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ORIGINAL ARTICLE

Fungal communities associated with *Picea abies*, *Pinus sylvestris* and *Larix* sp. seeds of different geographic origin: Implications for disease management

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Abstract

In Europe, *Pinus sylvestris* and *Picea abies* are the most common coniferous tree species used in commercial forestry, which rely on high-quality reproductive material for successful reforestation. Clear-cut harvested forest sites are often replanted using tree seedlings, which are produced in forest nurseries using seeds from seed orchards. However, incidences of fungal diseases in seedling production show that a better knowledge of seedborne fungi, including fungal pathogens, is needed to manage diseases in forest nurseries. This study aimed to assess seedborne fungal communities associated with commercial seeds of *P. abies*, *P. sylvestris* and *Larix* sp. seeds originated from geographically separated regions in Sweden, Belarus, Finland and Poland. Fungal communities were obtained first from the seed surface and then from the seed tissue. These were analysed using high-throughput sequencing of the ITS2 rDNA region. The results showed that fungal diversity and community composition differed between the seed surface and the seed tissue. *Picea abies* accommodated a higher fungal diversity than *P. sylvestris*. In addition, a strong host affinity of the fungal community composition on the seed surface and a weaker association in the seed tissue was found. Fungal communities on *P. abies* and *P. sylvestris* seed surface differed significantly between geographical regions, whereas no regional differences were found in the seed tissue. The seedborne fungal communities included a high proportion of plant pathogens, among which the most abundant were *Sydowia polyspora* (13.3%), *Phoma herbarum* (11.2%) and *Sirococcus conigenus* (3.8%). In conclusion, the results showed (a) characteristic fungal diversity and community composition between the seed surface and the seed tissue; (b) a host-specific fungal community composition on the seed surface and in the seed tissue; (c) regional difference in fungal communities on *P. abies* and *P. sylvestris* seed surface, thus the movement of seeds between different regions can contribute to the spread of fungal diseases; and (d) the presence of a high incidence of seedborne fungal pathogens which suggest a potential need of preventative or control measures to reduce the occurrence of these fungi on the seed surface.

KEYWORDS
Cladosporium sp., conifers, fungal diseases, pathogens, *Phoma* sp., seed orchard, *Sydowia polyspora*

1 | INTRODUCTION

Boreal forests, which are one of the largest biomes on Earth, are dominated by coniferous trees and provide important ecosystem services, including timber production, carbon sequestration, local climate regulation, recreation and ecotourism (Millennium Ecosystem Assessment, 2005). In northern Europe, *Pinus sylvestris* and *Picea abies* are the most common coniferous tree species used in forestry. Other conifer tree species such as *Larix* spp., *Picea* spp., *Pinus* spp. and *Pseudotsuga menziesii* are less common but still important for their specific wood properties or tolerance to harsh environmental conditions (Mataruga et al., 2023; Pâques et al., 2013). The forest industry relies on high-quality reproductive material for successful reforestation, and most of the coniferous tree planting stock is produced using seeds (Farjon, 2018; Pâques, 2013; Solvin et al., 2021). Forest tree seeds are usually collected from seed orchards or seed stands of local origin. However, forest seeds are frequently traded among European countries to meet the high demand for tree seedlings (Franić et al., 2023; Solvin et al., 2021). The trade and the production of forest tree seeds and seedlings within the EU member states is regulated by the EU Directive 1999/105/EC. Despite the strict regulation of phenotypical and genetical aspects of forest seeds, phytosanitary aspects currently only concern *Pinus* sp., *Pseudotsuga menziesii* and *Prunus avium* (EU Directive 2019/2072) and quarantine pests, which are non-native within the EU (EU Directive 2019/2023). The limited regulation to sustain healthy seed material can be problematic as seeds can be the source of several seedborne pathogens that can cause significant damage in seedling production and pose a risk for the spread of diseases into the forests (Cleary et al., 2019; Cram & Fraedrich, 2010; Franić et al., 2019; Mangwende et al., 2021).

Seeds for seedling production in Swedish forest nurseries either originate from forest stands or they are produced in seed orchards. Seed orchards are plantations of superior trees from the breeding programme used for the mass production of improved seeds, often established on former agricultural land, while seed stands are forest stands of good quality selected for seed harvesting (Pâques, 2013). Cones from seed stands or orchards are collected during certain years of a high yield. The cones are dried to about 10% moisture content and tumbled to extract the seeds (Belcher & Lowman, 1982; Fennessy, 2002). Seeds are then cleaned, sorted for size and quality, and tested for the germination rate. Good seed hygiene during the cone collection and seed processing has been suggested as the main approach to avoid or lower the risk of seed-transmitted diseases and the spread of fungal pathogens to the nurseries and forests (Lilja & Poteri, 2013; Martín-García et al., 2019). This includes, among other things, the removal of infested cones during cone collection. Chemical treatments can often effectively reduce surface contamination but will not reduce fungi colonizing the seed tissue. At the same time, the chemical treatments of seeds pose a risk of eliminating beneficial microbes or even damaging the seeds (Allen et al., 2004; Lilja & Poteri, 2013).

Seeds harbour many different microorganisms, including plant pathogenic fungi, saprotrophs and endophytes (fungi residing inside of the plant tissue) (Cleary et al., 2019; Franić et al., 2019; Mangwende et al., 2021; Martín et al., 2022). However, seeds are generally thought to harbour fewer microbial species compared to other parts of the plant (Abdelfattah et al., 2023). Several studies have reported host species as an indicator of fungal community characteristics associated with conifers, especially for the endophytic communities, for example, in needles (Apigo & Oono, 2022; Higgins et al., 2007) or in seeds (Franić et al., 2020). Furthermore, seeds of gymnosperms showed lower fungal richness than angiosperms (Franić et al., 2019, 2020). Fungal diversity (here referred to alpha diversity, i.e., species richness and diversity indices with related effective species numbers) and community composition in seeds can be influenced by several factors, including maternal effects and local environmental variables (e.g., temperature, precipitation, altitude, vegetation structure, seasonal variations), as well as the interaction between the fungi and their hosts (Fort et al., 2021; Franić et al., 2019, 2020; Oliva et al., 2013). Additionally, fungal communities can differentiate between geographically separated regions following the general biogeographic pattern (i.e., the species diversity increases towards the equator) (Arnold, 2007; Tedersoo et al., 2014). Thus, the coniferous seeds could be expected to harbour a fungal community reflected by the geographical location, the host tree species and the site characteristics shaped by the uniformity of a seed orchard or seed stand.

Many seed-transmitted diseases are caused by fungi (Martín et al., 2022). Fungi in seeds are considered to spread via two pathways. On the one hand, they can be carried externally on the seed surface and transmitted horizontally through different environmental factors (e.g., vegetation, soil, water, air dispersion, seed processing) (Shade et al., 2017). On the other hand, they can be present inside the seeds and transmitted vertically, either by colonizing the seed surface and internalize within the seed tissue or by residing internally in the seed tissue from the mother plant across generations (Gaur et al., 2020; Neergaard, 1977). Although several seedborne fungi, for example, saprotrophs, do not particularly impact seed performance when used in forest nurseries, certain fungal pathogens can cause significant losses. Several fungal pathogens have been reported as seed-transmitted after severe disease outbreaks in forest nurseries in North America. For example, shoot dieback caused by *Sirococcus conigenus* or damping-off diseases caused by *Fusarium* sp. on conifers (Cram & Fraedrich, 2010). Seedling cultivation can

also fail due to the reduced seed germination and post-emergence diseases of tree seedlings (Lilja & Poteri, 2013). The recent advancement of sequencing technologies enabled studies on seedborne fungi to assess a broader range of species, both within and on seeds (Nelson, 2018). However, many previous studies have focused on seedborne fungi associated with agricultural plant species (i.e., radish, rapeseed or bean), while fungal community studies associated with forest tree seeds are still sparse (Simonin et al., 2022). Furthermore, differentiating between fungi residing in the seed tissue and those assembled on the seed surface is necessary to integrate directed mitigation methods. For example, if harmful fungal pathogens mainly harbour the seed surface, they could be managed with common seed treatments (e.g., fungicides or biological control). However, other means of control would be needed if the pathogens internalize into the seed tissue.

This study aimed to assess the diversity and composition of seedborne fungi, especially fungal pathogens, of commercial coniferous seeds used for tree seedling production and reforestation in Sweden. Seeds of *P. abies*, *P. sylvestris* and *Larix*. sp. collected from seed orchards and seed stands of different geographic origins in northern Europe were included in the study. We hypothesize that the fungal diversity and community composition will differentiate between the seed surface and the seed tissue of each tree species. The fungal diversity will be higher on the seed surface than in the seed tissue and differ between tree species. Furthermore, the species diversity will be higher in the south and decrease northwards. We further hypothesize the relative importance of host tree species in shaping the fungal communities to be higher for communities associated with the seed tissue than those associated with the seed surface, as generalist fungal taxa might dominate seed surface communities. In addition, the fungal community composition will differ between geographical regions and over the years of seed collection.

2 | MATERIALS AND METHODS

2.1 | Seed material

In this study, all seeds were from commercial seed batches, and *Picea abies* originated from Sweden, Finland, Poland and Belarus, while *P. sylvestris* and *Larix* sp. seeds originated from Sweden and Finland (Figure 1; Table S1). All seed batches were harvested from seed orchards, except for *P. abies* seeds from Belarus and Poland, which were harvested from seed stands. Swedish *P. abies* and *P. sylvestris* seeds were harvested in 2015 and Polish seeds in 2006. The remaining seed batches were harvested between 1998 and 2016 (Table S1). The Swedish seeds of different origins were grouped into three geographical areas (regions) (northern, central and southern) based on locations of seed orchards, as the different areas were expected to be characterized by different climatic conditions. From each of these Swedish regions, additional seed samples of *P. abies* and *P. sylvestris* were collected to investigate the impact of seed-collection year on fungal communities within the same seed orchard (Table S1).

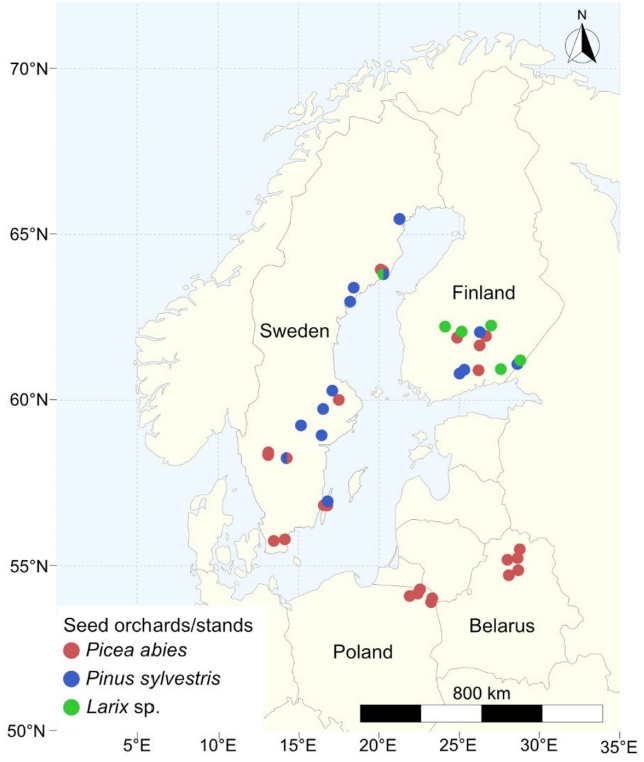

FIGURE 1 Map of northern Europe showing the location of the seed orchards in Sweden and Finland, and the seed stands in Poland and Belarus. Seed orchards/stands of different tree species are indicated using different colours.

Three *P. abies* origins (Björkebo: 2002, 2006, 2012, 2018; Nedra Sandby: 2004, 2007, 2011, 2017; Saleby: 1998, 2006, 2011, 2017) and three *P. sylvestris* origins (Alvik: 2003, 2006, 2012, 2018; Gotthardsberg: 2009, 2014, 2016, 2018; Hade: 1997, 2004, 2011, 2018) were included. All seed batches were designated for commercial use (i.e., for reforestation of forest land or cultivation of forest tree seedlings) and were kept stored at −18°C prior to the study. In total, 68 seed batches were used and ca. 30 mL of seeds per batch were received for the study (Table S1).

2.2 | DNA extraction, amplification and sequencing

To assess fungal communities on the seed surface and in the seed tissue, a sample containing 0.70 g of seeds (between 52 and 146 seeds) was randomly collected from each seed batch. The seeds were washed by vortexing in 1.5 mL 0.01% Tween20 solution to collect spores on the seed surfaces. After washing, the liquid was transferred into a new 1.5-mL tube, and the spores were pelleted by centrifugation at 16.2 *g* for 2 min. The supernatant was removed, and the pellet was used for the analysis of the fungal community on the seed surface. After washing, the same seeds were placed in a tea-strainer surface-sterilized in 0.5% sodium hypochlorite for 5 min and then rinsed twice in sterile Milli-Q

water (Sutherland et al., 2002). The seeds were placed on individual sterile filter papers to air dry and then homogenized using a mortar and pestle. For each sample, 50 mg of homogenized seed mass was transferred into 2-mL screw cap tubes and further homogenized together with four glass beads for 5000 ppm for 30 s using Precellys® 24 Tissue Homogenizer (Bertin instruments, Montigny-le-Bretonneux, France). In total, DNA was extracted from 136 samples collected from seed surfaces (68 samples) and seed tissue (68 samples) following the protocol of NucleoMag® Plant kit (Macherey-Nagel, Düren, Germany). DNA concentrations were determined using a NanoDrop™ One spectrophotometer (Thermo Scientific, Rochester, NY, USA) and diluted to 0.5 ng μL^{-1}. The ITS2 rDNA region was amplified by polymerase chain reaction (PCR) using the fITS7 (Ihrmark et al., 2012) and ITS4 primers tagged with unique identifier sequences. PCRs and cycling programme followed the protocol of Larsson et al. (2023) using an optimized number of PCR cycles. The AMPure kit (Beckman Coulter, Indianapolis, IN, USA) was used to clean PCR products, and DNA concentration was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, MA, USA). An equivalent molar mix of purified PCR products was pooled into two pools and cleaned using E.Z.N.A. Omega cycle pure kit (Omega Biotek, Norcross, GA, USA). Amplicon quality and size distribution were controlled using BioAnalyser DNA 7500 (Alignment Technologies, Boulder, CO, USA), and pooled libraries were sequenced on the PacBio RSII platform using two SMRT cells by SciLifeLab NGI (Uppsala, Sweden).

2.3 | Bioinformatics

The SCATA pipeline was used for quality filtering and clustering of sequences (Brandström-Durling et al., 2011). In the filtering process, sequences of low quality were removed, that is, too short (<200 bp), containing low read quality or missing a sample tag or a primer, along with primer dimers. Homopolymers of the sequences were collapsed into three base pairs. After quality filtering, sequences were clustered into operational taxonomical units (OTUs) by single linkage clustering with a minimum of 98.5% similarity (Tedersoo et al., 2022). The OTUs with less than nine sequences and which appeared in less than three samples were removed from further analyses. The OTUs were taxonomically classified using PROTAX-fungi and massBLASTer (UNITE/INSD fungi) databases implemented in the PlutoF biodiversity platform (Abarenkov et al., 2010). For the PROTAX software, a threshold value of 0.5 (plausible classification) was used for each taxonomic level (Abarenkov et al., 2018). From the massBLASTer-output, fungal OTUs were kept if the sequence similarity was >80% for identification at the phylum level. Fungal OTUs with lower similarity than 80% were considered as "non-fungal" and removed before further analysis (Menkis et al., 2016; Stenström et al., 2014). Identification criteria for genus level were set to at least 94% sequence similarity and for taxon level identification to more than 98% sequence similarity. Fungal OTUs with a high match to several species were assigned with their shared genus. Finally, taxonomies were assigned to the likely fungal OTUs by manually comparing the output files from massBLASTer and PROTAX-fungi. In case of disagreement between the output files, the fungal OTU was further verified using BLASTN algorithm in the GenBank (NCBI) database. Species hypotheses were assigned to fungal OTUs through SH Matching (v2.0.0) using the PlutoF biodiversity platform (Abarenkov et al., 2010). Sequences of fungal OTUs are available from GenBank database under accession numbers OR769607–OR769665 and PP759430–PP759619.

2.4 | Statistical analysis

All statistical analyses were performed using R Statistical Software v 4.3.0 and RStudio (Posit Team, 2022; R Core Team, 2022). The number of fungal OTUs (species richness), the Shannon diversity index and the Simpson's evenness index were used to assess the fungal diversity on the seed surface and in the seed tissue (McCune & Grace, 2002). The effect of sample type (seed surface and seed tissue), tree species and region, as well as the interaction between sample type and tree species and region, respectively, on the fungal diversity was tested by constructing general linear models. The square root of the total number of reads for each sample was used as the first factor in the models to account for variance in sequencing depth among samples (McMurdie & Holmes, 2013; Tedersoo et al., 2022). Data were transformed using a Box–Cox transformation to improve the distribution of residuals within the models. Residual plots were further compared to the Anderson–Darling normality test using the R package nortest (Gross & Ligges, 2015). Differences between sample types, tree species or regions were analysed using pairwise comparisons on estimated marginal means in the R package Emmeans v. 1.7.4.1 (Lenth, 2022).

The fungal community composition was investigated using the Phyloseq and Vegan R packages (McMurdie & Holmes, 2013; Oksanen et al., 2022). The permutational multivariate analysis of variance (PERMANOVA), on Bray–Curtis dissimilarity matrix with 999 permutations, was used in downstream analyses of group effects of sample type (seed surface and seed tissue), tree species, region and their interactions, on the fungal community composition. Additionally, the effects of seed orchard, year and the interaction between seed orchard and year were tested on the fungal community composition for seed samples collected over several years from the same *P. abies* and *P. sylvestris* seed orchard, respectively. To test for the assumption of PERMANOVA, the multivariate homogeneity of group dispersion (variance) in species composition between sample types was tested by a permutational analysis of multivariate dispersion (Anderson et al., 2006). The square root of the total number of reads was again used to account for variance in sequencing depth among samples (McMurdie & Holmes, 2013; Tedersoo et al., 2022). Fungal communities were analysed on a relative abundance table adjusted using a Hellinger transformation, and group differences were compared using pairwise comparisons in the R package Adonis v. 0.4

(Martinez Arbizu, 2017). Variations in fungal community compositions were visualized in ordination plots using principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity matrix.

Primary lifestyles were assigned to fungal OTUs taxonomically classified at the genus or species level using the FungalTraits database (Abarenkov et al., 2010; Põlme et al., 2020). The output of FungalTraits assigned 16 different lifestyles to fungal OTUs identified at the genus or the species level. The primary lifestyles were grouped into plant pathogen, saprotroph (dung, litter, nectar/tap, soil, sooty mould, wood, unidentified), mycoparasite and others (root endophyte, lichenized, lichen parasite, foliar endophyte, ectomycorrhizal, animal parasite, epiphyte), and fungal OTUs with unidentified lifestyles were set as unassigned. Relative abundances were calculated for all fungal OTUs, per tree species based on non-rarefied datasets for the seed surface and the seed tissue, respectively. The 20 most common fungal OTUs were plotted separately using the R packages reshape2 and ggplot2 (Wickham, 2007, 2016), while the remaining fungal OTUs were grouped into "Others." Additionally, the 20 most common fungal OTUs for *P. sylvestris* and *P. abies*, respectively, were plotted per region.

Indicator species associated with seeds were obtained for group combinations of sample type (seed surface and seed tissue) and tree species. A multilevel pattern analysis (multipatt) with 999 permutations was used to assess the indicator species using the Indicspecies R package (De Cáceres & Legendre, 2009). Indicator species were selected if they were identified at the genus or species level and had a high group association (indicator value >0.70).

Temperature and precipitation were used to compare regions, as they were expected to be characterized by different climatic conditions. Monthly average temperature and monthly total precipitation for each year of seed harvest were retrieved from the Climate data store from the Copernicus climate change service (Muñoz Sabater, 2019). Climatic data were re-calculated into annual mean temperature, converted into Celsius degrees (°C), and annual total precipitation, converted into millimetres (mm), for each year and location (Table S1). Climatic differences were tested between regions for *P. abies* and *P. sylvestris*, respectively, by constructing general linear models using the R package Vegan (Oksanen et al., 2022). Data were adjusted using a sample log-transformation to improve the distribution of residuals within the models, and differences were analysed using pairwise comparisons on estimated marginal means in the R package Emmeans v. 1.7.4.1 (Lenth, 2022).

3 | RESULTS

In total, 195,702 high-quality sequences were generated from 136 samples (68 seed surface and 68 seed tissue). The high-quality sequences were clustered into 970 global clusters (OTUs) and 906 singletons, which were excluded from further analysis. After removing "non-fungal" OTUs, the dataset included 771 fungal OTUs represented by 191,885 sequences. Further filtering (removal of fungal OTUs smaller than 10 reads and represented by less than three samples) resulted in a final dataset including 249 fungal OTUs represented by 179,920 sequences across 136 samples (Table S2). The detected fungal OTUs belonged to either Ascomycota (65.5%), Basidiomycota (34.1%) or Mucoromycota (0.4%) and were taxonomically classified at the following levels: 34.9% species, 39.0% genus, 4.8% family, 12.4% order, 3.2% class and 5.6% phylum (Table S2).

3.1 | Variation of seedborne fungal diversity

The fungal diversity was estimated by number of fungal OTUs, Shannon diversity index and Simpson's evenness index. The number of fungal OTUs differed between the seed surface and the seed tissue (Figure 2; Table S3, adj.$R^2 = .81$, $p < .001$) and was higher on the seed surface than in the seed tissue among all three tree species (pairwise comparisons, $p < .01$). However, the number of fungal OTUs did not differ between tree species compared within the seed surface or the seed tissue (Figure 2; pairwise comparisons, $p > .05$). The sequencing depth was significantly different for the effect on number of fungal OTUs (Table S3). The Shannon diversity index and Simpson's evenness index showed similar result, were significantly different between sample type (seed surface and seed tissue) and tree species, and had an interaction between sample type and tree species (Shannon diversity index, adj.$R^2 = 0.62$, $p < .001$; Simpson's evenness index, $R^2 = 0.50$, $p < .001$) (Figure 2; Table S3). The Shannon diversity index was higher on the seed surfaces of *P. abies* and *P. sylvestris* seeds than in the seed tissue (pairwise comparisons, $p < .01$), and the *P. abies* seed surface showed higher diversity than the *P. sylvestris* seed surfaces (pairwise comparisons, $p < .05$). In contrast, the Simpson's evenness index did not differ between sample type of *P. sylvestris* (Figure 2; pairwise comparisons, $p > .05$). The Shannon diversity index and Simpson's evenness index did not differ between sample type of *Larix* sp. or between *Larix* sp. and the other tree species compared within sample types (pairwise comparisons, $p > .05$). No regional differences of fungal diversity were found (Figure 2; Table S3).

In addition, the impact of seed-collection year on the fungal diversity was tested on *P. abies* and *P. sylvestris* seeds from northern, central and southern Sweden. Seeds collected between 1997 and 2018 within the same seed orchard were used. However, no impact of seed orchard nor seed-collection year was found on the fungal diversity (Table S4, $p > .05$).

3.2 | Variation of seedborne fungal community composition

A PERMANOVA analysis showed a significant separation ($p < .001$) of fungal communities between the seed surface and the seed tissue (Figure 3; Table S5). The sample type explained 10.8% of the variation, whereas tree species explained 13.7% of the variation. In addition, the fungal community composition was less dispersed among the seed surface samples than among the seed tissue samples

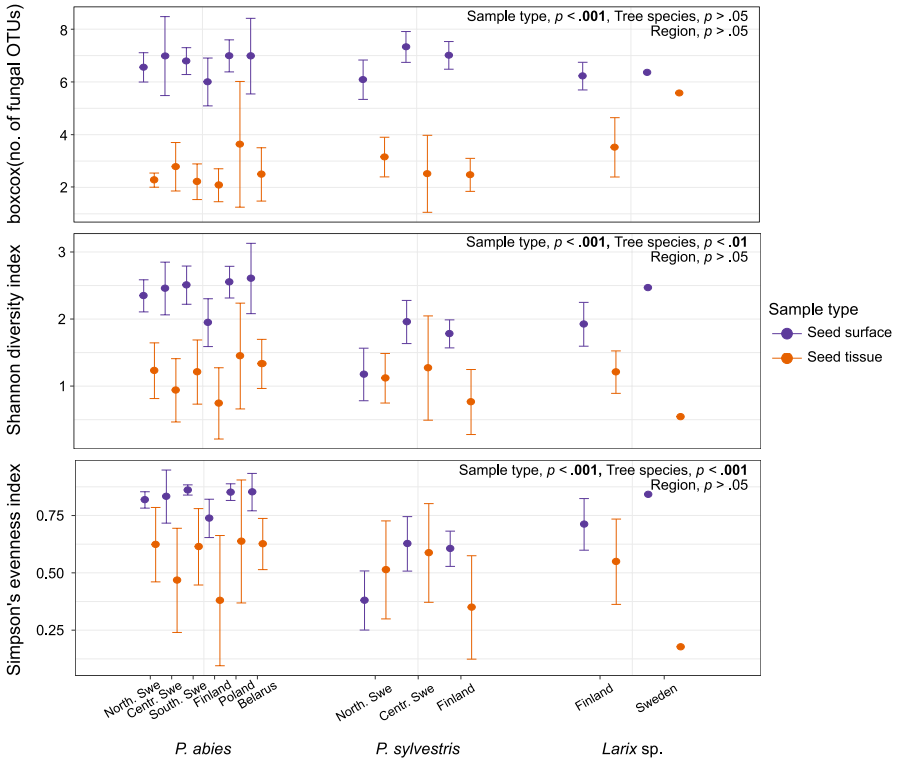

FIGURE 2 The number of fungal OTUs, the Shannon diversity index and the Simpson's evenness index from the seed surface and the seed tissue of fungal communities detected from *P. abies*, *P. sylvestris* and *Larix* sp. seeds collected from different geographical regions. The mean values with standard error are indicated with points, and the sample type is indicated using different colours.

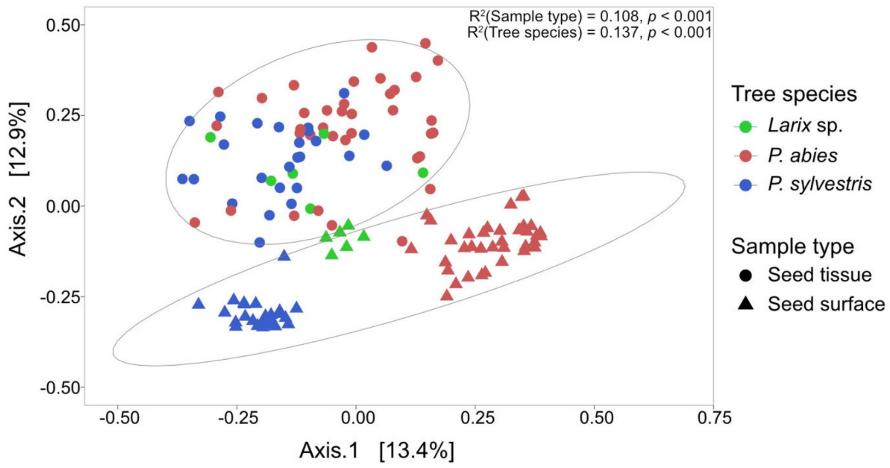

FIGURE 3 Principal coordinate analysis (PCoA) plot of fungal community sampled from the seed surface and the seed tissue of *P. sylvestris*, *P. abies* and *Larix* sp. seeds. Sample type is indicated using different symbols and tree species using different colours. The ellipses represent a 95% confidence interval around the group centroids of the sample types.

(betadisper, $F = 205.8$, $p < .001$) (Figure S1). A pairwise comparison among *P. abies*, *P. sylvestris* and *Larix* sp. showed that their seeds hosted significantly different fungal communities, both on the seed surface and in the seed tissue (Figure 3; Table S6). Furthermore, an interaction between sample type and tree species was found, as well as a regional difference explaining 8.1% of the variation in fungal community composition (Table S5).

The variation in fungal community composition explained by tree species was 39.6% on the seed surface ($p < .001$) and 8.5% in the seed tissue ($p < .001$) when analysing the sample types separately (Table 1). Sequencing depth across samples for the fungal communities only explained 2.3% of the variation on the seed surfaces and 3.5% of the variation in the seed tissue (Table 1). Geographical regions explained 15.1% of the variation in the fungal community composition on the seed surface, whereas 14.5% of the variation in the seed tissue (Table 1). As geographical regions were not found to be significantly different in the seed tissue (Table 1; Figure S2; $p > .05$), regional differences were only investigated for the fungal community composition on the seed surface (Table 1, $p < .01$). A pairwise comparison showed that fungal community composition on the seed surface of *P. sylvestris* differed significantly between all regions ($p.adj < .05$) and between several regions on the *P. abies* seed surface (Table S7; Figure S2). For example, *P. abies* seeds from Poland hosted a different fungal community composition than all other regions except for the seeds from Belarus ($p.adj > .05$). Additionally, northern Sweden hosted a different fungal community composition than seeds from central and southern Sweden ($p.adj < .05$). Seeds from Finland had a different fungal community composition than seeds from central Sweden, while seeds from Belarus had a different

TABLE 1 Permutational multivariate analysis of variance (PERMANOVA) of the fungal community composition on the seed surface and in the seed tissue of *Picea abies*, *Pinus sylvestris* and *Larix* sp. seeds of different origin.

Sample type	Variable	df	SumOfSqs	R^2	*F*	Pr(>*F*)
Seed surface	Tot. reads	1	0.190	.021	1.571	.099
	Tree species	2	3.650	.396	15.112	**<.001**
	Region	6	1.391	.151	1.919	**<.01**
	Residual	33	3.985	.432		
	Total	42	9.216	1.000		
Seed tissue	Tot. reads	1	0.541	.033	1.493	.073
	Tree species	2	1.381	.085	1.906	**<.001**
	Region	6	2.352	.145	1.082	.263
	Residual	33	11.955	.737		
	Total	42	16.229	1.000		

Note: Significant differences ($p < .05$) are indicated in bold.

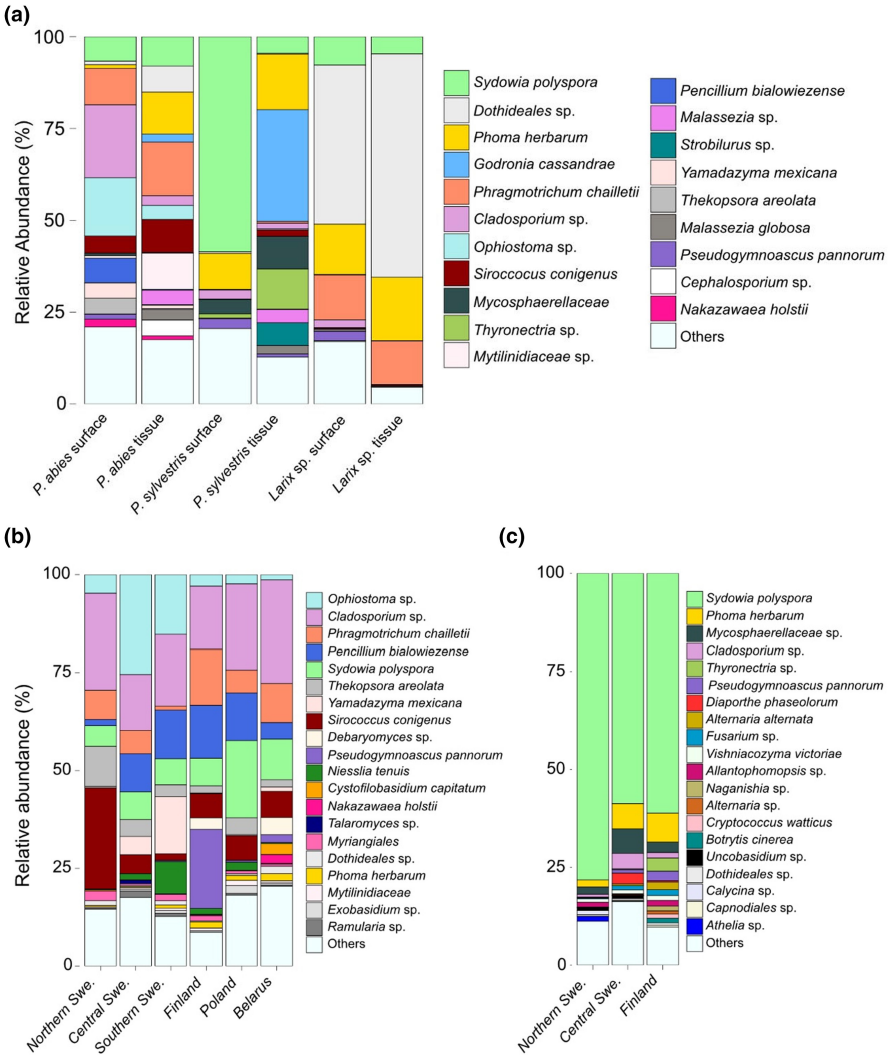

FIGURE 4 The relative abundance of the 20 most common fungal OTUs associated with (a) the seed surface and the seed tissue of *Picea abies*, *Pinus sylvestris* and *Larix* sp. seeds, and the geographical regions of (b) *P. abies* and (c) *P. sylvestris* seed surfaces. Remaining fungal OTUs are grouped into "Others."

fungal community composition compared to seeds from Sweden (Table S7; Figure S2; *p*.adj < .05).

The mean annual temperature correlated with seed orchard locations, being different between geographical regions of both *P. abies* ($R^2 = .94$, $p < .001$) and *P. sylvestris* ($R^2 = .82$, $p < .001$) seed origin (Figure S3). Similarly, the total annual precipitation was different between geographical regions of both *P. abies* ($R^2 = .38$, $p < .001$) and *P. sylvestris* ($R^2 = .30$, $p < .05$) (Figure S3).

The impact of seed-collection year was tested on the fungal community composition of *P. abies* and *P. sylvestris* seeds from northern, central and southern Sweden. The fungal community composition on the *P. abies* seed surface was significantly different between

seed orchards, which explained 25.1% of the variation, and between the seed-collection year, which explained 12.5% of the variation (Table S8; Figure S4). The interaction between the seed orchards and the years explained 11.4% of the variation. Similarly, the fungal community composition differed significantly between seed orchards on the *P. sylvestris* seed surface, which explained 21.3% of the variation, whereas the seed-collection year explained 6.2% and the interaction between the seed orchards and the years explained 13.6% of the variation (Table S8; Figure S4). Additionally, differences in sequencing depth were found to explain 11.5% of the variation of the fungal community composition found on the *P. sylvestris* seed surface. The fungal community composition in the seed tissue of different seed orchards was not significantly different ($p > .05$) but explained between 12.4% and 15.7% of the variation (Table S7). However, differences in sequencing depth explained a large part of the variation (10.3%–13.0%) and were significant from *P. abies* seed tissue samples.

3.3 | Seedborne plant pathogens detected on seeds of different host species and origin

Primary lifestyles of the 249 fungal OTUs on seed surface and/or in seed tissue were either plant pathogens (22.5%), saprotrophs (38.2%), mycoparasites (4.4%) and others (6.0%) or not taxonomically classified to be assigned a lifestyle (28.9%) (Table S2). Several fungal OTUs were frequently detected from both the seed surface and in the seed tissue (65.1%), and these were often among the most abundant fungal OTUs (Table S2). The remaining fungal OTUs were detected either on the seed surface (31.7%) or in the seed tissue (3.2%). The most common fungal OTUs detected on both sample types were *Sydowia polyspora* (13.3%), *Dothideales* sp. (13.2%), *Phoma herbarum* (11.2%), *Phragmotrichum chailletii* (8.4%) and *Cladosporium* sp. (7.0%) (Table S2).

Among the 20 most common fungal OTUs, all but four OTUs were detected in all tree species (Table S2). From *P. abies* and *P. sylvestris* seeds, most of the common fungal OTUs showed different relative abundance between the seed surface and the seed tissue (Figure 4; Table S2). Similar differences in relative abundance were not found between the seed surface and the seed tissue of *Larix* sp. seeds. The large geographical spread of the *P. abies* and *P. sylvestris* seed sources made it possible to investigate regional patterns of the common fungal OTUs, which was not possible for *Larix* sp. seeds. Furthermore, since the fungal community composition was significantly different between regions from the seed surface, but not the seed tissue (Table 1), the relative abundance of the 20 most common fungal OTUs for the seed surface is illustrated in Figure 4. *Cladosporium* sp. was evenly distributed between regions of *P. abies* (14.2%–26.3%), while *Ophiostoma* sp. were predominant in central (25.4%) and southern (15.3%) Sweden. *Sirococcus conigenus* had a high relative abundance in northern Sweden (25.8%), while low in central (4.8%) and southern (1.6%) Sweden (Figure 4). *Sydowia polyspora* was the most predominant fungal OTU on *P. sylvestris* in northern (78.2%) and central (58.7%) Sweden, as well as in Finland (61.1%).

An indicator species analysis resulted in twelve fungal OTUs being strongly associated with *P. abies* seed surface, eleven fungal OTUs with *P. sylvestris* seed surface and two fungal OTUs with *Larix* sp. seed surface (Table 2). *Sirococcus conigenus* was an indicator species for both the seed surface and the seed tissue of *P. abies* and in the seed tissue of *Larix* sp., while *Fusarium* sp. was an indicator species on the seed surface of *Larix* sp. and *P. sylvestris*. Seven fungal OTUs were strongly associated with all three tree species, indicating that they are all common fungal OTUs among tested *P. abies*, *P. sylvestris* and *Larix* sp. seeds (Table 2). *Phoma herbarum* and *S. polyspora* were two of these common fungal OTUs being frequent in seeds of all tree species (Table S2; Table 2). However, their relative abundance varied between the seed surface and the seed tissue. *Sydowia polyspora* showed a high relative abundance on *P. sylvestris* seed surface (58.6%), while *P. herbarum* showed a low relative abundance in *P. abies* seed surface (1.0%) (Figure 4; Table S2). *Dothideales* sp. (seed surface 43.3%; seed tissue 60.8%) were mainly detected on *Larix* sp. seeds, whereas *P. chailletii* on both *P. abies* (seed surface 9.9%; seed tissue 14.6%) and *Larix* sp. (seed surface 12.2%; seed tissue 11.8%) seeds. The fungus *Godronia cassandrae* (30.4%) was mainly detected in the seed tissue of *P. sylvestris* (Figure 4; Table S2).

4 | DISCUSSION

This study investigated seedborne fungi of three commercial coniferous tree species (*P. abies*, *P. sylvestris* and *Larix* sp.) using high-throughput sequencing. Seedborne fungal communities were distinguished between the seed surface and the seed tissue with host-specific fungal community composition. Additionally, fungal community composition on *P. abies* and *P. sylvestris* seed surface was defined by geographical regions. Seedborne fungal communities were also found to include a high proportion of fungal pathogens.

4.1 | Seedborne fungi on the seed surface and seed tissue of different host species

The present study showed a clear difference in fungal diversity and community composition between the seed surface and the seed tissue, as hypothesized. The fungal diversity was higher on the seed surface than in the seed tissue, especially prominent for the number of fungal OTUs observed from the seeds. Although many detected fungal OTUs were common to the different host tree species, the results showed a clear host-specific seedborne fungal community composition. The fungal community composition in the seed tissue was characterized by the host species, which was similar to previous reports on endophytic fungal communities from coniferous seeds (Franić et al., 2020), wood tissue (Romeralo et al., 2022) and needles (Arnold & Lutzoni, 2007). However, only about 9% of the variation of the community composition in the seed tissue was explained by

TABLE 2 Indicator species associated with seed surface and/or tissue of *Picea abies*, *Pinus sylvestris* and *Larix* sp., and with the combination of the sample types and the tree species.

		P. abies		*P. sylvestris*		*Larix* sp.		
OTU	Fungal taxon	Surface	Tissue	Surface	Tissue	Surface	Tissue	Ind. value
5765_15	*Penicillium bialowiezense*	x						0.98***
5765_9	*Thekopsora areolata*	x						0.92***
5765_81	*Lapidomyces* sp.			x				0.89***
5765_42	*Exobasidium* sp.	x						0.87***
5765_51	*Ramularia* sp.	x						0.86***
5765_127	*Phaeotheca fissurella*			x				0.84***
5765_97	*Capnodiales*			x				0.83***
5765_47	*Ramularia* sp.	x						0.83***
5765_6	*Ophiostoma* sp.	x						0.83**
5765_193	*Dothideomycetes*			x				0.82***
5765_164	*Helotiales*			x				0.81***
5765_28	*Niesslia tenuis*	x						0.81***
5765_68	*Allantophomopsis* sp.			x				0.81***
5765_54	*Dothideales*	x						0.80***
5765_165	*Rhizosphaera kalkhoffii*	x						0.77***
5765_176	*Collophora* sp.			x				0.77***
5765_208	*Aequabiliella* sp.	x						0.77***
5765_82	*Herpotrichiellaceae*			x				0.75***
5765_115	*Penicillium* sp.	x						0.74**
5765_161	*Microbotryomycetes*					x		0.74***
5765_14	*Yamadazyma mexicana*	x						0.73**
5765_225	*Cyphobasidiales*			x				0.72***
5765_184	*Ascomycota*			x				0.71***
5765_250	*Ascomycota*			x				0.70***
5765_122	*Microbotryomycetes*					x		0.70***
5765_45	*Cystobasidium* sp.					x	x	0.75**
5765_74	*Vishniacozyma tephrensis*			x			x	0.71**
5765_35	*Acremonium sclerotigenum*	x				x		0.85***
5765_38	*Fusarium* sp.			x		x		0.72**
5765_162	*Cyphellophora* sp.			x		x		0.72***
5765_5	*Cladosporium* sp.	x		x		x		0.92***
5765_65	*Chaetothyriales*	x		x		x		0.76***
5765_2	*Dothideales*	x				x	x	0.95**
5765_4	*Phragmotrichum chailletii*	x				x	x	0.88***
5765_72	*Yunzhangia*			x		x	x	0.84***
5765_11	*Mycosphaerellaceae* sp.	x		x	x			0.78*
5765_7	*Sirococcus conigenus*	x	x				x	0.74**
5765_10	*Mytilinidiaceae*	x	x			x	x	0.73*
5765_33	*Vishniacozyma victoriae*	x		x		x	x	0.75**
5765_52	*Diaporthe phaseolorum*	x		x		x	x	0.71**
5765_46	*Alternaria alternata*	x		x		x	x	0.70**
5765_1	*Sydowia polyspora*	x		x	x	x	x	0.92***
5765_0	*Phoma herbarum*	x		x	x	x	x	0.88*

Note: The indicator species analysis is based on non-rarefied filtered dataset of 249 fungal OTUs. Indicator species with an indicator value >0.70 are represented in the table. Significant values are indicated with *$p < .05$, **$p < .01$ and ***$p < .001$.

the tree species, whereas most of the variation (about 74%) was not explained by any tested factor. Thus, other variables not included in this study would probably be more important to explain the variation of fungal community composition in the seed tissue. For example, seed chemical composition, host-defence mechanisms, nutrition availability or interactions with other organisms could have been important contributions (Baldrian, 2017; Lebeis, 2015). Opposite to our hypothesis, host-specific fungal community composition was also detected on the seed surface, where tree species explained close to half of the variation in fungal community composition. A part of the fungal colonization of commercial coniferous seeds is suggested to occur in the seed orchards, or the seed stands, during the cone development (de la Bastide et al., 2019; Fraedrich & Miller, 1995). Since conifer cones are usually harvested directly from the tree branch while the cone scales are still closed, seed contamination from the forest floor (i.e., when cones are in direct contact with the ground) is likely to be small (Mittal & Wang, 1987). However, fungal contamination during the seed processing is expected to occur, for example, by *Penicillium* spp. (Mittal & Wang, 1987; Whittle, 1977). Nevertheless, if the impact from seed processing had been strong, the fungal community composition detected on the seed surface was expected to be more similar between the different tree species, which was not the case in this study. The strong host affinity in fungal community composition from the seed surface of these coniferous seeds suggests host-specific fungal colonization, probably occurring during cone development before cone harvest. Thus, the fungal colonization of the seed surface is probably influenced by horizontal transmission during cone development shaped by the uniformity of the seed orchards. Additionally, this result further suggests that the impact of contamination from the seed processing on fungal community composition might be limited to a few species.

The fungal community composition obtained from the seed tissue showed a more dispersed distribution among samples than from the seed surface samples which clustered more closely together. This could indicate that more specialized species are involved in the colonization of the seed tissue, introducing more variation than for the seed surface. Fungal colonization of the seed tissue would require the fungi to either penetrate the seed coat to enter the seed tissue or to be vertically transmitted from the mother plant into the seed tissue (Abdelfattah et al., 2021; Fort et al., 2021; Guan et al., 2020). Hence, the fungal colonization of the seed tissue partly relies on the success of the fungi in entering and establishing inside the seed tissue. In contrast, the fungal colonization of the seed surface could be any fungi actively or passively attached to the seeds.

The fungal diversity detected on the seed surface showed some differences between host species, indicating higher diversity on *P. abies* seeds than on *P. sylvestris* seeds. However, number of fungal OTUs were not different between tree species on the seed surface and no differences in fungal diversity were detected in the seed tissues. Additionally, several indicator species were detected for both *P. abies* and *P. sylvestris* seed surfaces, whereas *Larix* sp. seed surface only had a few indicator species associated with them. Plant species can have a significant influence on the diversity and structure of seedborne fungi, previously reported from a broad spectrum of plant families, that is, *Brassicaceae*, *Fabaceae* and *Poaceae* (Simonin et al., 2022). From conifers, seeds mature under the protected structure of their cones and the different cone structures between tree species could determine the fungal colonization of coniferous seeds (Tomlinson & Takaso, 2002).

4.2 | Geographical regions and yearly variations of seedborne fungal communities

In this study, we report that fungal communities found on *P. abies* and *P. sylvestris* seed surfaces change over a latitudinal gradient. As hypothesized, differences between geographical regions were found. All three geographical regions of *P. sylvestris* showed distinct differences in fungal community composition. In addition, several regions of *P. abies* seeds showed distinct fungal community composition. Similar trends were previously reported for endophytic fungal communities from *P. sylvestris* trees and needles (Arnold, 2007; Millberg et al., 2015; Terhonen et al., 2011). The assembly and structure of seedborne fungal community composition can be influenced by several factors, that is, temperature, precipitation, latitude, vegetation, interactions with host or other organisms, or nutrition availability, which can contribute to the observed regional differences (Baldrian, 2017; Lebeis, 2015; Ridout et al., 2019; Würth et al., 2019). Previously, specific site characteristics and local environmental variables have been suggested to impact the fungal communities in functional tissues (seeds, roots, shoots, and needles) of *P. sylvestris* and *P. abies* (Franić et al., 2020; Marčiulyniene et al., 2022; Mishcherikova et al., 2023). Thus, environmental variables could be a contributing factor in the present study, as seeds were collected from geographically separated seed orchards or seed stands. Although this study gives an indication of an impact of seed-collection year, a deeper sampling from the seed-collection years would be necessary due to uneven sequencing depth to further investigate the influence of seasonal variation on the fungal community composition.

The geographical differentiation of fungal communities suggests that traded seed lots could be the source of introducing new fungal species into an area, as previously reported by Franić et al. (2020). Given the strong host-associated fungal community detected in this study, the main concern would probably be the transmission of fungi between trees or seedlings of the same host species. In forest nurseries, where millions of seedlings of the same tree species are produced, even a small number of seed-transmitted infections can lead to a rapid spread of fungal diseases (Cram & Fraedrich, 2010; Lilja & Poteri, 2013; Mataruga et al., 2023). However, as no regional differences in fungal communities of *P. abies* and *P. sylvestris* seed tissue were found, the contribution and movement of seedborne fungi between regions are more likely to concern fungi harbouring the seed surface.

4.3 | Seedborne fungal pathogens

Seedborne fungi were mainly Ascomycota or Basidiomycota. The majority possessed a primary lifestyle as either plant pathogen or saprotroph, which was consistent with earlier reports on seedborne fungi from conifers (Cleary et al., 2019; Franić et al., 2020). To our knowledge, this is the first study to investigate tree-seed fungal communities using the PacBio platform. Here, the fungal OTUs often had high identification matches to either species or genus level, indicating that many detected fungi were common and/or well-studied species. The saprotrophs detected in the current study probably have a low impact on seed quality and seedling health as they in general are expected to be harmless to seeds. For example, two saprotrophic fungal OTUs detected in this study, *P. chailletii* and *Baeospora myosura*, are both common cone fungi of *Picea* species, but can also occur on *Larix* or *Pinus* species (Jeppson & Nilsson, 2005; Koukol et al., 2023). Several common plant pathogenic fungi, *S. polyspora*, *P. herbarum* and *S. conigenus*, identified in this study are known to infect commercial seeds or seedlings in forest nurseries. These species have been shown to prevent germination, causing damping-off or disease outbreaks among young seedlings (Cram & Fraedrich, 2010; Lilja & Poteri, 2013).

Sydowia polyspora was the most common fungus detected in the majority of samples, but with a predominant abundance on the seed surface of *P. sylvestris*, irrespectively of seed origin. *Sydowia polyspora* is often recognized as a foliar endophyte in conifers but it can also act as an opportunistic pathogen and cause infection in cankers or needles (Eo & Eom, 2022; Talgø et al., 2010). Previous studies have reported *S. polyspora* to be common on seeds of several *Pinus* sp. and to cause a strong reduction in germination of *P. ponderosa* seeds (Cleary et al., 2019; Ridout & Newcombe, 2018). Recently, *S. polyspora* was reported to occur in several *P. contorta* seed orchards and appeared in association with pollen, in needles, on cones and seeds after surface sterilization (de la Bastide et al., 2019). Even though *S. polyspora* often is considered a weak pathogen, it could be problematic given the high prevalence on the seeds.

Phoma herbarum, a widely distributed plant pathogen with a broad host range (Deb et al., 2020), was detected from all tree species. This fungus has been reported to occur in both the rhizosphere and the phyllosphere of nursery-grown *P. sylvestris* and *P. abies* seedlings (Larsson et al., 2023; Menkis et al., 2016; Okorski et al., 2019). *Phoma herbarum* was confirmed viable from the seeds (unpublished data) and was reported to cause infection in 1-year-old *P. abies* (Lilja et al., 2005) and *P. sylvestris* nursery-grown seedlings (Larsson et al., 2023). Still, there is no confirmed evidence of seed transmission of *Phoma* sp. into seedlings (Motta et al., 1996). However, seeds have been considered as an important source of inoculum and spread of several *Phoma* spp., for example, *Phoma koolunga* causing ascochyta blight of filed pea (Avila-Quezada & Rai, 2022; Khani, 2015). Given the high frequency of *P. herbarum* on seeds in the current study and previous reports on seedling infections in nurseries (Lilja et al., 2005; Okorski et al., 2019), the potential risk of seed-transmitted *P. herbarum* should be further investigated.

In the current study, *S. conigenus* was detected from all tree species, but predominantly from *P. abies* seeds, which is consistent with previous findings. *Sirococcus conigenus* is an important fungal pathogen that can infect several coniferous tree species but is reported as a seedborne fungal pathogen causing shoot die-back to mainly *Picea* spp. in forest nurseries (Cram & Fraedrich, 2010; Lilja et al., 2005; Motta et al., 1996). Seeds have been suggested as the main source of inoculum in containerized nursery seedlings, and a previous study reported the presence of *S. conigenus* throughout the endosperm and embryo tissue of diseased seeds (Cram & Fraedrich, 2010; Sutherland et al., 1981). Similarly, we found *S. conigenus* on both the seed surface and in the seed tissue of *P. abies* seeds.

A few, less abundant, fungal pathogens important for seed health and seedling production were detected in this study. For example, *Diplodia sapinea* appeared on the seed surface of both *P. sylvestris* and *P. abies* seeds. *Diplodia sapinea* is an opportunistic and latent pathogen of economic importance to many *Pinus* spp. and can occasionally infect *Abies* sp., *Picea* sp., *Larix* sp. and *Pseudotsuga* sp. as well (Stanosz et al., 1999). Severe incidence of *D. sapinea* was reported in North America, causing shoot blight and cankers on *Pinus resinosa* seedlings in forest nurseries (Palmer et al., 1988; Stanosz et al., 2005). Recently, *D. sapinea* was reported to infect *P. sylvestris* seedlings in European forest nurseries (Larsson et al., 2021). Furthermore, previous studies have reported *D. sapinea* on seeds of several *Pinus* spp. (Cleary et al., 2019; Decourcelle et al., 2015; Smith et al., 2015). Although Decourcelle et al. (2015) could not detect a seed transmission of *D. sapinea* to *Pinus nigra* subsp. *laricio* seedlings, the risk of seed transmission should not be excluded. The level of infected seeds can vary among different host species, and even a low infection level could result in a high number of seedling infections in forest nurseries.

Other less abundant fungal pathogens were *Fusarium* sp. and *Alternaria* sp., detected mainly on the seed surface of all tree species. Seedling infection caused by *Fusarium* sp. has been reported from several coniferous species in North America, South Africa and elsewhere in the world (Gordon et al., 2015). In forest nurseries, *Fusarium* sp. or *Alternaria* sp. are among the most important damping-off pathogens and are often seed-transmitted (Cram & Fraedrich, 2010; Lilja & Poteri, 2013). The disease incidence in nurseries does not necessarily correspond to the level of contaminated seeds since the pathogenicity of *Fusarium* spp. can vary between different species (Motta et al., 1996). However, correct species identification can be crucial. For instance, *Fusarium circinatum* is currently a quarantine pest on conifer seeds in the EU and should not be spread via seeds (EU Directive 2019/2023), while there are many *Fusarium* spp. that are not regulated. To separate different *Fusarium* species from each other can be difficult based on ITS region alone and may require the use of additional markers or species-specific primers (Chehri et al., 2015).

Thekopsora areolata, a fungus that infects mainly spruce cones and destroys seed production, appeared abundant on the seed surface of *P. abies* seeds. This fungal pathogen can cause severe infections of cones resulting in significant losses in seed production

(Kaitera & Tillman-Sutela, 2014). However, it is not recognized to be transmitted to seedlings and the risk of the spread via the nurseries is probably low.

4.4 | Implications for seedborne disease management

Good seed hygiene during seed collection and processing practices has been suggested as the main approach to avoid and lower the risk of seed-transmitted diseases and the spread of fungal pathogens (Lilja & Poteri, 2013; Martín-García et al., 2019). However, accurate preventative control measurements or curative control treatments require appropriate knowledge of when and where fungal pathogens are expected to colonize seeds. Seeds can be cleaned to remove surface pathogens by using water or mild disinfectants. This method can be effective against surface pathogens like *S. conigenus* (Neergaard, 1977). Additionally, hot water at specific temperatures can be used to eliminate both surface and internal pathogens, including *P. herbarum*, without damaging the seeds (Maude, 1996). Chemical fungicides that penetrate the seed coat can eradicate a range of fungal pathogens, for example, *S. polyspora* (Agrios, 2005). Another effective approach is to coat the seed surface with beneficial microorganisms that compete with or antagonize fungal pathogens (Harman, 2006). Treatment with plasma under atmospheric conditions has been shown to be effective against a broad spectrum of pathogens (Selcuk et al., 2008). Choosing an appropriate seed treatment method requires a good understanding of both the pathogens present and the ecological roles of associated fungal communities. Diagnostic assessments of fungal diversity before and after treatment are important for optimizing these methods (McDonald, 1998).

5 | CONCLUSION

Increased knowledge of seedborne fungi and more precise species identification of seedborne fungi in seed production and trade are necessary to avoid introducing seed- and seedling-damaging fungal pathogens into forest nurseries and forests. Seeds infected with fungal pathogens are easily overlooked without thorough examination since the seeds often look healthy through visual observations (Cram & Fraedrich, 2010). In conclusion, our results suggest the seedborne fungal diversity and community composition to be characterized by the seed surface and the seed tissue. Furthermore, the results showed a high host affinity of fungal communities associated with the seed surface of *P. abies*, *P. sylvestris* and *Larix* sp. The large geographical spread of the seed sources of *P. abies* and *P. sylvestris* enabled an investigation into the regional dependence of the fungal community composition. Our findings showed significant regional differences of both *P. abies* and *P. sylvestris* seed surfaces. Thus, the movement of seeds between different regions could contribute to the introduction of fungal pathogens into forest nurseries. The seeds of all tree host species were found to harbour a high proportion of important plant pathogens such as *S. polyspora*, *P. herbarum* or *S. conigenus*. However, using accurate preventative control measurements or curative control treatments, the occurrence of pathogenic fungi on the seed surface can be reduced.

AUTHOR CONTRIBUTIONS

Rebecca Larsson: conceptualization, sampling and laboratory work, data analysis and visualization, interpretation of the result, writing the first draft, and reviewing. Audrius Menkis and Åke Olson: conceptualization, interpretation of the result, writing and reviewing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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DATA AVAILABILITY STATEMENT

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

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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