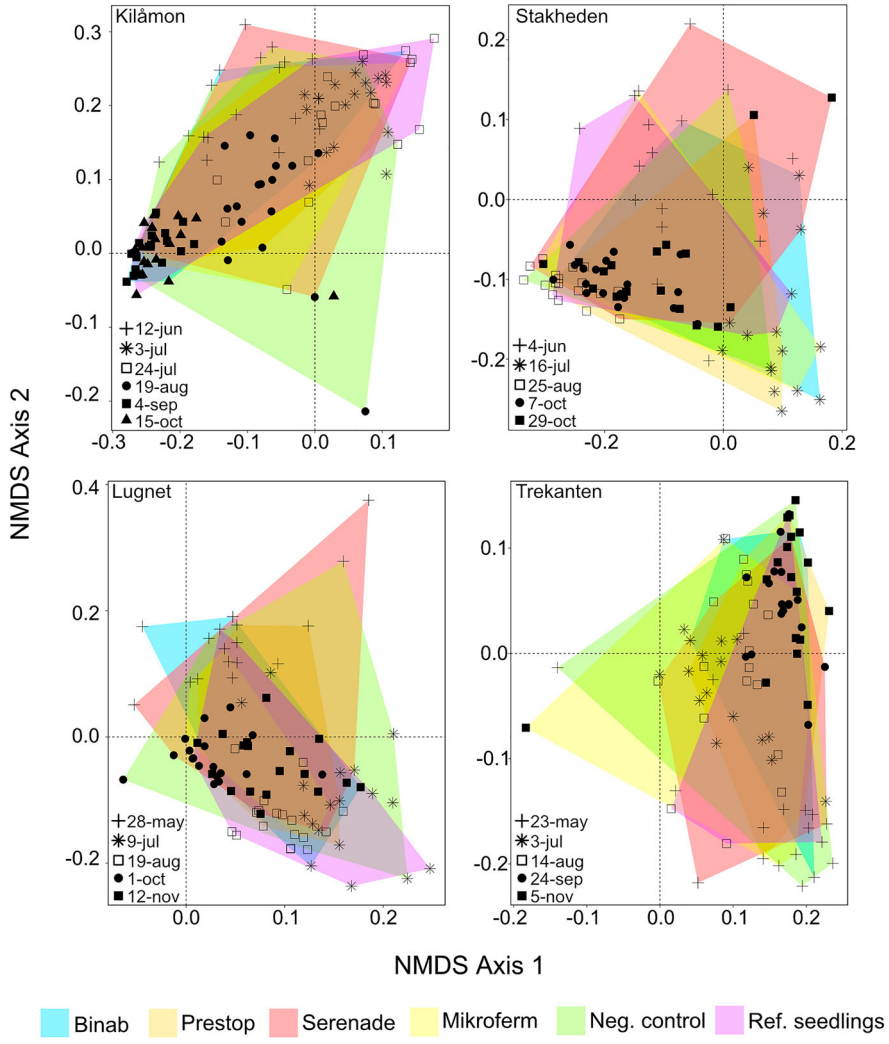

Figure 4. Nonmetric multidimensional scaling (NMDS) of the foliar fungal communities on non-symptomatic *Pinus sylvestris*-needles from four forest nurseries (Kilåmon, Stakheden, Lugnet and Trekanten). Plots are based on Bray-Curtis dissimilarities (no. dimensions = 3, stress value = 0.149). Symbols represent communities in individual sampling time-points (+ first, * second, □ third, • fourth, ■ fifth, and ▲ sixth) and communities connected to same treatment are colour coded. Degree of separation was significant different between each time point, except between fourth and fifth time points in Stakheden and Lugnet nurseries (permanova, $p<0.001$).

Primary lifestyles of foliar fungi in forest nurseries

The relative abundances of fungal OTUs grouped into different primary lifestyles shifted over the season (Figure 7). The proportion of fungal OTUs grouped into the category of plant pathogens reached high relative abundances (32.9–72.9%) in all four nurseries. At Kilåmon nursery, pathogenic OTUs had a high relative abundance throughout the whole season (36.4–67.4%) but these occurred most frequently in July and August. Saprotrophic OTUs were more abundant in June (53.5%), became less abundant in July and August, with the lowest relative abundance being in September (4.6%) and October (8.6%). At the same time, unidentified fungal OTUs became more abundant in September (52.3%) and October (52.1%) (Figure 7). Stakheden nursery had the lowest relative abundance of pathogenic OTUs among the nurseries (9.8–32.9%). In contrast to the other nurseries, Stakheden also had a high relative abundance of ectomycorrhizal fungal OTUs appearing in July (27.7%). The relative abundance of saprotrophic OTUs was high in June (42.8%) and July (31.6%) but very low in August (7.7%), when the group of unidentified OTUs contributed with 78.1% of all fungal OTUs. In October, the group of unidentified OTUs occurred less (49.2–29.2%) while pathogenic (19.5–32.9%) and saprotrophic (23.3–30.5%) OTUs were more abundant. Lugnet nursery had the highest relative abundance of mycoparasitic OTUs among the nurseries (4.6–38.4%). The relative abundance of pathogenic OTUs was highest in May (72.9%) and lowest in August (16.0%) when the relative abundance of mycoparasites was highest. The relative abundance of pathogenic OTUs increased again in October (55.7%) and remained high in November (47.6%). The relative abundance of saprotrophic OTUs was even from July (43.9%) to November (41.7%). Trekanten nursery had the highest relative abundance of saprotrophic OTUs (47.7%) at the beginning of the growing season, while the relative abundance of pathogenic OTUs was the lowest (26.1–35.8%). From August, the pathogenic OTUs (47.0%) became more abundant than the saprotrophic OTUs (32.8%) and remained more abundant throughout the rest of the season (67.1–70.2%).

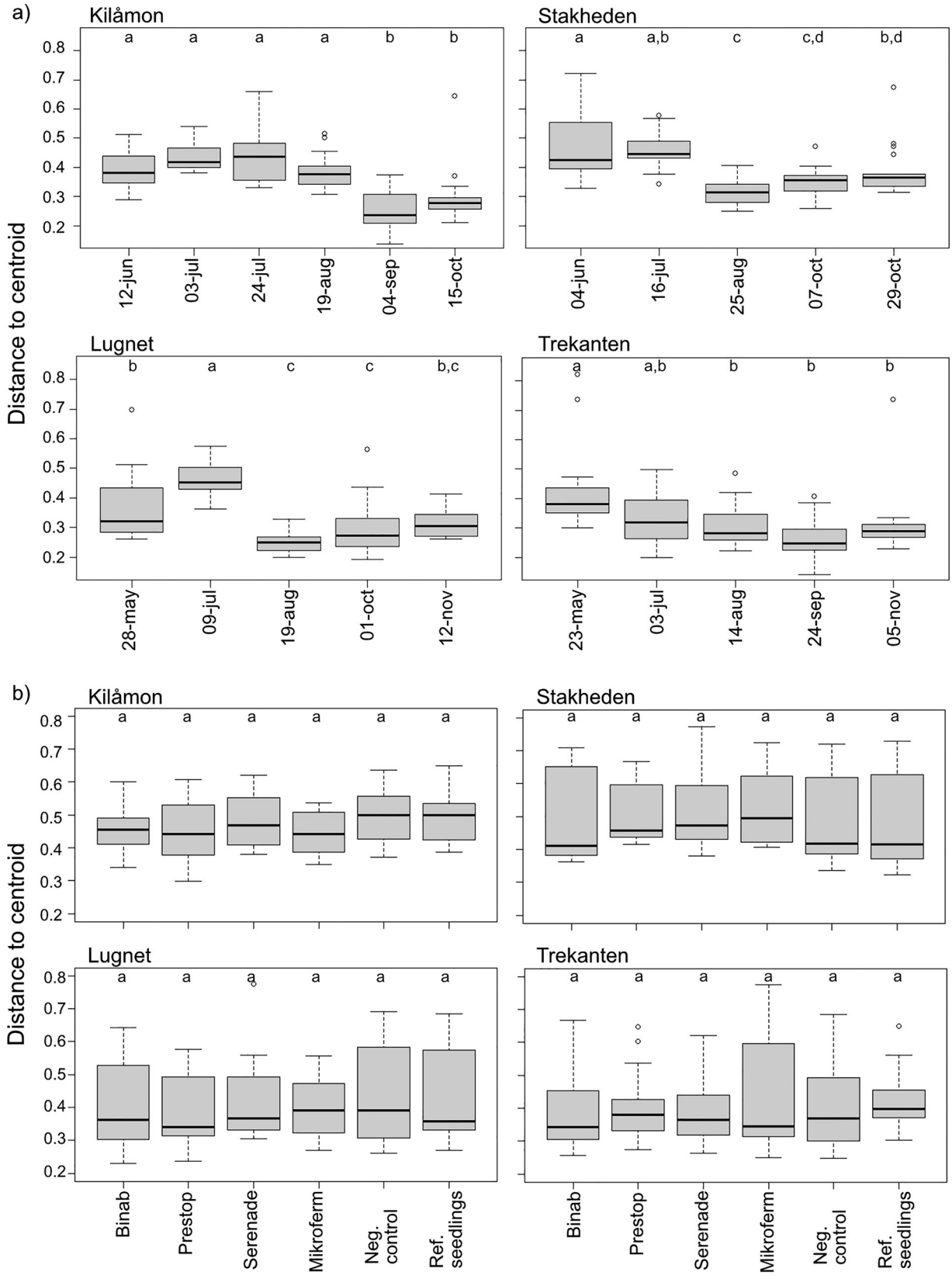

Figure 5. Boxplots presenting the distances to the centroid of foliar fungal communities from samples of non-symptomatic *Pinus sylvestris*-needles from each nursery within (a) sampling time-points and (b) treatments. The distances are based on Bray-Curtis dissimilarity matrices, with permutational analysis of multivariate dispersion (PERMDISP) test results. Different letters indicate significant different values ($p < 0.01$), based on pairwise comparison of group mean dispersions.

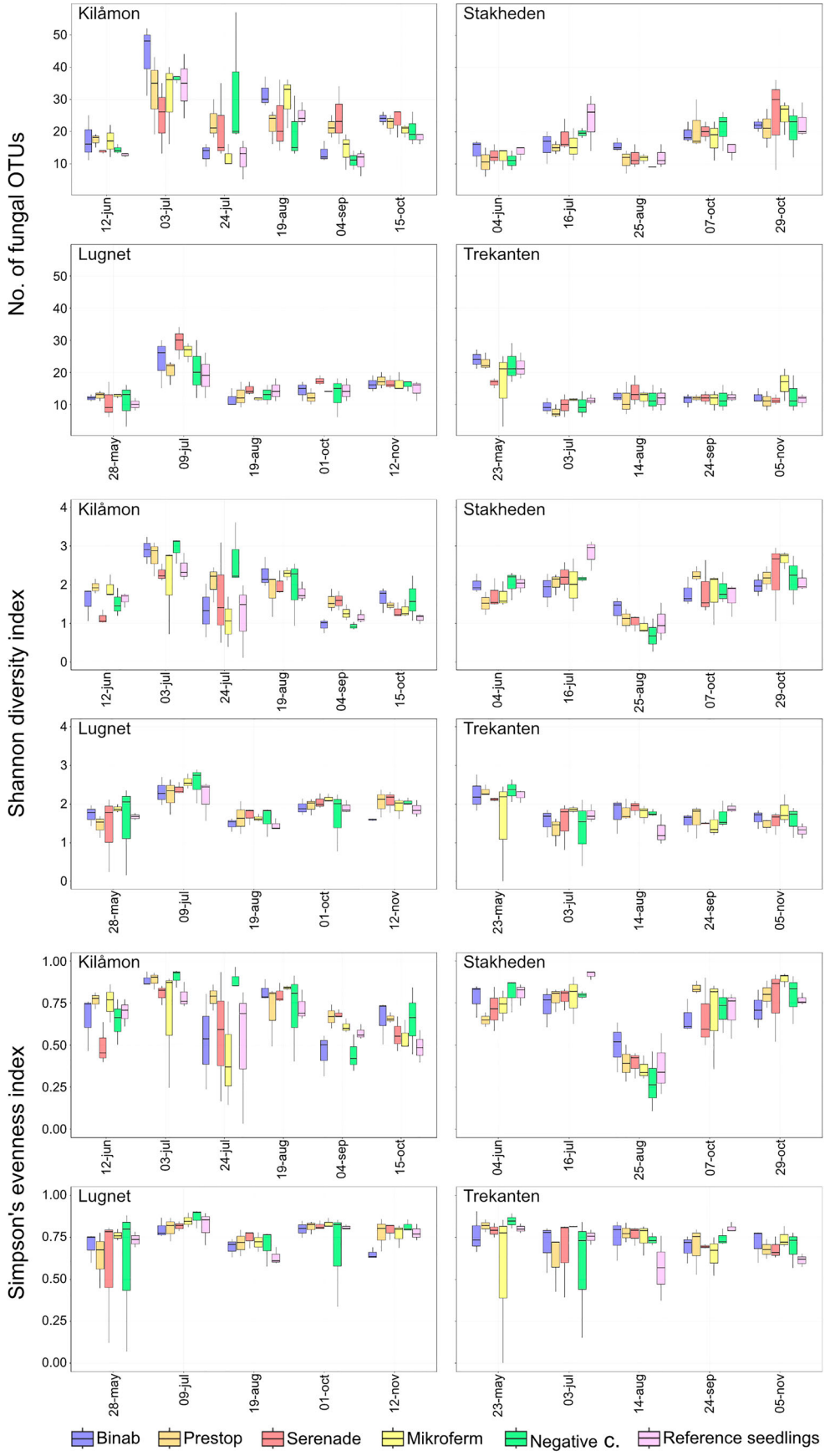

Figure 6. Boxplots presenting (a) Fungal OTU richness, (b) Shannon diversity index, and (c) Simpson's evenness index from non-symptomatic *Pinus sylvestris*-needles separated by sampling time-point, where different colours indicate different treatments. Based on rarefied datasets.

Table 2. Effects of treatments, sampling time points, and their interaction on (a) No. of fungal OTUs, (b) Shannon diversity index, and (c) Simpson's evenness index.

Nursery	Kilåmon			Stakheden			Lugnet			Trekanten		
	df	F	*P*	df	F	*p*	df	F	*P*	df	F	*p*
(a) No. of fungal OTUs												
treatment	5	1.47	0.21	5	0.52	0.76	5	1.39	0.24	5	0.094	0.99
time points	5	13.11	**<0.001**	4	10.41	**<0.001**	4	16.76	**<0.001**	4	10.11	**<0.001**
treat*time	25	1.39	0.14	20	0.84	0.66	20	0.61	0.98	20	0.71	0.80
(b) Shannon diversity index												
treatment	5	1.26	0.29	5	0.38	0.86	5	2.16	0.071	5	0.11	0.99
time points	5	17.70	**<0.001**	4	16.51	**<0.001**	4	14.15	**<0.001**	4	6.29	**<0.001**
treat*time	25	1.56	0.075	20	0.68	0.83	20	0.31	0.10	20	1.17	0.31
(c) Simpson's evenness index												
treatment	5	1.31	0.27	5	0.79	0.56	5	2.072	0.082	5	0.073	0.10
time points	5	12.63	**<0.001**	4	18.12	**<0.001**	4	9.37	**<0.001**	4	2.30	0.070
treat*time	25	1.34	0.17	20	0.86	0.64	20	0.34	0.10	20	1.21	0.28

Note: Based on rarefied dataset adjusted using a sample rank transformation in general linear models. Significant values ($p < 0.05$) indicated in bold.

Foliar fungal OTUs in forest nurseries

The most common fungi were *Cladosporium sp.* (15.1%), *P. herbarum* (14.5%), unidentified sp. 5320_4 (11.0%), and *Gjaerumia minor* (10.8%) (Table 3). The presence and frequency of abundant fungal OTUs varied over the season and among forest nurseries (Table 3, Figure 8). However, the treatments did not affect the most abundant fungal OTUs, except in a few cases. At the beginning of the experiment, different fungal OTUs appeared more frequent among nurseries, followed by a clear shift in fungal communities over the growing season (Figure 8). For example, Kilåmon nursery had a high relative abundance of *S. polyspora* (38.2 ± 7.6%) (mean ± SD), Lugnet nursery of *P. herbarum* (39.7 ± 15.2%), and Trekanten nursery of *Cladosporium sp.* (34.8 ± 8.2%). Following the growing season, the occurrence of many fungal OTUs shifted between sampling occasions (Figure 8). For example, *P. herbarum* had a high relative abundance early in the growing season at Kilåmon nursery (41.0 ± 18.7%), while it reached a high relative abundance later in the growing season at Trekanten nursery (29.9 ± 13.2%). In contrast, *Cladosporium* sp. appeared evenly abundant over the whole growing season at Lugnet and Trekanten nursery, yet with a scattered appearance at Kilåmon and Stakheden nursery. The presence of unidentified sp. 5320_4 was high in August at Stakheden nursery (76.6 ± 5.1%) and in September at Kilåmon nursery (53.4 ± 14.9%), but low throughout the growing season at Lugnet and Trekanten nursery (Table 3). Other abundant fungal OTUs observed in the study were *B. cinerea* (high relative abundance at Kilåmon and Stakheden nurseries) and *Alternaria alternata* (high relative abundance at Lugnet and Trekanten nurseries) (Table 3, Figure 8). Furthermore, the fungal component of two of the microbial additives, *Trichoderma* sp. (<0.02%) and *C. roesa* (0.08%) were observed in the foliar fungal communities from the needle samples (Table S2).

Discussion

This study investigated foliar fungal communities in *P. sylvestris* seedlings from four forest nurseries. Fungal diversity and community composition changed over time. Known pathogenic fungi of *P. sylvestris* were identified on healthy seedlings, and their presence observed in the different nurseries. Microbial additives were not shown to impact seedling growth, survival, disease incidence, or fungal community composition.

Foliar fungal community composition and OTU richness

The results demonstrated a high number of fungal OTUs associated with non-symptomatic needles of nursery-grown *P. sylvestris* seedlings. The OTU richness found in forest nurseries was comparable to those in forest stands of *P. sylvestris* growing in northern Europe, where around a thousand fungal OTUs were observed in both managed and unmanaged forest stands (Lynikienė et al. 2020). The number of fungal OTUs was also comparable to those reported in previous studies on soil mycobiomes in forest nurseries (Menkis et al. 2016; Marciulyniene et al. 2021). Fungal OTU richness varied among forest nurseries, with the highest number of fungal OTUs observed in the two northernmost forest nurseries and lower numbers further south (Figure 3). This result contrasts with the general biogeographic patterns of increasing species richness towards the equator, for example observed in both foliar endophytes and soil fungi in natural systems (Arnold 2007; Tedersoo et al. 2014). However, Tedersoo et al. (2014) also observed a deviation from these patterns by functional group, with saprotrophic, pathogenic, and parasitic fungi increasing in diversity at lower latitudes. Thus, the patterns of fungal richness observed in the forest nurseries may reflect the functional groups that dominate the communities. However, several conditions differed between the nurseries (e.g. surrounding vegetation, seed origin, seedling size and cultivation density) and a part of the observed species richness in the north could also be induced by the dense conifer forests around the nurseries (Eusemann et al. 2016).

In this study, the fungal community composition and species diversity changed during the growing season in all four forest nurseries (Figures 4 and 6). The development of foliar fungal community structure is influenced by several factors, e.g. environmental variables, host interactions, interspecific competition, or nutrition availability (Baldrian 2017; Wurth et al. 2019; Ata et al. 2022). An important factor for the fungal community dynamics in the present study could be rapid changes in the foliar system due to seedling growth and aging of needles, as fungal colonisation increases with the increase of needle age

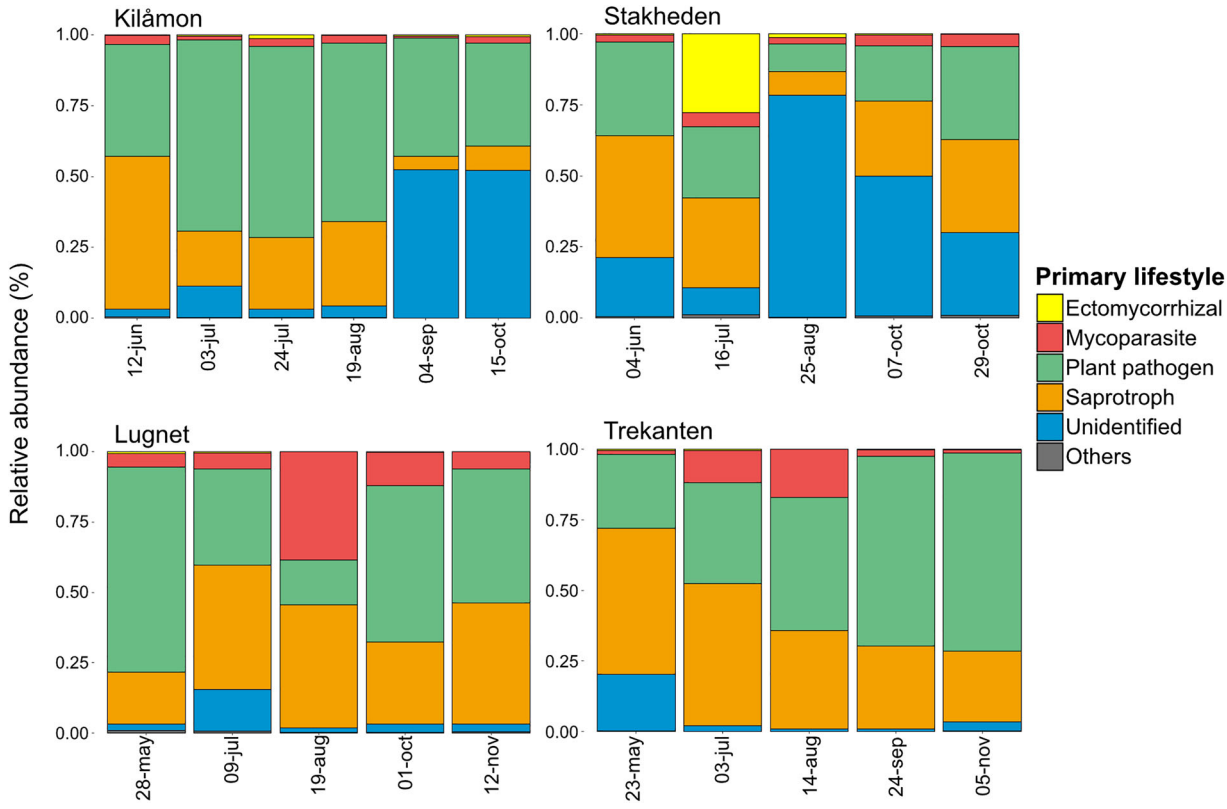

Figure 7. The relative abundance of primary lifestyles for fungal OTUs in non-symptomatic needles of *Pinus sylvestris* that were assigned with a genus (742 OTUs), separated by nursey. Primary lifestyles represented by less than 3% of fungal OTUs in all nurseries, respectively, are grouped into "Others".

(Sieber 2007). This pattern has been previously reported to occur between different needle age-classes of mature *P. sylvestris* and our findings could be a result of the aging of the seedlings (Agan et al. 2021). Furthermore, in all nurseries, fungal community diversity varied among nurseries and fluctuated over the growing season, and no distinct pattern for changes in fungal diversity was found. The assembly of fungal communities is sensitive to temporary stress events; for example temperature or precipitation (Lebeis 2015; Baldrian 2017), and the intensive nursery management practices (i.e. regular fertilisation, irrigation systems, cultivation systems) could influence the fungal community composition.

Table 3. Relative abundance of the 19 most common fungal OTUs from needles of non-symptomatic *Pinus sylvestris*-needles from four Swedish forest nurseries.

				Relative abundance (%)				
OTU	Phylum	Reference	Similarity*, (%)	Kilåmon	Stakheden	Lugnet	Trekanten	All nurseries
Cladosporium sp.	Ascomycota	MW44908	243/243 (100)	4.27	9.52	25.76	26.14	15.07
Phoma herbarum	Ascomycota	MG888615	249/249 (100)	18.39	3.54	10.98	22.19	14.52
Unidentified sp. 5320_4	Basidiomycota	KU188676	344/345 (99)	13.42	30.03	0.42	0.83	11.01
Gjaerumia minor	Basidiomycota	NR_138402	317/319 (99)	18.54	2.88	11.35	5.31	10.83
Alternaria alternata	Ascomycota	MZ670760	253/253 (100)	0.53	0.83	8.46	14.60	5.50
Sporobolomyces roseus	Basidiomycota	KX067834	300/300 (100)	0.74	3.11	13.48	6.85	5.49
Botrytis cinerea	Ascomycota	MT573470	240/240 (100)	8.21	7.51	2.93	1.52	5.39
Sydowia polyspora	Ascomycota	MN636228	256/256 (100)	10.70	0.52	0.05	0.40	3.94
Vishniacozyma sp.	Basidiomycota	MN913595	234/234 (100)	0.99	0.55	2.42	1.61	1.36
Cryptococcus sp.	Basidiomycota	MW765143	318/320 (99)	2.57	1.18	0.01	0.33	1.21
Aureobasidium pullulans	Ascomycota	MW449063	249/249 (100)	0.14	1.45	1.30	2.35	1.15
Thelephora terrestris	Basidiomycota	MT644883	313/313 (100)	0.08	5.23	0.10	0.08	1.12
Rhodotorula sp.	Basidiomycota	MK186928	302/302 (100)	2.03	0.57	0.06	0.46	0.94
Helotiales sp.	Ascomycota	MH858280	233/242 (96)	0.18	0.23	0.95	2.71	0.92
Entyloma sp.	Basidiomycota	MF482854	328/330 (99)	0.16	0.06	3.15	0.50	0.91
Rhodotorula mucilaginosa	Basidiomycota	LC473094	311/311 (100)	0.13	2.88	0.00	0.00	0.63
Ustilentyloma sp.	Basidiomycota	KX067827	316/316 (100)	1.59	0.01	0.03	0.24	0.62
Lophodermium pinastri	Ascomycota	KY742603	239/239 (100)	0.83	0.64	0.23	0.55	0.59
Venturia sp.	Ascomycota	KU220965	237/243 (98)	0.50	0.53	0.23	0.96	0.54
Total of 19 fungal OTUs				83.99	71.26	81.90	87.65	81.74

Notes: Data from different treatments and sampling occasions are combined within each forest nursery.
*Similarity column shows a comparison of base pairs between the query sequence and the reference sequence from the NCBI databases, with sequence similarity expressed as a percentage.

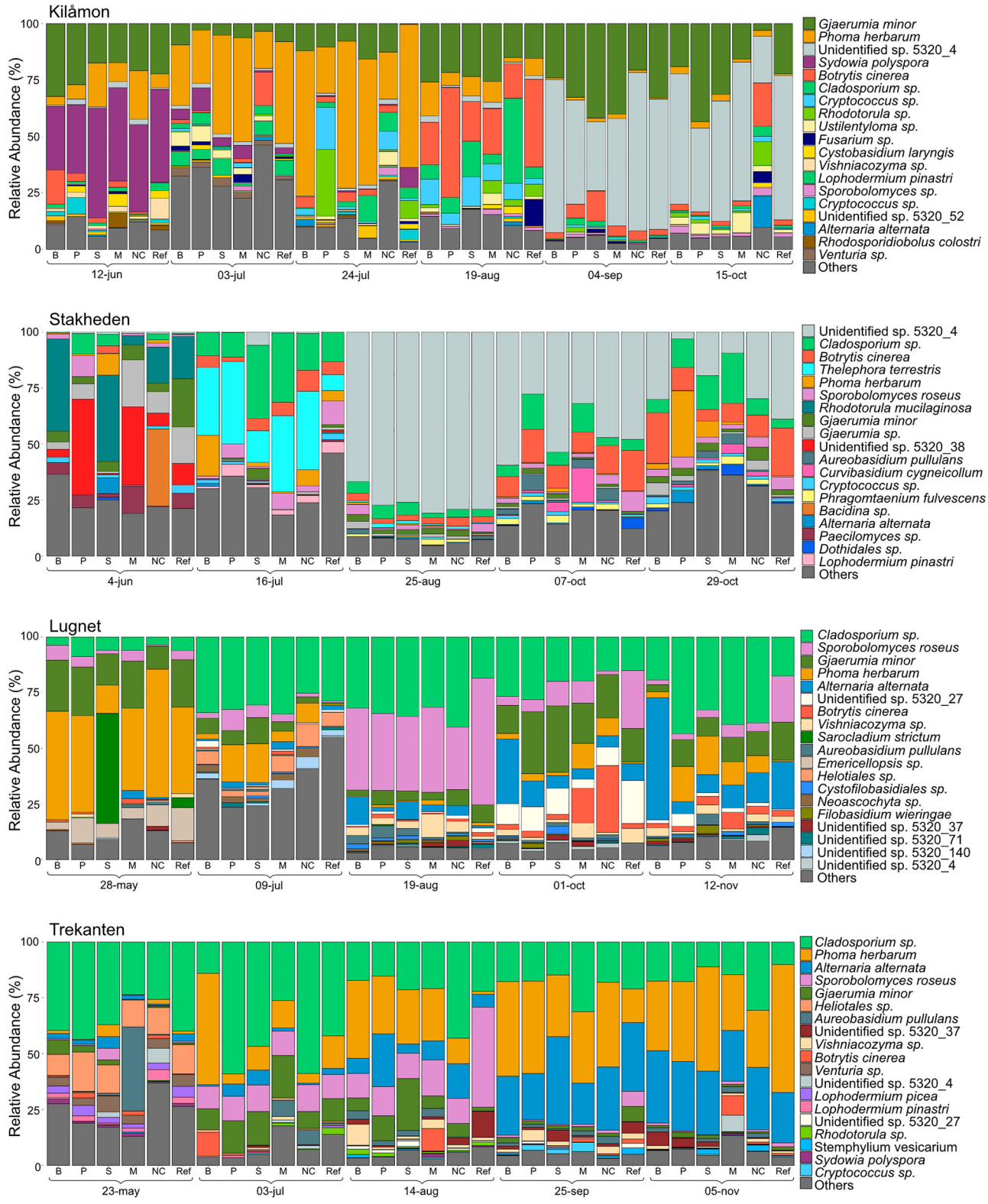

Figure 8. The relative abundance of the 19 most common fungal OTUs in non-symptomatic needles of *Pinus sylvestris*, separated by nursey. Remaining fungal OTUs after the 19 most abundant fungal OTUs are grouped together as "Others". Bars are presented by treatments per time point, where B = Binab, *P* = Prestop, S = Serenade, M = Mikroferm, NC = negative control and Ref = reference seedlings.

Development and local distribution of foliar fungal pathogens

Climatic conditions influence the local abundance and distribution of fungal species (Millberg et al. 2015), which was also observed in this study as a variation in the occurrence and distribution of fungal OTUs among nurseries and over the growing season. Although only about ten percent of the total number of fungal OTUs was shared among all four nurseries (Figure 3), the most frequently observed fungal OTUs

occurred in two or more nurseries (Figure 8). The foliar fungal communities were mainly composed of plant pathogens, saprotrophs, and mycoparasites (Figure 7). Although pathogenic fungi were abundant in the present study, previous reports on rhizosphere fungal communities from the same forest nurseries showed the predominance of saprotrophs, endophytes, and mycorrhizal fungi (Stenström et al. 2014; Menkis et al. 2016).

Some of the detected fungal pathogens were probably resting spores from the needle surface. Still, some could be fungal pathogens with an endophytic life stage inside the needles as they were obtained from asymptomatic needles (Petrini 1991). Similarly, a high proportion of plant pathogens was recently reported in both stems and roots of nursery-grown *P. sylvestris* seedlings, indicating that such plant pathogens can be latent in forest nurseries (Okorski et al. 2019).

Cladosporium sp., *P. herbarum*, *A. alternata*, *B. cinerea* and *S. polyspora,* known pathogens on *P. sylvestris* seedlings, were among the most frequently detected fungal OTUs in all nurseries. *Botrytis cinerea*, *A. alternata* and *S. polyspora* are plant pathogens commonly observed in forest nurseries in northern Europe (Capieau et al. 2004; Lilja et al. 2010; Okorski et al. 2019). Although *B. cinerea* is a common species causing disease outbreaks in forest nurseries, it was not the most frequently observed species in our study. In this study, *P. herbarum* and *Cladosporium* sp. were highly abundant and widely distributed in different forest nurseries. *Phoma herbarum* is a widespread plant pathogen that causes leaf spot on a broad range of plant species and important crops (Deb et al. 2020). Similarly, *Cladosporium* spp. are common fungi with a worldwide distribution found in many different environments and many species of this genus are plant pathogens (Heuchert et al. 2005). *Phoma* sp. and *Cladsoporium* sp. have previously been detected in forest nurseries (Stenström et al. 2014; Menkis et al. 2016; Okorski et al. 2019; Sheller et al. 2020), but Swedish forest nurseries have only recently experienced disease outbreaks caused by these fungal pathogens (personal communication). In this study, we confirmed the presence of several fungal pathogens and the predominant occurrence of *P. herbarum* and *Cladosporium* sp. in forest nurseries and provided information on their spatio-temporal distribution within each nursery. Despite the high relative abundance of these plant pathogenic fungi, a limited number of disease outbreaks were reported during the study, indicating that the pathogens were latent or present as propagules on the needle surface. However, the occurrence of fungal pathogens poses a risk of disease outbreak under conditions favourable to the fungal pathogens, e.g. when seedlings are stressed. Furthermore, the presence of latent pathogenic fungi can also limit the success of seedling storage and their establishment following outplanting due to opportunistic species (e.g. *Botrytis cinerea*, *Phoma herbarum*, or *Cladosporium* sp.) (Lilja and Rikala 2000; Petäistö 2006; Lilja et al. 2010). Fungal diseases can rapidly develop from symptomless seedlings when seedlings experience a stressful environment, e.g. failure during storage or drought, frost, and flooding in the forest.

Other common foliar fungal OTUs in forest nurseries

Among other fungi, the unidentified sp. 5320_4 was a common fungus, mainly present in the two northern forest nurseries (Figure 8, Table 3). The taxonomic affiliation of this fungus could not be established except that it belonged to the phylum Basidiomycota. In the prospect of finding a suitable BCA candidate for forest nurseries, this could be an interesting fungus to investigate for possible antifungal activity, as it appeared with very high abundance while other fungal OTUs appeared in low abundance (Sivanandhan et al. 2017; Gholami et al. 2019; Prospero et al. 2021). Another fungal OTU observed with high abundances at Stakheden nursery was *Thelephora terrestris*, an ectomycorrhizal fungus known to dominate growth substrates in forest nurseries (Stenström et al. 2014; Menkis et al. 2016). However, this result probably reflects the dispersal of spores detected on the needle surface.

Microbial additives – effects on seedling growth and diseases incidence

Microbial treatments were tested under field conditions for seedling growth, survival, disease incidence, as well as possible target and non-target effects on foliar fungal communities. Opposite to our hypothesis, treatments were not found to impact seedling growth, while the disease control was not possible to evaluate due to the low level of infection. However, the experimental design of replicates of individual treatments in single blocks, which was a consequence of the automated management of procedures in the nurseries, may have reduced the possibility of identifying such effects. Another important factor that may have influenced the chance to detect treatment effects was the warm and dry weather conditions in the previous year (2018) and the year of the study (2019) (SMHI 2022), which resulted in very few disease incidents reported overall. Further testing should be repeated over several growing seasons to include seasonal variations with a high risk of disease outbreaks. The results of this study suggest that in years with unfavourable climatic conditions for fungal infections, control measures could be restricted to in first hand mechanical control and in second hand to chemical control as a part of an integrated disease management. Furthermore, microbial treatments were not found to have neither a positive nor a negative impact on the fungal community composition or species richness. The applied microbial agents *Trichoderma* spp. and *C. rosea* were detected in the fungal communities after application but were not among the most abundant fungal OTUs. This could indicate a short-lived state of the added microorganisms or failure to establish on the seedlings. The establishment of microbial agents depends on several aspects (i.e. climatic constraints, interactions with native organisms, lack of hosts, common cultivation practices), and the intensive irrigation during warmer weather conditions probably had a negative impact on the establishment of microbial additives (Schulz et al. 2019). Biological management of fungal pathogens in forest nurseries still lags behind agricultural production systems, and better targeted BCAs for forest seedlings are needed. Uncertainties around the efficacy of

available biological products may not be economically justifiable, and the use of microbial additives under field conditions needs to be thoroughly evaluated for successful implementation in forest nurseries (Prospero et al. 2021).

Conclusion

In conclusion, our results suggest that the foliar fungal community of nursery-grown *P. sylvestris* seedlings harboured a high fungal OTU richness, and that the community composition underwent dynamic changes over time. Furthermore, the occurrence of dominant fungal taxa was influenced by the forest nurseries and the development of fungal communities. Under appropriate environmental conditions and nursery management strategy, seedlings can remain healthy even in the presence of fungal pathogens.

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Disclosure statement

Four authors are affiliated with Sveaskog AB, but this does not impact our adherence to Scandinavian Journal of Forest research policies on sharing materials and data. Commercialised microbial products were used in this study, but no collaboration or financing were granted from the manufacturer.

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Data availability statement

Relevant data are provided either within the paper or as supplementary files.

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