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Spatial and temporal dynamics of seedborne, airborne, and foliar fungal communities in Swedish forest nurseries

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Cover: View of Stakheden nursery with tree seedlings in the foreground and greenhouses in the background (photo by Åke Olson).

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Abstract

Fungal infections in forest nurseries can significantly affect the economic sustainability of tree seedling production. Increased knowledge of local fungal communities, especially prevalent pathogens, could improve preventative and targeted control strategies, ultimately reducing the usage of fungicides. This thesis aimed to acquire new knowledge and advance a general understanding of seedborne, airborne, and foliar fungal communities in Swedish forest nurseries. The studies were conducted in five forest nurseries. Fungal communities were assessed from *Picea abies*, *Pinus sylvestris*, and *Larix* sp. seeds differentiated between the surface and the tissue (paper I), non-symptomatic *P. sylvestris* needles (paper II), and deposited spores (paper IV) using high-throughput sequencing of the ITS2 rDNA region. Results showed that seedborne fungal communities were distinguished between the seed surface and the seed tissue, where fungi from the seed surface showed a strong host-affinity and regional dependence. This result suggests that the movement of seeds between different geographic regions, including frequently traded seeds, could be a potential source of the spread and introduction of fungal species. The foliar and airborne fungal communities comprised high species richness and showed clear temporal shifts over the seedling growing seasons. The experiments included microbial treatments to control fungal pathogens and stimulate seedling growth on *P. sylvestris* seedlings. However, the use of microbial treatments was not found to impact the seedling growth or the disease incidence, which was generally low, and had no impact on the foliar fungal communities. All the fungal communities showed a high prevalence of nursery fungal pathogens, e.g., *Cladosporium* sp., *Botrytis cinerea*, *Phoma herbarum*, and *Sydowia polyspora*. In this thesis, the disease incidence of *Diplodia sapinea* in *P. sylvestris* seedlings was confirmed (paper III). Additionally, low abundances of *D. sapinea* were detected from the seeds and the deposited airborne spores. The findings in this thesis may contribute to developing disease management strategies in forest nurseries owing to

a better understanding of fungal communities, thereby highlighting the importance of managing fungal pathogens to maintain healthy tree seedling production.

Keywords: conifers, fungal disease, high-throughput sequencing, Norway spruce, pathogens, Scots pine, seed orchard, spore trap, tree seedling.

Rumslig och tidsmässig dynamik hos svampsamhällen som är fröburna och luftburna, samt härrör från tallbarr, i svenska skogsplantskolor

Sammanfattning

Svampinfektioner i skogsplantskolor kan avsevärt påverka den ekonomiska hållbarheten vid produktion av skogsplantor. Ökad kunskap om lokala svampsamhällen, särskilt vanliga skadesvampar, skulle kunna förbättra förebyggande och riktade kontrollstrategier, vilket i slutändan kan minska användningen av fungicider. Den här avhandlingen syftade till att generera ny kunskap och främja en allmän förståelse för svampsamhällen som är fröburna och luftburna, samt som härrör från tallbarr, i svenska skogsplantskolor. Studierna har genomförts i fem skogsplantskolor. Svampsamhällen från gran- (*Picea abies*), tall- (*Pinus sylvestris*) och lärkfrön (*Larix* sp.), fröytan respektive frövävnaden (artikeln I), icke-symptomatiska tallbarr (artikel II) och svampsporer (artikel IV) analyserades med hjälp av sekvensering av ITS2 rDNA-regionen. Resultaten visade att fröburna svampsamhällen skiljde sig åt mellan fröytan och frövävnaden, där svampsamhällena från fröytan dessutom var åtskilda mellan trädslag och region. Dessa resultat tyder på att förflyttning av frön mellan olika geografiska regioner, inklusive kommersiella frön, kan vara en potentiell källa till spridning och introduktion av svamparter. De barr- och luftburna svampsamhällena bestod av hög artrikedom och visade tydliga strukturella skiftningar över växtsäsongerna. Försöken inkluderade även biologiska behandlingar som verkar mot skadesvampar och kan stimulera planttillväxt. Användningen av biologiska behandlingar visade sig dock varken påverka planttillväxten eller andelen sjuka plantor, som generellt var låg. Det fanns inte heller någon påverkan på tallbarrens svampsamhällen från behandlingarna. Samtliga svampsamhällena visade en hög förekomst av skadesvampar som är vanliga i plantskolor, t.ex. *Cladosporium* sp., *Botrytis cinerea*, *Phoma herbarum* och *Sydowia polyspora*. I den här avhandlingen bekräftades infektion orsakad av *Diplodia sapinea* i tallplantor. En låg förekomst av *D. sapinea* bekräftades även från frön och de luftburna sporerna. Resultaten i denna avhandling

bidrar med kunskap om svampsamhällen i skogsplantskolor och belyser vikten av att hantera skadesvampar i plantskolorna för att upprätthålla en frisk produktion av skogsplantor.

Nyckelord: barrträd, fröplantage, gran, lärk, skadesvamp, skogsplanta, sporfälla, svampsjukdom, tall.

Dedication

To my family

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Larsson, R.**, Menkis, A., Olson, Å. (2024). Fungal communities associated with *Picea abies*, *Pinus sylvestris*, and *Larix* sp. seeds of different geographical origin: implications for disease management. (*submitted*)

II. **Larsson, R.**, Menkis, A., Skogström, O., Espes, C., Brogren-Mohlin, E-K., Larsson, M., Olson, Å. (2023). Seasonal dynamics in foliar fungal communities of nursery-grown *Pinus sylvestris* seedlings. Scandinavian Journal of Forest Research. doi: 10.1080/02827581.2023.2277745. (*published*)

III. **Larsson, R.**, Menkis, A., Olson, Å. (2021). *Diplodia sapinea* in Swedish forest nurseries. Plant Protection Science, 57 (1), pp. 66–69. doi: 10.17221/68/2020-PPS. (*published*)

IV. **Larsson, R.**, Menkis, A., Olson, Å. (2024). Temporal dynamics of airborne fungi in Swedish forest nurseries. (*manuscript*)

The contribution of Rebecca Larsson to the papers included in this thesis was as follows:

I. Contributed to conceptualisation and experimental design, coordinated and performed sampling and laboratory work, was the lead author of data analysis, visualisation, and writing. Responsible for correspondence with the journal.

II. Contributed to conceptualisation and experimental design, coordinated and performed field and laboratory work, was the lead author of data analysis, visualisation, and writing. Responsible for correspondence with the journal.

III. Contributed to laboratory work, was the lead author of visualisation and writing. Responsible for correspondence with the journal.

IV. Contributed to conceptualisation and experimental design, coordinated and performed field and laboratory work, was the lead author of data analysis, visualisation, and writing.

List of figures

Abbreviations

HTS	High-throughput sequencing
IPM	Integrated pest management
ITS	Internal transcribed spacer
NMDS	Nonmetric multidimensional scaling
OTU	Operational taxonomic unit
PacBio	Pacific BioSciences
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational multivariate analysis of variance
qPCR	Quantitative PCR

1. Introduction

Boreal forests, dominated by coniferous tree species, belong to one of the largest biomes on the Earth, covering vast parts of North America and Eurasia (Farjon 2018). Providing several important ecosystem services (e.g., wood and fibre production, local climate regulation, carbon sequestration, ecotourism), boreal forests have a high impact on the environment and human welfare (Millennium Ecosystem Assessment 2005; Farjon 2018).

For centuries, Sweden has utilised forest raw materials to sustain important industries, from steel and iron production to the modern saw timber and pulp industry (Royal Swedish Academy of Agriculture and Forestry 2015). Today, productive forests possess a high economic value and cover more than 50% of the total land area (Hallsten & Jensen 2022; Skogsstyrelsen 2024b). The main tree species in the production forests are the native conifers Norway spruce (*Picea abies* (L.) Karst.) and Scots pine (*Pinus sylvestris* L.), used for their high timber quality and growth capacity (Pâques 2013). Together, they contribute to 77% of the standing volume (Riksskogstaxeringen 2023). In addition to *P. abies* and *P. sylvestris*, *Betula* spp. contribute to 15% of the standing volume, and the remaining 8% consist of 22 conifer and broadleaved tree species (e.g., *Larix* spp., *Pinus* spp., and *Pseudotsuga menziesii* (Mirb.) Franco).

The production forests are mainly managed using an even-age approach, including harvesting and reforestation with long rotation times (Royal Swedish Academy of Agriculture and Forestry 2015). Since 1993, environmental and conservation considerations of forestry practices have been added to the forest policy to ensure sustainable forest management. Regulated by the Forestry Act (SFS 1979:429), forest owners are obligated to ensure reforestation after clear-cutting, which is often achieved by planting nursery-produced tree seedlings. Consequently, the annual

production of forest tree seedlings now exceeds 400 million seedlings, mainly consisting of *P. abies* and *P. sylvestris* (Mataruga *et al.* 2023).

Fungal diseases are among the biggest challenges in forest tree seedling production and can jeopardise forest reforestation, as well as cause tremendous economic losses in forest nurseries. Furthermore, disease management can be expensive and often requires fungicide treatments. Repeated use of fungicides poses environmental hazards and the risk of resistance developed by pathogens, which is an ongoing obstacle in forest nurseries (Nielsen *et al.* 2024). Not only are disease outbreaks problematic for forest nurseries, but infected tree seedlings also pose a risk for the spread of diseases into the forests. Apart from well-known fungal diseases (e.g., grey mould or root-rot diseases), new and emerging diseases (e.g., Diplodia tip blight) can be expected to threaten tree seedling production and reforestation in the future (Stenlid *et al.* 2011).

Climate change and the transportation of goods and people are generally thought to be responsible for the arrival and spread of new diseases (Stenlid *et al.* 2011). A future climate, including higher frequency and intensity of dry and warm summers, as well as changed precipitation patterns, can be expected to create other challenges for disease management in forest nurseries (IPCC 2014; Anderegg *et al.* 2022). Furthermore, tree seedlings under more stressful conditions are more likely to be affected by opportunistic pathogens, which increases the risk of infection and disease development.

Increased knowledge of fungal communities could give a better understanding of fungal dynamics associated with tree seedling production. Investigating the seedborne fungal communities could explain the relative contribution of fungi residing on the seed surface to those residing in the seed tissue of different tree species. Furthermore, improved knowledge of the spread of fungal spores could explain the temporal dynamics of airborne inoculum over the growing seasons. Finally, the foliar communities of tree seedlings could reveal the presence of fungi on the needles that are important for seedling health. Thus, early detection of fungal infection and prediction of disease outbreaks could be achieved by identifying the potential introduction and transmission of fungal pathogens in forest nurseries. In this thesis, I provide new knowledge of seedborne, airborne, and foliar fungal communities associated with nursery-grown conifer seedlings in Swedish forest nurseries.

2. Background

2.1 Fungal communities

2.1.1 Functional groups and interactions of fungi

Fungi are heterotrophic organisms which have evolved several strategies to acquire nutrients. These strategies include different traits (e.g., morphological, physiological, genetic, and enzymatic), characterising fungi into functional groups. These groups are often referred to as guilds, such as plant pathogens, saprotrophs, mycorrhiza, or mycoparasites (Treseder & Lennonb 2015; Zanne *et al.* 2020). The functional groups of fungi can be associated with different impacts on plant health. Plant pathogens affect plant health by retrieving nutrients from their host. In a biotrophic mode, they obtain their nutrition directly from living plant tissue (Hamelin 2022), whereas in a necrotrophic mode, they obtain their nutrients by degrading dead plant tissue (Govrin & Levine 2000). Saprotrophs are often harmless to living plants as they obtain their nutrients by decomposing dead organic matter (Baldrian & Valásková 2008), while mycorrhizal fungi are beneficial to plants by forming mutualistic symbioses with their host to exchange nutrients (Marschner & Dell 1994).

The transition of fungal species among functional groups is highly variable and difficult to define. Many fungi can switch between different strategies and change their lifestyle in response to environmental conditions (Promputtha *et al.* 2007; Zanne *et al.* 2020). This is often recognised among the group of endophytes, which includes fungi residing inside the host tissue. The term “endophyte” usually implies fungi that colonise the plant tissue asymptomatic, i.e., without causing visible disease symptoms, but also

includes fungi that reside within healthy plant tissue with the ability to infect under favourable conditions (Petrini 1991; Schulz & Boyle 2005). Thus, a fungus could have a latent endophytic life stage inside its host until conditions become favourable for the fungus, often under conditions stressful for the host, and then turn into a pathogenic life stage and cause disease. In contrast to endophytes, fungi residing on the surface of the host tissue are considered epiphytes. In conifers, epiphytes are suggested to be dominated by nonspecific generalists (Legault *et al.* 1989), whereas endophytes would be dominated by a few host-specific species (Sieber 2007). Endophytes and epiphytes play a vital role in plant health, as they can include both harmful plant pathogens and beneficial symbionts. Moreover, fungal pathogens can interact with their host in a latent phase without causing symptoms, only to infect the host when conditions change. These pathogens are commonly considered as "opportunistic" pathogens.

The plant-associated microbiomes are complex, and a single tree provides several habitats, such as the foliage, the seeds, the wood, and the roots (Baldrian 2017). Foliage (from shoots or needles) and seed-inhabiting fungi play various ecological roles. Fungal-fungal interactions can be beneficial for plants and modify disease development. For example, non-pathogenic and pathogenic fungi can form interactions by hyper-parasitism, antibiosis, or competition (Busby *et al.* 2016). Antagonistic interactions in tree foliar systems have previously been reported to reduce disease severity. For example, the disease severity of *Melampsora* leaf rust in *Populus* spp. was reduced by *Stachybotrys* sp. and *Trichoderma atroviride* P. Karst. (Raghavendra & Newcombe 2013). Similarly, *Penicillium* sp. was shown to modify and lower the disease severity of *Dothistroma* pine needle blight in *Pinus ponderosa* Dougl. ex. Law. (Ridout & Newcombe 2015). Plant-fungal interactions can also be important through several mechanisms, e.g., by increased nutrient uptake, stress tolerance, and plant growth (Hardoim *et al.* 2015). In forest nurseries, the tree seedling root-system can harbour beneficial microorganisms, such as mycorrhizal fungi, which can provide nutritional benefits to the seedlings and potentially improve seedling growth and survival in nurseries and after outplanting (Menkis *et al.* 2007).

2.1.2 Spread and assembly of fungi in forest nurseries

In forest nurseries, fungi can be introduced and spread through different pathways. For example, fungi can be introduced into nurseries as seedborne

fungi and, if seed-transmitted, spread among seedlings. Furthermore, fungi can spread via spores from the surrounding environment, e.g., infected seedlings, other vegetation, production facilities, or equipment. Many fungal pathogens spread via sexual and asexual spores, and their dispersal serves as a primary process in transmitting various diseases.

Fungi can be seedborne by having spores externally attached to the seed surface, internalising within the seed tissue, or residing internally from the mother plant (Shade *et al.* 2017; Gaur *et al.* 2020). Conifer-associated seedborne fungi are likely influenced by spores already spread during cone development (Whittle 1977). They could also be induced by fungal pathogens that colonise and reproduce on cones, e.g., *Diplodia sapinea* (Fr.) Fuckel [syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.) Dyko & Sutton] (Oliva et al. 2013) or *Sirococcus conigenus* (Pers.) P.F. Cannon & Minter (Smith et al. 2003). Many seedborne saprotrophs are not expected to influence seed performance when used in forest nurseries, while seedborne pathogens can be seed-transmitted and reduce seed germination or cause damping-off diseases (Cram & Fraedrich 2010; Lilja & Poteri 2013).

In addition to seeds, spores can spread to and within forest nurseries by air. Specific characteristics of the fungi will define the presence of spores in the air, e.g., the production and release mechanism of the spores, the size and shape of spores, and the distance to the spore source (Van der Heyden *et al.* 2021). Large and small spores are expected to have different dispersal distances, and some spores can spread up to several kilometres (Brown & Hovmøllller 2002; Golan & Pringle 2017).

The assembly of fungal communities is influenced by both biotic (e.g., surrounding vegetation, host species, needle age) and abiotic (temperature, precipitation, latitudinal gradients) factors. Furthermore, previous studies have reported host species to characterise conifer-associated fungal communities, both in needles (Higgins *et al.* 2007; Apigo & Oono 2022) and seeds (Franić *et al.* 2020). Thus, the nursery location could potentially reflect associated fungal communities due to local environmental and climatic differences. Fungal communities are suggested to differentiate between geographically separated regions following the general biogeographical pattern (i.e., the fungal diversity increases towards the equator) (Arnold 2007; Tedersoo *et al.* 2014). However, this is not always straightforward, and previous reports have shown contrasting results (Higgins *et al.* 2007).

The association between latitude and fungal community structures is not necessarily directly linked. Instead, other parameters determined by location, e.g., temperature and precipitation, could contribute more to the assembly of fungal communities. Furthermore, seasonality and variations in weather conditions during the growing season could cause temporal variation in fungal community composition within the forest nurseries (Peay & Bruns 2014; Crandall & Gilbert 2017).

2.1.3 Methods to study fungal communities

Today, fungal communities are commonly studied using metabarcoding, which gives relative abundance and DNA-based identification of taxa in environmental samples containing a mix of species. In general, the procedure of metabarcoding includes the following steps: 1) sampling and DNA extraction and 2) amplification of a genetic marker using polymerase chain reactions (PCR) and tagged primers for a specific organism group, followed by 3) high-throughput sequencing (HTS) (Tedersoo *et al.* 2022).

In fungal community studies, sequencing of the ITS region has become the most frequently used genetic marker in metabarcoding (Begerow *et al.* 2010; Nilsson *et al.* 2019). The ITS region includes part of the small subunit, the 5.8S gene, and part of the large subunit of the ribosomal RNA, interlinked by ITS1 and ITS2 (Schoch *et al.* 2012; Nilsson *et al.* 2019). To achieve species-level identification for fungal communities, ITS1 and/or ITS2 can be used as these regions are highly variable between different species (Schoch *et al.* 2012). However, the ITS2 region has been recommended for broader taxonomic coverage (Nilsson *et al.* 2019).

The rapid development of HTS technologies has given higher resolution to fungal community studies and increased the potential of analysing diversity, ecology, function, and distribution attributes. Several HTS platforms are currently available, although providing different capacities and precision to sequence DNA fragments of different lengths (Nilsson *et al.* 2019; Castaño *et al.* 2020). Since the ITS2 region is considered to include a size variation across groups of fungi, the Pacific Biosciences (PacBio) platform has been recommended for better representation of sequences of different lengths (Castaño *et al.* 2020).

2.2 Production of forest tree seedlings

2.2.1 From seed to seedling

Tree seeds used for forest seedling production in Sweden either originate from selected forest stands of good quality or are produced in seed orchards (Haapanen *et al.* 2015). Seed orchards are intensively managed plantations of selected tree genotypes targeted for multi-traits, including better growth capacity, good wood quality, improved vitality, and higher survival (Rosvall 2011). Cones from seed stands or orchards are collected during years of high yield, and those years vary between regions and tree species. For example, *P. abies* often has an irregular flowering, which causes uneven seed production compared to *P. sylvestris*, which has a more regular seed production as it flowers yearly (Jansson *et al.* 2013; Krakau *et al.* 2013). Consequently, seeds are often traded between countries within Northern Europe, especially in years with low *P. abies* seed production (Solvin *et al.* 2021). After the harvest, cones are dried to about 10% moisture content (Belcher & Lowman 1982; Fennessy 2002). Seeds are then extracted and cleaned, sorted for size and quality, and tested for the germination rate (Wennström *et al.* 2016). High-quality seeds are grouped into different seed batches based on their origin and year of seed collection.

In Sweden, forest nurseries have become a highly advanced industry producing forest seedlings of different tree species and seedling sizes. The production of *P. sylvestris* seedlings in 2022 corresponded to 50.6% of the total seedling production (422 million), whereas the production of *P. abies* seedlings was 45.6% (Skogsstyrelsen 2024a). The remaining tree species produced were *Pinus contorta* Dougl. ex. Loud. (1.8%), *Larix* sp. (0.8%), *Betula* spp. (0.4%), and other conifer and broadleaved tree species (0.6%).

Most seedlings are produced as containerised seedlings, i.e., densely cultivated in multi-cell growing trays elevated from the ground (**Figure 1)**

Figure 1. Procedure of forest nursery practices. Cultivation trays are transported by trucks after sowing (top) and placed in the green houses (middle). When seedlings are 5 – 10 weeks old they are transported outdoor for further cultivation (bottom). Photos by Audrius Menkis (top and middle) and Rebecca Larsson (bottom).

(Lilja *et al.* 2010; Menkis *et al.* 2016). The tray cells vary in volumes of 30–115 cm^3, giving cultivation densities of 400–1300 seedlings m^2 (Lilja *et al.* 2010; Wennström *et al.* 2016). The seeds are sown from March to June using automatic sawing machines in either wet or dry *Sphagnum* peat (Lilja *et al.* 2010). For the first five to ten weeks, seedlings are cultivated in greenhouses under controlled environmental conditions before being moved to outdoor cultivation (**Figure 1)**. All transportation of the seedlings around the nurseries is done by trucks, with the seedling containers positioned in large metal frames (**Figure 1**).

During the cultivation period, the seedlings are subjected to several management practices, e.g., regular fertilisation, irrigation from above, fungicide treatments and occasional herbicide treatments (Lilja *et al.* 2010). Cultivation of the seedlings proceeds until they are packed in carton boxes, ranging from August to December. The seedlings are then either delivered directly into the forest for direct outplanting or stored at – 4°C before being delivered frozen for outplanting from April to June of the following year (Lilja *et al.* 2010; Wennström *et al.* 2016). In addition, seedlings can be kept outdoors the whole winter. They are then protected by a snow cover applied on top of the seedlings.

2.2.2 Nursery-associated fungal diseases

Before the containerised production system was introduced in Sweden during the 1970s (Nyström 1983), several associated diseases have caused problems in forest nurseries (Unestam & Beyer-Ericson 1990; Lilja & Poteri 2013; Poteri *et al.* 2021). Forest nurseries, owing to the intensive seedling production, stand out as highly favourable environments for fungal pathogens. The dense cultivation systems create conditions favourable for the establishment of fungal pathogens, and the large monocultures increase the risk for rapid spread of fungal infections among the seedlings (Menkis *et al.* 2006).

The most damaging fungal pathogen in the forest nursery is the worldwide distributed *Botrytis cinerea* Pers.:Fr., causing shoot and needle infections in forest seedlings (Mittal *et al.* 1987; Poteri *et al.* 2021). It is a facultative parasite with a broad host range, though most seriously affecting conifer seedlings in forest nurseries. *Botrytis cinerea* can be seedborne or airborne. Seed-transmitted grey mould often results in poor or lack of seed germination, whereas initial symptoms of airborne transmission commonly

are found on dead needles on the lower part of the stem of the densely cultivated seedlings (Mittal *et al.* 1987). Other frequently occurring symptoms include top dieback (**Figure 2**), necrotic wounds on the stem, or yellow spots on the needles (Lilja & Poteri 2013).

Damping-off and root rot diseases are often seed-transmitted and reduce germination or damage young emerging seedlings (Cram & Fraedrich 2010; Lilja & Poteri 2013). The most important pathogens causing damping-off diseases in conifer seedling production belong to the genera of *Fusarium*, *Phytophthora*, *Pythium*, *Rhizoctonia*, *Cylindrocladium*, and *Neonectria* (Sutherland *et al.* 2002; Menkis *et al.* 2006; Cram & Fraedrich 2010; Lilja & Poteri 2013). Shoot blight, caused by *S. conigenus* is another seed-transmitted disease that can also damage current-year shoots (**Figure 2**) (Sutherland *et al.* 2002). Once seedlings get infected and spores are produced, these can easily spread to new seedlings of several conifer tree species (Sutherland *et al.* 2002; Lilja *et al.* 2010).

Figure 2. A one-year-old *P. sylvestris* seedling infected by *B. cinerea* (left, photo by Rebecca Larsson) and one-year-old *P. contorta* seedlings infected by *S. conigenus* (right, photo by Audrius Menkis).

Other important pathogens are *Lophodermium seditiosum* Minter, Staley & Millar, *Gremmeniella abietina* (Lagerb.) M. Morelet, and *Melampsora populnea* (Pers.) P. Karst. [syn. *Melampsora pinitorqua* Rostr.], mainly damages *P. sylvestris* seedlings in the Nordic region. Infection by *L. seditiosum* causes needle cast and is favoured by high precipitation during late summer and autumn, when the airborne ascospores can travel long distances (Diwani & Millar 1986; Stenström & Ihrmark 2005). Even though *L. seditiosum* does not always kill its host; years of severe infections can cause substantial needle loss (Lilja & Poteri 2013). Furthermore, the actual disease symptoms are often not visible in the one-year-old seedlings in the forest nurseries. They are often recognised the following spring when the infected needles turn brown and fall off, generally observed after the spring planting.

Similarly, the infection process by *G. abietina* in both *P. abies* and *P. sylvestris* seedlings occurs during the summer. Still, symptoms are often not visible until the end of the growing season and most often not until the next spring (Petäistö & Laine 1999; Petäistö 2008). *Gremmeniella abietina* infects shoots and causes rot at the base of needles, which eventually fall off. This fungus is favoured by two consecutive years of cool and rainy summers (Lilja & Poteri 2013).

Rust diseases on conifers are a minor problem for the nurseries. Still, under the right conditions (i.e., warm and humid conditions during May and June), infection of *M. populnea* can occur, causing pine twisting rust in *P. sylvestris* seedlings (Martinsson 1985). However, since *M. populnea* is alternating with poplars (*Populus* spp.), the disease incidence can be reduced by removing alternating hosts from the vicinity of the nurseries (Lilja & Poteri 2013).

Climatic change and stronger regulations of available control products for disease management pose risks for new and emerging diseases in forest nurseries. Previous reports have shown the non-symptomatic presence of *Phoma* sp. in both roots and stems of nursery-grown seedlings (Menkis *et al.* 2016; Okorski *et al.* 2019), as well as infected *P. abies* seedlings (Lilja *et al.* 2005). Despite these findings, only in recent years have the reports of increased disease outbreaks caused by *Phoma* sp. come from the nurseries. Species of *Phoma* cause tip blight or dieback in conifer seedlings as they infect the stem and eventually cause needle loss (Kliejunas *et al.* 1985).

Diplodia sapinea is another potential threat. This is a common fungal pathogen mainly found in *Pinus* spp. but occurs on several other coniferous hosts (Stanosz *et al.* 1999). *Diplodia sapinea*, causing tip blight, has severely damaged *Pinus resinosa* Sol. ex. Aiton and *Pinus banksiana* Lamb. seedlings in North American forest nurseries (Stanosz *et al.* 2001; Stanosz *et al.* 2007). Still, no large disease outbreak has been observed among *P. sylvestris* seedlings. However, recent reports suggest that *D. sapinea* is expanding northwards, and a warmer climate could potentially increase the risk of infections in the Swedish forest nurseries (Oliva *et al.* 2013; Brodde *et al.* 2019).

2.2.3 Integrated pest management

Legislation in the EU enforces plant production to follow the Integrated Pest Management (IPM) principles (EU DIRECTIVE 2009/128/EC). IPM includes several components and addresses the following aspects of pest management options: the required knowledge and resources needed to develop management strategies, the importance of monitoring and timely action, and the communication to transfer knowledge for the benefit of everyone (Dara 2019).

As part of the IPM strategy, forest nurseries should avoid or reduce the use of fungicides as much as possible. Primarily, preventative control methods against fungal infections should be used during seed processing and seedling production, e.g., good hygiene, cleaning of equipment and greenhouses, removal of infected cones or seedlings, and reduced humidity among the densely cultivated seedlings (Sutherland *et al.* 2002; Cram & Fraedrich 2010; Lilja & Poteri 2013; Poteri *et al.* 2021). The predisposition to fungal infections could be avoided through increased seedling spacing. However, in current practice, a reduction in seedling spacing is not applied due to economic constraints.

Fungicidal applications are used in forest nurseries to reduce the infection and prevent the spread of diseases (Lilja & Poteri 2013; Poteri *et al.* 2021). However, while fungicides reach the desired effect on targeted fungal pathogens, they can also substantially reduce the abundance of non-target fungal species (Noel *et al.* 2022). In addition, the use of fungicides involves a high risk of environmental hazards (Carisse 2010). Consequently, some products previously used in forest nurseries are currently prohibited or strongly restricted within the EU (Kemikalieinspektionen 2019;

Kemikalieinspektionen 2023). For example, available means of disease control against infection caused by *L. seditiosum* is prohibited (Tilt®) and the control of *S. conigenus* is reduced (Amistar®). Furthermore, the reduced effectiveness of fungicides due to the resistance development in fungal pathogens is a continuous problem. A recent study found fungicide resistance to several commonly used product formulations in *B. cinerea* isolated from nursery-grown *P. abies* seedlings (Nielsen *et al.* 2024).

Adding beneficial microbes could be a sustainable option to improve seedling growth conditions and inhibit disease outbreaks in forest nurseries. For example, fermented soil amendment, including beneficial microorganisms, may increase seedling growth and survival following outplanting (Jaramillo-López *et al.* 2015). Biological control, as an alternative to chemical control, is another option which may inhibit fungal pathogens and suppress disease development. Numerous plant-beneficial microorganisms (e.g., *Bacillus subtilis* (Ehrenberg) Cohn, *Clonostachys rosea* (Link) Schroers & al., *Trichoderma* spp.) are applied as biological control agents for their abilities to control pathogens, improve growth conditions, and stimulate defence mechanisms (Benítez *et al.* 2004; Fravel 2005). Although biological control against *B. cinerea* has been used in nurseries over the last two decades, the efficiency is still unclear, and the use has not yet been fully implemented (Capieau *et al.* 2004).

3. Aims and Objectives

Knowledge about fungal communities, especially fungal pathogens, associated with conifer seedling production can potentially improve preventative control measures and decision-making to reach the goals of IPM. This thesis aimed to acquire new knowledge and advance a general understanding of seedborne, airborne, and foliar fungal communities in Swedish forest nurseries. The thesis focused on five geographically separated forest nurseries in Sweden. Different aspects of fungal spread were investigated by studying the spatial and temporal dynamics of fungal communities following the growing period of the seedlings. The specific objectives were:

In **paper I**, to assess the diversity and composition of seedborne fungal communities from commercial *P. abies*, *P. sylvestris*, and *Larix* sp. seeds of different origins. Seedborne fungal communities were hypothesised to differentiate between the seed surface and the seed tissue for each tree species. Furthermore, the relative importance of tree species would be higher for fungi associated with the seed tissue than for the seed surface, as generalists might dominate the seed surface. Fungal communities were expected to be different between tree species and to differentiate based on the geographical location of their seed origin.

In **paper II**, to assess the diversity, composition, and dynamics of foliar fungal communities in *P. sylvestris* seedlings in four forest nurseries. In this experiment, four commercial microbial products were applied, and seedling growth, survival, and disease incidence were analysed. *Pinus sylvestris* seedlings were hypothesised to host a high diversity of foliar fungi, which would alter from lower to higher diversity with the ageing of the seedlings.

In addition, a higher growth rate and lower disease incidence were expected among seedlings subjected to microbial treatments. Within the same experiment, in **paper III,** *P. sylvestris* seedlings infected with *D. sapinea* were described in two forest nurseries through morphological identification and species-specific PCR assay.

In **Paper IV**, to assess the fungal diversity and community composition of airborne spread in four forest nurseries. Deposited spores were obtained using spore traps following two growing seasons. The occurrence of fungal pathogens relevant to forest nurseries was estimated using relative abundance data and quantitative measures of the deposited spores and correlated to local weather conditions. Temperature and precipitation were hypothesised to impact the airborne spread of the fungal pathogens. Furthermore, the fungal diversity and community composition were expected to be influenced by changes in climatic conditions and vegetation following the growing season, as well as to differ between forest nurseries and seasons.

4. Materials and Methods

4.1 Study sites and sampling

Seeds (**paper I)** were sampled from commercial seed batches of *P. abies* (Sweden, Finland, Poland, and Belarus), *P. sylvestris* (Sweden and Finland), and *Larix* sp. (Sweden and Finland) (**Figure 3**). The seeds of Swedish origin were categorised into three geographical regions (Northern, Central, and Southern) according to the locations of the seed orchards. This categorisation was intended to address the expected differences in climatic conditions among these regions. A total of 68 seed batches were sampled in **paper I**.

Five forest nurseries belonging to the forest company Sveaskog, and its business unit Svenska Skogsplantor, were included in the studies presented in this thesis: Kilåmon (**paper II**), Stakheden (**paper II** and **IV**), Lugnet (**paper II**, **III**, and **IV**), Vibytorp (**paper IV**), and Trekanten (**paper II**, **III**, and **IV**) (**Figure 3**). The area surrounding these nurseries varies from *P. sylvestris*-dominated forests (Kilåmon and Stakheden), and mixed *P. sylvestris*/*P. abies* forests and agricultural fields (Lugnet and Vibytorp) to agricultural-dominated land with patches of conifers and broadleaved tree species (Trekanten). In addition, Vibytorp and Trekanten nurseries are located next to small towns.

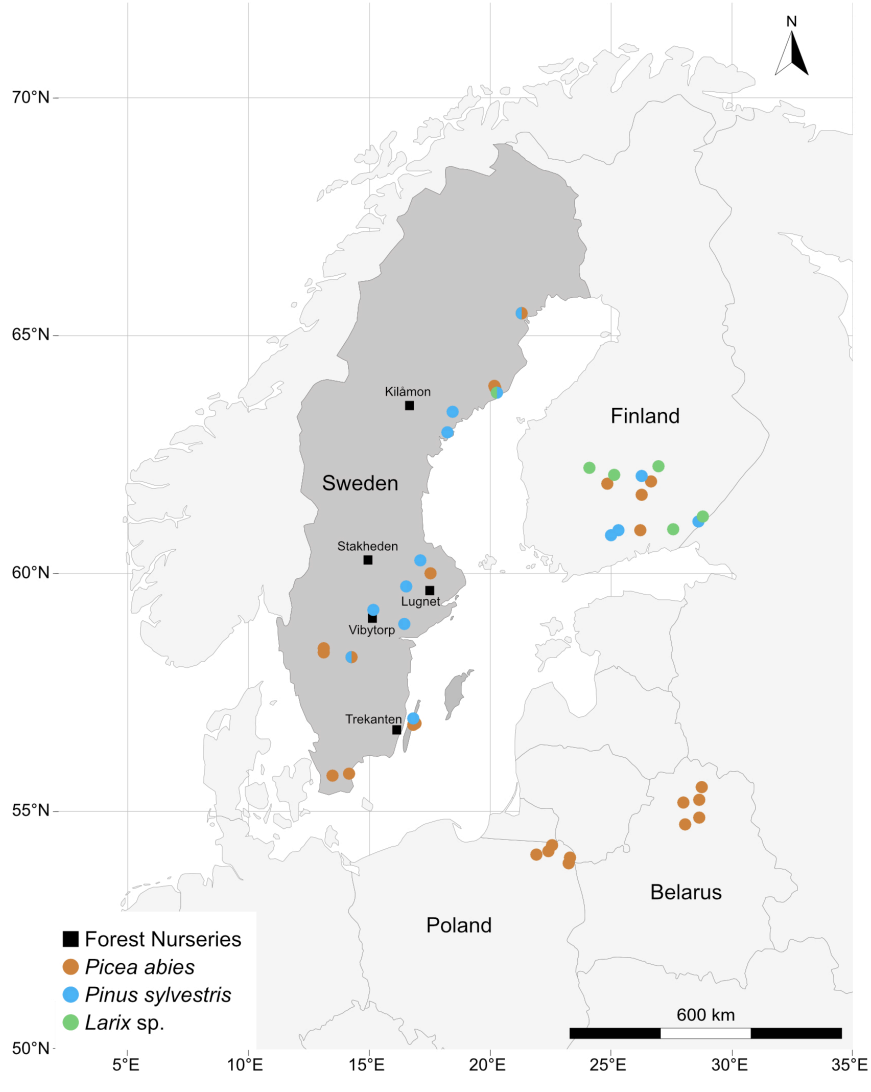

Figure 3. Map of Sweden (shaded area) and northern Europe showing the location of Swedish forest nurseries (Kilåmon, Stakheden, Lugnet, Vibytorp, and Trekanten) and seed orchards in Sweden, Finland, Poland, and Belarus. Seed orchards of different tree species are indicated with different colours.

Random sampling of non-symptomatic *P. sylvestris* needles, followed by microbial treatments (Binab®, Mikroferm®, Prestop®, Serenade®), were carried out every third week during the 2019 growing season (**paper II**). The production systems in each forest nursery differed, particularly in the design of cultivation trays, the seed provenance, and the seedling size. However, the number of trays in the study was carefully determined to capture a representative number of seedlings from each nursery and treatment. In total, the study included 33,683 seedlings and 378 needle samples from four nurseries. A detailed description of experimental design and microbial products is described in **paper II**.

Seedlings grown at the nurseries (**paper II)** were regularly monitored for disease infection during the study. Seedling growth and survival were measured by the end of the growing season. As a consequence of the disease monitoring, five *D. sapinea*-infected seedlings were detected (**paper III**) during the study. In addition, 30 cones, 20 shoots from mature *P. sylvestris*, and 82 asymptomatic seedlings were collected to test for the presence of *D. sapinea*.

Spores were sampled weekly from early May until the end of November using passive spore traps in 2020-2021 (**paper IV**). The spore traps comprised one horizontally fixed filter paper attached ca. 1.2 m above the ground (Garbelotto *et al.* 2008; Zhang *et al.* 2022). Three traps were distributed around the nurseries to cover the whole production area. In this study, 567 filters with fungal spores from four nurseries were collected.

4.2 Morphological and DNA-based identification of fungal pathogens

In this thesis, fungal pathogens of diseased seedlings were morphologically characterised based on their fruiting bodies and, if necessary, based on spores, followed by fungal isolation into pure culture from symptomatic needles (**paper II**, **III**). If multiple cultures were diagnosed with the same species, mycelia from a representative isolate was used for DNA extraction. The ITS1 and ITS2 regions were amplified from the DNA by PCR using the fungal-specific primer ITS1f (Gardes & Bruns 1993) and the universal primer ITS4 (White *et al.* 1990). Sequences were verified by blasting them against the NCBI nucleotide database in accordance with Menkis and colleagues (Menkis *et al.* 2005).

Differentiating between closely related species can sometimes be challenging using morphology or the fungal-specific primer (ITS1f) due to high similarities in the ITS sequence. Therefore, a species-specific DpF/BotR primer pair (Smith & Stanosz 2006) was used in PCR assays to verify the presence of *D. sapinea* in diseased seedlings (**paper II**, **III**) and test for presence in asymptomatic seedlings (**paper III**) and cones and shoots of mature *P. sylvestris* (**paper III**). The PCR products were analysed on 1% agarose gels using gel electrophoresis.

4.3 DNA-based fungal community analysis

4.3.1 Metabarcoding

To obtain the fungal communities from the seed surfaces, DNA was extracted from a pellet obtained after washing the seeds in Tween20 solutions (**paper I**). Then, the seeds were sterilised using 0.5% sodium hypochlorite to destroy DNA from the seed surfaces, followed by grinding the seeds to extract DNA from the seed tissue. Foliar fungal communities were obtained by DNA extraction from non-sterilised, lyophilised needles (**paper II)**, while airborne fungal communities were obtained by DNA extraction from spores obtained after washing the filter papers (**paper IV**), following the protocol of Zhang et al. (2022). DNA extraction was done using extraction kits, which is further explained in the individual papers.

Fungal communities (**papers I**, **II**, **IV**) were detected by amplifying the ITS2 region using the fungal-specific primer fITS7 (Ihrmark *et al.* 2012) and the universal primer ITS4 (White *et al.* 1990) with unique identification tags, following an in-house protocol (Clemmensen *et al.* 2016). PCR products were pooled into two (**paper I**), four (**paper II**), and seven (**paper IV**) libraries, which were then sequenced on the PacBio RSII platform at the SciLifeLab NGI (Uppsala, Sweden).

4.3.2 Quantitative PCR

The sequencing data obtained through high-throughput sequencing, which can give the relative abundance of species, can sometimes challenge the interpretation of temporal shifts in fungal community composition. For example, if the relative abundance of one species fluctuates over time, these changes will correspond with changes of the relative abundnace in other

species in the community (Alteio *et al.* 2021). To address this challenge, incorporating quantitative PCR (qPCR) together with sequencing data to get absolute abundance can help to characterise observed shifts in fungal community structure (Alteio *et al.* 2021). This approach was used to detect dynamic changes in airborne fungal communities in **paper IV**. To achieve correct absolute abundance, identical primer pairs (fITS7/ITS4) were used for the qPCR assay and amplicon sequencing. Before the qPCR assays, potential PCR inhibition was tested by spiking the samples with a circular pGEM plasmid and running the qPCR using plasmid-specific primers. A detailed description of the qPCR assays and the PCR inhibition test are provided in **paper IV**.

4.4 Bioinformatic pipeline

The sequences generated using the PacBio platform in **paper I**, **II**, and **IV** were processed using the Sequence Clustering and Analysis of Tagged Amplicons (SCATA) pipeline (Brandström-Durling *et al.* 2011). The sequences were filtered for quality (removal of low-quality sequences) and clustered into operational taxonomic units (OTUs). To achieve a fair compromise between intraspecific variance, variance between closely related species, and possible sequencing errors, the clustering was done by single linkage clustering with a minimum of 98.5% similarity (Tedersoo *et al.* 2022). The taxonomic assignment followed the same procedure for **paper I**, **II**, and **IV**. In brief, the OTUs were filtered for criteria of sample representation and cluster size, further described in each paper. The OTUs were then taxonomically classified using the PROTAX-fungi (**paper I** and **II**) and the UNITE (**paper I**, **II**, and **IV**) databases implemented in the PlutoF biodiversity platform (Abarenkov *et al.* 2010). Furthermore, the Ribosomal Data Base (RDP) pipeline classifier was used in **paper II** (Wang *et al.* 2007). Additionally, the largest clusters were manually blasted against the NCBI nucleotide database. Finally, the taxonomic assignment was based on a consensus of the output from these searches. Primary lifestyles (**paper I**, **II**, and **IV**) were assigned to fungal OTUs identified at the genus level using the FungalTraits database (Põlme *et al.* 2020).

4.5 Fungal community statistics

The fungal community composition and diversity were used to describe the characteristics of fungal communities. Several methods are currently available to explore the fungal community composition. Two common methods used in this thesis were the principal coordinate analysis (PCoA) (**papers I** and **IV**) and the nonmetric multidimensional scaling (NMDS) (**paper II**) (Paliy & Shankar 2016). In **papers I**, **II**, and **IV**, models of permutational multivariate analysis of variance (PERMANOVA) were used to test for the effect of different parameters of interest on the fungal community composition (Paliy & Shankar 2016; Tedersoo *et al.* 2022). Based on this model, factors explaining variation in fungal community composition could be attained. All analyses were done on relative abundance tables adjusted using a Hellinger transformation, and to account for differences in sequencing depth, the square root of the number of reads per sample was used as the first factor in the models of **paper I** and **paper IV**.

Species diversity of fungal communities is commonly studied using OTU richness (**paper II**), Shannon diversity index (**papers I**, **II**, and **IV**), and Simpson's evenness index (**papers I**, **II**, and **IV**). The diversity indexes are used to expand the measure of diversity by incorporating both species richness and evenness (relative abundance) (McCune & Grace 2002; Gotelli & Ellison 2018; Alberdi & Gilbert 2019). General linear models (**papers I** and **II**) and general linear mixed-effects models (**paper IV**) were constructed to investigate potential factors explaining any effects on fungal diversity. All analyses on species diversity indexes were performed using rarefied datasets. Detailed descriptions of the analyses and data transformations are presented in the individual papers.

5. Results and Discussion

The main objective of the thesis was to gain new knowledge of fungal communities and investigate the occurrence of fungal pathogens associated with the production of forest tree seedlings in Swedish forest nurseries. This chapter presents and discusses the key findings of **papers I-IV**.

5.1 Seedborne fungal communities

In **paper I**, seedborne fungi on the seed surface and in the seed tissue from 68 commercial *P. abies*, *P. sylvestris*, and *Larix* sp. seed samples were studied using high-throughput sequencing. Furthermore, the composition and diversity of the fungal community obtained from seeds of different geographical origins were investigated, and important seedborne fungal pathogens were identified.

5.1.1 Spatial dynamics of seedborne fungi

The PERMANOVA analyses showed significantly different fungal community compositions between the seed surface and the seed tissue. The fungal community composition varied more and showed a more dispersed distribution among samples from the seed tissue. Thus, the fungal colonisation of the seed tissue can involve more specialised species (Ganley & Newcombe 2006), introducing more variation in the fungal colonisation of the seed tissue than for the seed surface. Furthermore, the fungal community composition in the seed tissue was significantly different between tree species (**Figure 4**, **paper I**). However, only 8.7% of the variation was explained by the tree species, whereas a large part of the variation (73.3%) was not explained by the tested factors. This result indicates that other variables not included in the study probably contributed

to shaping the fungal community composition of the seed tissue. For example, host-defence mechanisms, nutrition availability, seed chemical composition, or interactions with other organisms could have been important factors (Lebeis 2015; Baldrian 2017; Würth *et al.* 2019).

Opposite to the hypothesis, the fungal community composition differed between the tree species and explained 51.3% of the variation on the seed surface (**paper I**). Similarly, the fungal diversity differed between tree species as well. The high host-specificity on the seed surface suggests that the seeds were mainly colonised before cone harvest during the cone development (Whittle 1977; Fraedrich & Miller 1995), and possible contamination during seed processing was small or limited to a few species. The surrounding environment likely influenced the seed development and, thereby, the transmission of fungi to the seeds. The fungal assembly probably occurred either before or after seed maturation while the cones were still attached to the trees (Deckert *et al.* 2019). Furthermore, it seems more likely that the main part of the fungal assembly will occur before the cones are fully developed. After the cones are mature, the fungi would have to penetrate the cone scales to colonise the seeds.

The large geographical spread of *P. abies* and *P. sylvestris* seed sources enabled an investigation of the regional differences in the fungal community composition and diversity (**paper I**). Pairwise post-hoc analyses showed that fungal community composition on the seed surface differentiated between all tested *P. sylvestris* regions and between several *P. abies* regions, whereas no geographical differentiation was found from the seed tissue (**Figure 4**). These results showed that fungal communities on the seed surface of *P. abies* and *P. sylvestris* change over a latitudinal gradient, which further suggests that environmental factors expressed by regional differences influence the fungal assembly of the seed surface. Similar observations were previously reported from *P. sylvestris* needles (Terhonen *et al.* 2011; Millberg *et al.* 2015).

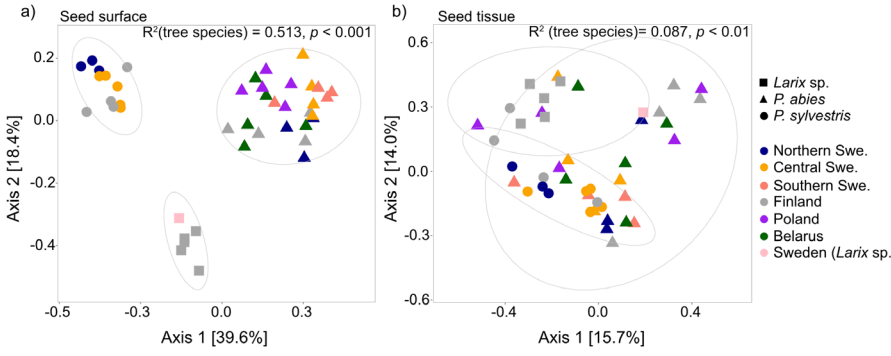

Figure 4. Principal coordinate analysis (PCoA) plot of fungal community sampled from (a) the seed surface and (b) the seed tissue of *P. sylvestris*, *P. abies*, and *Larix* sp. from geographically separated regions. Different tree species are indicated using different symbols, and geographical regions are indicated using different colours. The ellipses represent a 95% confidence interval around the group centroids of the tree species. Figure reproduced from **paper I**.

5.1.2 Seedborne fungal pathogens

Seedborne fungi obtained from *P. abies*, *P. sylvestris*, or *Larix* sp. seeds were mainly plant pathogens (39.0%) or saprotrophs (40.7%) belonging to either Ascomycota (72.9%) or Basidiomycota (27.1%) (**paper I**). Several nursery pathogens that could potentially prevent germination or cause damping-off and disease outbreaks among young seedlings were identified. Here, some of those fungal pathogens will be highlighted and discussed for their importance as seedborne pathogens in forest nurseries.

In this study, *Sydowia polyspora* (Bref. & Tavel) E. Müll. was the most common seedborne fungus (13.3%). *Sydowia polyspora* was found in every seed surface sample and over half of the seed tissue samples, showing a notably higher presence on the surface of *P. sylvestris* seeds, regardless of their origin (**Figure 5**). Previous studies have reported *S. polyspora* to be common in *Pinus* spp. seeds and to strongly reduce seed germination (Ridout & Newcombe 2018; Cleary *et al.* 2019; de la Bastide *et al.* 2019). Given the high prevalence of *S. polyspora* detected in this study, this pathogen can potentially be problematic during both seed germination and the growth of

young seedlings, even though *S. polyspora* is considered to be a weak pathogen.

Phoma herbarum was also among the most common fungi (11.2%), obtained from all three host species on the seed surface (**Figure 5**) and in the seed tissue. In addition, *P. herbarum* was confirmed viable from the seeds (**paper I**). Reports of disease incidence caused by *P. herbarum* have increased in forest nurseries over the last few years (personal communication), often associated with stressed seedlings. However, the pathway of *P. herbarum* to be introduced into forest nurseries is still unclear. Considering the high prevalence in this study, seeds could be a potential inoculum in forest nurseries. Seeds have previously been considered a source of inoculum for *Phoma* spp. in, e.g., sunflowers, lettuce, and citrus (Avila-Quezada & Rai 2022), which should be further investigated for tree seeds used in forest nurseries.

Sirococcus conigenus was predominant in *P. abies* seeds and was obtained on the seed surface (**Figure 5**) and in the seed tissue. Previous studies have suggested seeds as the main inoculum source of *S. conigenus*, showing symptoms of infection throughout the seed tissue into the embryo (Sutherland *et al.* 1981). Seedborne diseases are often recognised to infect during germination or over the first weeks when seedlings are very young. Thus, maintaining optimal conditions favouring rapid and even seed germination and lowering seedling stress become essential to prevent the infection of seedborne *S. conigenus*.

Moreover, *Cladosporium* sp. was among the most detected fungi on the *P. abies* seed surface (**Figure 5**). Similar to *P. herbarum*, reports of disease incidence caused by *Cladosporium* sp. have increased lately in forest nurseries (personal communication). Here, seeds as the potential source of inoculum could also be considered, especially as seedborne *Cladosporium* has previously been reported as the causal agent of disease outbreak (Hernandez-Perez 2006).

Except for the abundant fungal pathogens, *D. sapinea* was obtained from *P. sylvestris* and *P. abies* seed surfaces. *Diplodia sapinea* was later confirmed on cones and shoots collected from one of the *P. sylvestris* seed orchards included in the study (unpublished data). Thus, detecting *D. sapinea* on seeds, as in this study, suggests that it could be present in seed orchards as well. As a latent pathogen, *D. sapinea* can be a potential problem in seed

production. For example, a previous study reported severe damage in *Pinus nigra* J.F. Arnold. seed orchards caused by *D. sapinea*, followed by a hailstorm (Decourcelle *et al.* 2015). *Diplodia sapinea* has previously been detected as seedborne (Decourcelle *et al.* 2015; Cleary *et al.* 2019) and was also associated with diseased *Pinus* spp. seeds (Cram & Fraedrich 2010). Yet, the risk of seed-transmitted *D. sapinea*, i.e., from seed to seedling, is still under investigation. Decourcelle et al. (2015) could not verify seed transmission, even though the seed lot was moderately infected. However, even if the frequency of seed-transmitted *D. sapinea* is low, the potential spread in forest nurseries could be large, considering the current scale of seedling production.

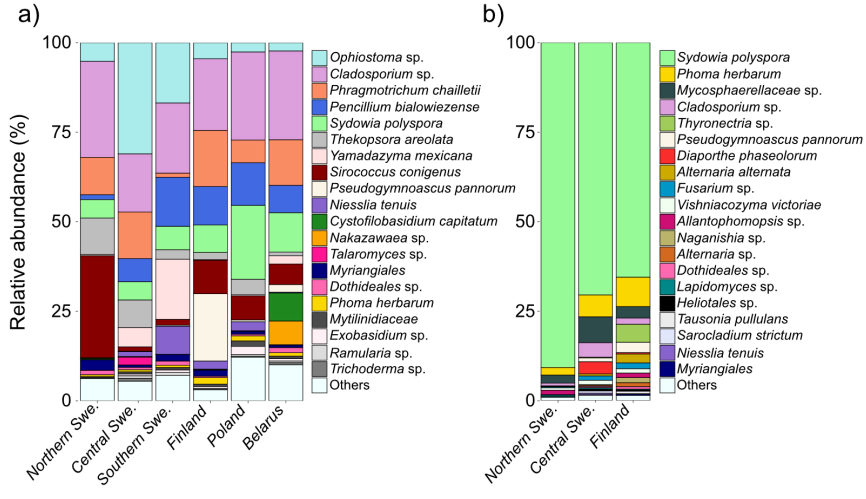

Figure 5. The relative abundance of the 20 most common fungal OTUs associated with the seed surface of (a) *P. abies* and (b) *P. sylvestris*. Remaining fungal OTUs are grouped into "Others". Figure reproduced from **paper I**.

5.1.3 Implications for disease management

Commercial seeds used in forest nurseries are often traded within and between countries to meet Northern Europe's high demand for tree seedlings (Solvin *et al.* 2021; Franić *et al.* 2023; Mataruga *et al.* 2023). The regional dependence described for fungal communities obtained from *P. abies* and *P.*

sylvestris seed surfaces suggests that frequently traded commercial seeds could be a potential source of the spread and introduction of fungal species, especially concerning fungal pathogens carried on the seed surface. Yet, the phytosanitary aspect of the seed-trade regulation in the EU (EU Directive 1999/105/EC) is limited to quarantine pests (non-native) only. Fungal pathogens carried by seeds without showing disease symptoms are difficult to detect without thorough examination, and diseased seeds are still easily overlooked by visual observations (Cram & Fraedrich 2010). The frequent trade of tree seeds suggests that phytosanitary aspects should receive more focus, perhaps through stronger regulations, to avoid the spread of fungal pathogens. Furthermore, the ongoing climate change can change prerequisite conditions to become more favourable for seedborne fungi moved with seeds. Even if the risk for seed-transmitted disease is considered low today, the regional movement of tree seeds could pose a risk in the future.

Furthermore, the health condition of uniformed and managed seed orchards becomes an important contributing factor to the spread of seedborne fungi. The risk of spreading fungal pathogens through seed material could be reduced by avoiding fungal infections of host trees within and near the seed orchards. However, latent pathogens that do not show disease symptoms would still be difficult to prevent by management practises. Thus, DNA-based molecular verification is probably necessary to verify or estimate the frequency of latent pathogens in seeds and seed orchards.

The strong fungal association with host species indicated that host availability could limit spread of fungal disease, as previously reported by Franić et al. (2020). However, in forest nurseries where tree seedlings are densely cultivated in large monocultures, the spread is likely not limited by the host availability. Only a few infected seedlings could cause the rapid spread of diseases. In this study, the healthy commercial conifer seeds obtained many common seedborne nursery pathogens (**paper I**). To lower the risk of seed-transmitted disease, seed producers could combine preventative and sustainable control strategies as part of the integrated pest management (IPM) strategy. For example, the approach used in this study to obtain fungal communities from the seed surface (**paper I**) involved washing the seeds with a detergent-like product. While the seedborne fungi included several nursery fungal pathogens, some were easily washed off and collected for the seed surface samples. This result indicates that washing the seeds could be a preventative method to reduce the number and frequency of fungi

on the seed surfaces. However, fungi strongly attached to the seed coat or integrated with the seed tissue would need other means of control. Combining several environmentally sustainable approaches could be an efficient alternative to controlling for seedborne fungal pathogens, e.g., washing the seeds prior to non-chemical treatments such as thermotherapy (Koch & Roberts 2014).

5.2 Foliar fungal communities

Using high-throughput sequencing, **paper II** investigated the temporal dynamics of foliar fungal communities from four forest nurseries (Kilåmon, Stakheden, Lugnet, and Trekanten) in 2019. Fungal community composition and diversity were obtained from 378 asymptomatic *P. sylvestris* needle samples, and important nursery fungal pathogens were identified.

5.2.1 Temporal dynamics of foliar fungi

Following the outdoor season of the forest tree seedlings, dynamic changes in the foliar fungal communities could be investigated while the seedlings were growing. The foliage represents an environment exposed to several dynamic changes, such as rapid changes in air moisture, temperature, or solar radiation, as well as the foliage's own development and activity (Baldrian 2017). This affects the assembly of foliar fungi and suggests that the fungal community will likely shift over the growing season following changes in the surrounding environment and seedling growth. Thus, early in the season, when the seedlings were young and considerably small, fewer fungi and less diverse fungal communities were expected to harbour the needles. However, as the seedlings grew, the fungal richness and diversity were expected to increase.

The PERMANOVA analyses showed a significant shift in fungal community composition over time, where the sampling time-point explained 61 – 64% of the variation in all nurseries (**Figure 6**). The observed shift could reflect the ageing and growth of the seedlings, as hypothesised. However, in this study, the needles were not surface sterilised to allow for the detection of plant pathogens residing on the needles, which could be potentially harmful to *P. sylvestris* seedlings. Thus, except for the impact of seedling ageing, the fungal community composition was probably influenced by fungal spores spread around the seedlings. This might further explain why,

contrary to the hypothesis, this study did not detect any clear patterns for fungal richness and diversity following the growing season. However, significant temporal fluctuations were observed (**paper II**).

Local influencing factors, such as changes in weather or vegetation, probably contributed to the observed shifts of foliar fungal communities over the growing season. In contrast to natural systems, tree seedlings in forest nurseries are exposed to nursery management practices, which can further influence foliar fungal communities. For example, the seedlings were exposed to regular irrigation, which increased the moisture around the needles, especially on warm and dry summer days. Moreover, the seedlings were regularly fertilised, which kept their nutrient levels high. These conditions could benefit fungi, where some species can become more dominant, especially later in the season when the seedlings are larger and grow more densely.

5.2.2 Foliar fungal pathogens

High species richness of foliar fungi was observed at each nursery (**paper II**), which mainly belonged to either Ascomycota (54.2%) or Basidiomycota (44.7%). The largest functional groups detected were plant pathogens (44%) and saprotrophs (31%). Among these, several fungal pathogens with the potential to infect nursery-grown tree seedlings (Lilja & Poteri 2013) were detected from asymptomatic *P. sylvestris* needles. Here, emphasis is placed on important fungal pathogens, and their potential to cause fungal diseases among nursery-grown tree seedlings is discussed.

Although *B. cinerea* is a well-known nursery pathogen causing disease outbreaks in forest nurseries (Capieau *et al.* 2004; Lilja & Poteri 2013; Nielsen *et al.* 2024), it was not among the most abundant fungal pathogens in this study (5.4%). However, 57 seedlings infected by *B. cinerea* were collected during the growing season, making it the most prevalent species causing disease symptoms during the study (**paper II**). *Botrytis cinerea* was present at all nurseries, generally showing higher relative abundance from August and onwards (**Figure 7**). These findings are consisten with the

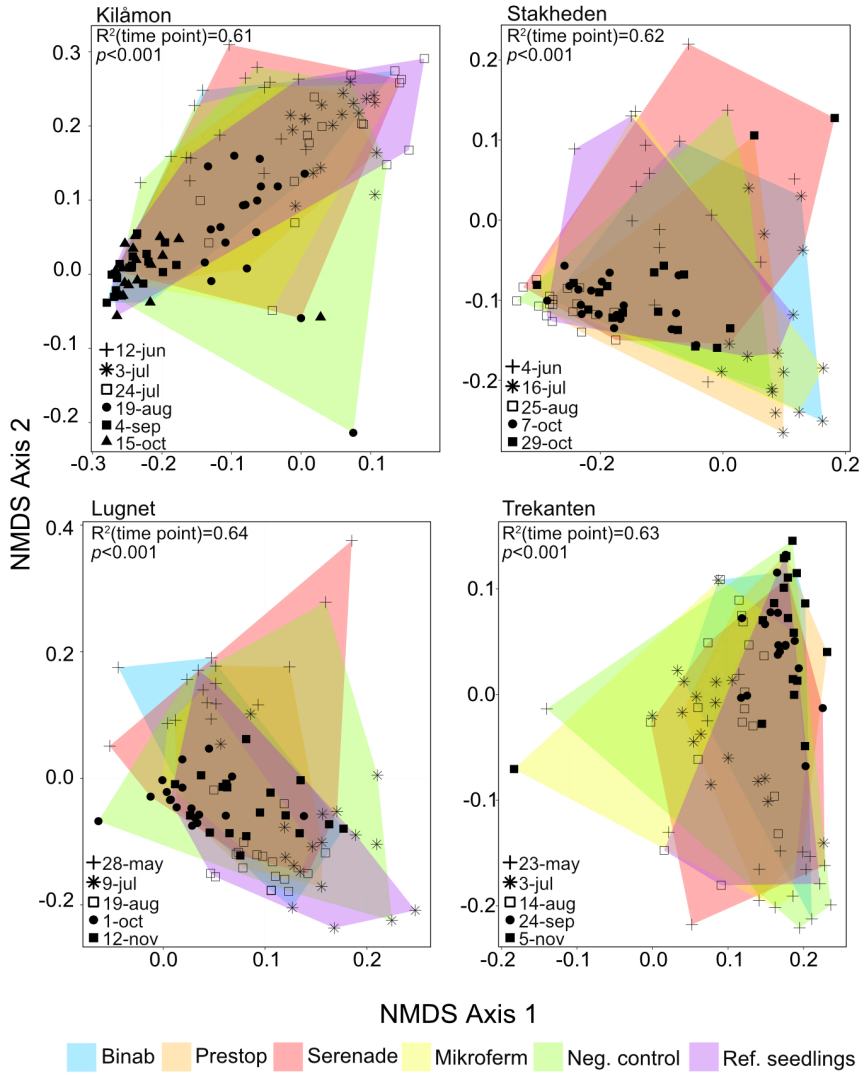

Figure 6. Nonmetric multidimensional scaling (NMDS) of the foliar fungal communities on non-symptomatic *P. sylvestris* needles from four forest nurseries (Kilåmon, Stakheden, Lugnet, and Trekanten). Plots are based on Bray-Curtis dissimilarities (no. dimensions = 3, stress value = 0.149). Sampling points are indicated using different symbols, and microbial treatment using different colours. Figure modified from **paper II**.

current view of *B. cinerea* as a common nursery pathogen causing disease in forest seedlings. *Botrytis cinerea* is observed as an opportunistic pathogen in forest nurseries, often causing disease when seedlings are exposed to stressful conditions (e.g., drought, frost, or failure during storage). However, in recent years, disease incidences caused by other opportunistic pathogens, e.g., *Cladosporium* sp. and *P. herbarum*, have increased in forest nurseries (personal communications). *Cladosporium* sp. was predominant among foliar fungi (15.1%) and present in all nurseries, particularly abundant at Lugnet and Trekanten nurseries (**Figure 7**). Similarly, *P. herbarum* was one of the most abundant foliar fungi (14.5%), whose appearance fluctuated over the growing season among the nurseries (**Figure 7**). These results correspond to previous reports which have confirmed the presence of *Cladosporium* sp. and *P. herbarum* in forest nurseries (Stenström *et al.* 2014; Menkis *et al.* 2016; Okorski *et al.* 2019). Furthermore, infection by *P. herbarum* was observed in *P. sylvestris* seedlings (**paper II**). Interestingly, these pathogens showed different relative abundance between nurseries and over time (**Figure 7**). For example, *P. herbarum* was more prevalent in July in the north (Kilåmon) while more prevalent later in the season in the south (Trekanten), while *Cladosporium* sp. showed higher abundance in the southern nurseries.

A high relative abundance of *S. polyspora* (3.9%) was observed at Kilåmon nursery at the beginning of the growing season (**Figure 7**). *Sydowia polyspora* is often recognised as a foliar endophyte (Ridout & Newcombe 2018). However, this fungus can act as a latent pathogen and was previously reported to cause necrosis in *Abies* spp. seedlings (Talgø *et al.* 2010).

The increased reports of disease incidences caused by *Cladosporium* sp. and *P. herbarum* from the nurseries might correspond to the stopped or reduced use of previously common fungicides (e.g., Tilt®). These fungicides could unintentionally have reduced the disease levels of these less familiar pathogens. Moreover, acting as opportunistic pathogens, the risk of disease outbreaks might increase under changed climatic conditions, e.g., warmer and drier summers or shifting seasons. Thus, working preventatively by

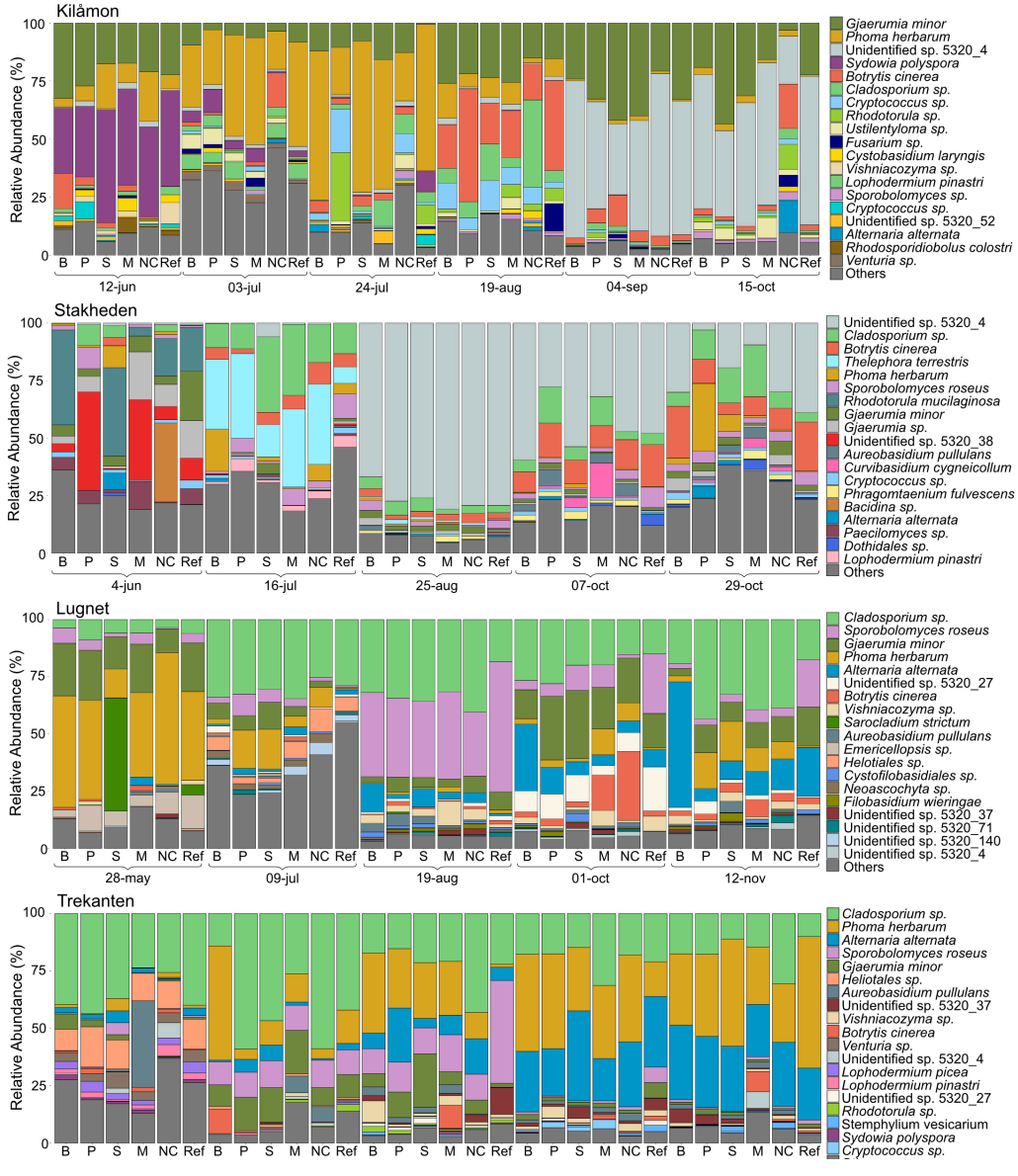

Figure 7. The relative abundance of the 19 most common fungal OTUs in non-symptomatic needles of *P. sylvestris*, separated by nursey. Remaining fungal OTUs after the 19 most abundant fungal OTUs are grouped together as "Others". Bars are presented by treatments per time point, where B = Binab, P = Prestop, S = Serenade, M = Mikroferm, NC = negative control and Ref = reference seedlings. Figure reproduced from **paper II**.

improving growth conditions and avoiding stressful conditions should be prioritised, given the high prevalence of opportunistic pathogens detected on the *P. sylvestris* needles. Consequently, this could lower the risk of fungal infection and reduce the need for fungicides followed by disease outbreaks.

Except for the common fungal pathogens observed among the foliar fungal communities, infections caused by *D. sapinea* in *P. sylvestris* seedlings were reported in this thesis (**papers II**, **III**). Morphological assessment and species-specific PCR assays confirmed the infection of *D. sapinea* (**Figure 8**).

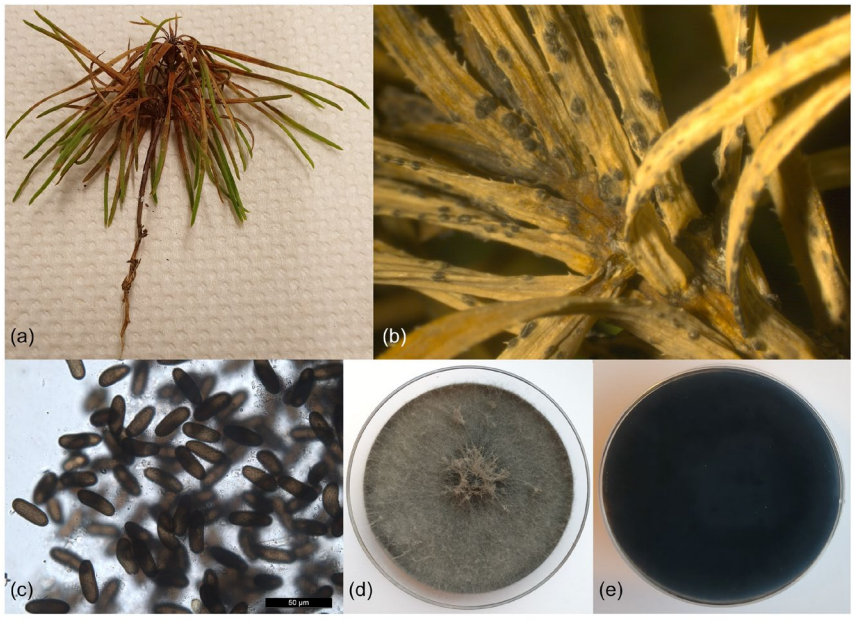

Figure 8. (a) One-year-old *P. sylvestris* seedling infected by *D. sapinea*; (b) characteristic pycnidia on needles and stem; (c) conidia of *D. sapinea*, 400× magnification; (d) *D. sapinea* culture on Hagem medium with dark-grey mycelium on the upper side and (e) black mycelium on the back side. Figure reproduced from **paper III**.

In addition, cones and shoots from mature *P. sylvestris* trees in the vicinity of the nurseries were confirmed with the presence of *D. sapinea*, indicating a possible source of infection (**paper III**). *Diplodia sapinea* has previously been reported to cause severe infections in nursery-grown *Pinus* spp. seedlings in North America (Stanosz *et al.* 2007). However, no large disease outbreak has yet been reported by Swedish forest nurseries.

5.2.3 Disease incidences and microbial additives

In **paper II**, *P. sylvestris* seedlings were subjected to four microbial treatments every third week during seedling growing season. Overall, low disease incidence levels were reported (**paper II**). Most of the seedlings surveyed, including those treated with only water as the negative control, did not show symptoms of fungal infection. In contrast to the hypothesis, microbial treatments did not impact seedling growth, and evaluation of disease control was not possible due to the low level of fungal infection (**paper II**). Similarly, the PERMANOVA analyses showed no target or non-target effects by the microbial treatments on the fungal community composition (**Figure 6**), and no impact on the fungal diversity was found (**paper II**).

The relatively low abundance of applied microorganisms among the foliar fungal communities indicated failure of establishment on the *P. sylvestris* needles. The applied microorganisms might not establish well on the needles, or they might be quickly transferred to the growth substrate by rapid runoff or regular irrigation. Further testing of microbial treatments on conifer seedlings should consider where and how well the applied organism/-s is established. Furthermore, testing should also consider achieving even product distribution by using a tractor sprayer in the field to ensure effective treatment. Sometimes, especially under warm and dry summer days, intensive irrigation cannot be avoided. Under those conditions, the manufacturer's instructions for microbial treatments might be impossible to follow, which causes the failure of the treatments. Better knowledge of the establishment and application effectiveness of microbial treatments would improve the use of microbial treatments as an alternative to fungicidal treatments. However, the overall healthy seedlings in this study suggest that disease management could first be restricted to mechanical control measures in years with conditions unfavourable for fungal infections and then to chemical control treatments as part of integrated pest management.

5.3 Airborne fungal communities

In **paper IV**, spatial and temporal dynamics of airborne fungal communities were investigated at four forest nurseries (Stakheden, Lugnet, Vibytorp, and Trekanten) following the seedling-growing seasons in 2020 and 2021. Fungal community composition and diversity were obtained from 567 spore filters using high-throughput sequencing. Important nursery pathogens were identified, and their distribution over time was further investigated.

5.3.1 Temporal and spatial dynamics of airborne fungi

Seasonal and spatial dynamics of the airborne fungal communities could be assessed by following two outdoor growing seasons from four forest nurseries. Temporal variations of fungal community composition were mainly explained by the specific time point (week) of spore trapping (21.2%), whereas the year of sampling (1.9%) and the forest nurseries (3.5%) explained a minor part (**Figure 9**, PERMANOVA). In addition, the interaction between the spore-sampling year and week explained another 8.3% of the variation. These results suggest that temporal shifts within the growing season influence the airborne fungal community composition, including a combined effect of seasonal variations on the deposited spores within and between years. However, a considerable large part of the variations was not explained by any of the tested factors. This result suggests that other factors not included in the study can have contributed to shaping the airborne fungal community composition. For example, solar radiation, soil temperature, and relative humidity can impact the composition and spread of airborne fungi (Crandall *et al.* 2020).

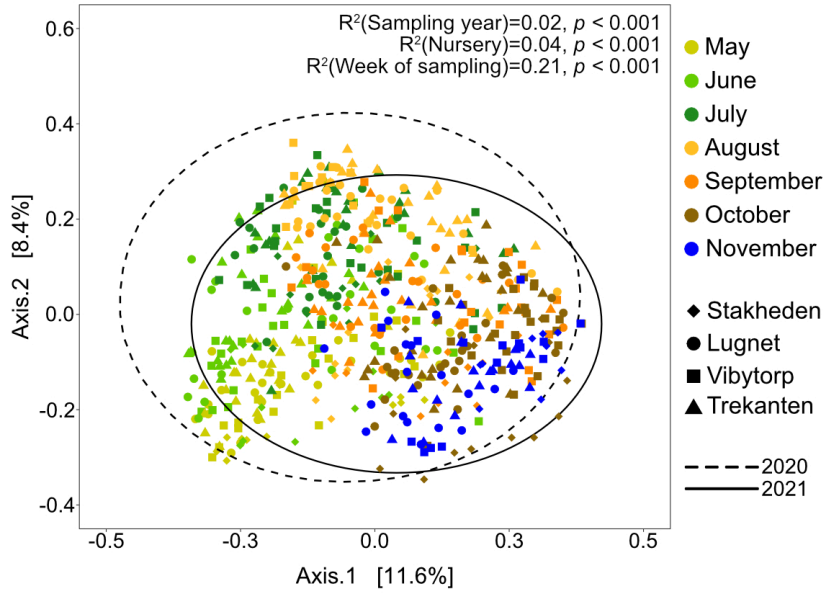

Figure 9. Principal coordinate analysis (PCoA) plot of fungal community sampled from spore traps at four forest nurseries. Different forest nurseries are indicated using different symbols, and different colours indicate the spore collection's month (weeks combined). The ellipses represent a 95% confidence interval around the group centroids of the year of spore collection. Figure reproduced from **paper IV**.

The fungal diversity obtained from deposited spores was found to differentiate between years and fluctuate throughout the growing seasons at all nurseries (**paper IV**). Furthermore, differences in fungal diversity were found between nurseries in 2021. Then, fungal diversity was higher in either one or two of the northern nurseries (Stakheden and Lugnet) than in the two more southern nurseries (Vibytorp and Trekanten) (**paper IV**).

Changed structures of the airborne fungal communities due to temporal shifts are common events reported on local (Castaño *et al.* 2019) and landscape scales (Bowers *et al.* 2013). This study indicates some local differences (e.g., nearby surroundings or local meteorological conditions) affecting the diversity of airborne fungal communities. However, the low effect of nursery location on fungal community composition could reflect the high similarities between the forest nurseries regarding management practices. Furthermore, these results suggest temporal variations throughout the growing season to be an important factor in shaping the airborne fungal

communities. Forest nurseries could expect the spread of fungi to change following the growing season from May until late autumn. Moreover, different groups of fungi are probably favoured by the different conditions that occur over the growing seasons. Thus, it could be that some fungal species are more prevalent earlier in the seasons, whereas others appear later.

5.3.2 Airborne fungal pathogens

The airborne fungal communities obtained from spore traps consisted mainly of Ascomycota (49.5%) and Basidiomycota (50.0%), which were functionally categorised into saprotrophs (38.5%), plant pathogens (28.0%), and ectomycorrhizal fungi (4.5%) being the largest groups (**paper IV**). Similarly to foliar fungal communities, several airborne fungal pathogens were identified for their potential to infect nursery-grown tree seedlings (Lilja & Poteri 2013). In addition, the fluctuation of the spore load for each fungal pathogen over the growing seasons could be investigated using the quantification of ITS copy number and the relative abundances obtained per sample. Here, emphasis is again placed on important nursery pathogens, focusing on their local distribution over the growing seasons for each forest nursery.

Cladosporium sp., *B. cinerea*, and *S. polyspora* were among the most abundant airborne fungal pathogens. *Cladosporium* sp. and *S. polyspora* showed seasonal differences, with a generally higher occurrence in 2021 than in 2020 (**Figure 10**, **paper IV**). In contrast, the general occurrence of *B. cinerea* did not differ between years. Furthermore, neither *B. cinerea* nor *S. polyspora* showed different deposited spore loads between the nursery locations. In contrast, the spore loads of *Cladosporium* sp. tended to be lower at the northern nurseries, especially at Stakheden nursery in 2021. These three nursery pathogens showed significant within-season variations by fluctuations over the growing season at all nurseries (**Figure 10**). Interestingly, *B. cinerea* peaked in week 20 at Stakheden nursery, while the other three nurseries showed higher peaks between weeks 31 and 39. Similarly, *S. polyspora* also had a high peak at week 20 at Stakheden nursery, indicating that conditions for spore spread of both *B. cinerea* and *S. polyspora* were favourable at Stakheden early in the season of 2021.

Often, infections by opportunistic pathogens such as *B. cinerea* or *Cladosporium* sp. are observed during autumn in forest nurseries, when

seedlings are grown more densely, which preserves moisture around the lower parts of the stems. Furthermore, autumn often includes more wet weather conditions and cooler temperatures. The airborne spread of *Cladosporium* sp. and *B. cinerea* observed in this study supports that picture, with more peaks of deposited spore loads in late summer and autumn (**Figure 10**).

Indications of the impact of temperature and precipitation on deposited spore loads were found for *Cladosporium* sp. and *B. cinerea*. The deposited spore loads of *Cladosporium* sp. were positively correlated with increased temperature and negatively correlated with increased precipitation (**paper IV**). Similarly, the deposited spore load of *B. cinerea* was also negatively correlated with precipitation. These patterns are in agreement with previous findings (Fernández *et al.* 1998; Blanco *et al.* 2006). However, a recent study showed a positive correlation with relative humidity and a negative correlation with air temperature for *B. cinerea* airborne inoculum (Leyronas & Nicot 2013). Thus, several factors are probably involved in the spore dispersal, and other weather parameters, such as air humidity or solar radiation, could contribute to explaining the airborne spread of spores.

A future climate, including warmer and dryer summers, as well as changed precipitation patterns, will likely impact the spread of nursery pathogens. For example, *Cladosporium* sp. tends to occur more under warm and dry conditions, and the frequency of airborne inoculum could increase northwards if the conditions become more favourable. However, unfavourable future conditions might also inhibit airborne spread of fungal pathogens. Following the airborne spread over time would allow for detecting changed dispersal patterns and thus improve the prediction of infection risk.

Melampsora populnea was among the most common airborne fungi in this study. This is a fungal pathogen causing pine twisting rust in *Pinus* spp., which has been reported to occur sporadically in forest nurseries while severely threatening young *P. sylvestris* seedling plantations (Lilja *et al.* 2010). No seasonal differences were found for *M. populnea* (**paper IV**). Meanwhile, within-season fluctuations were observed, showing significant differences

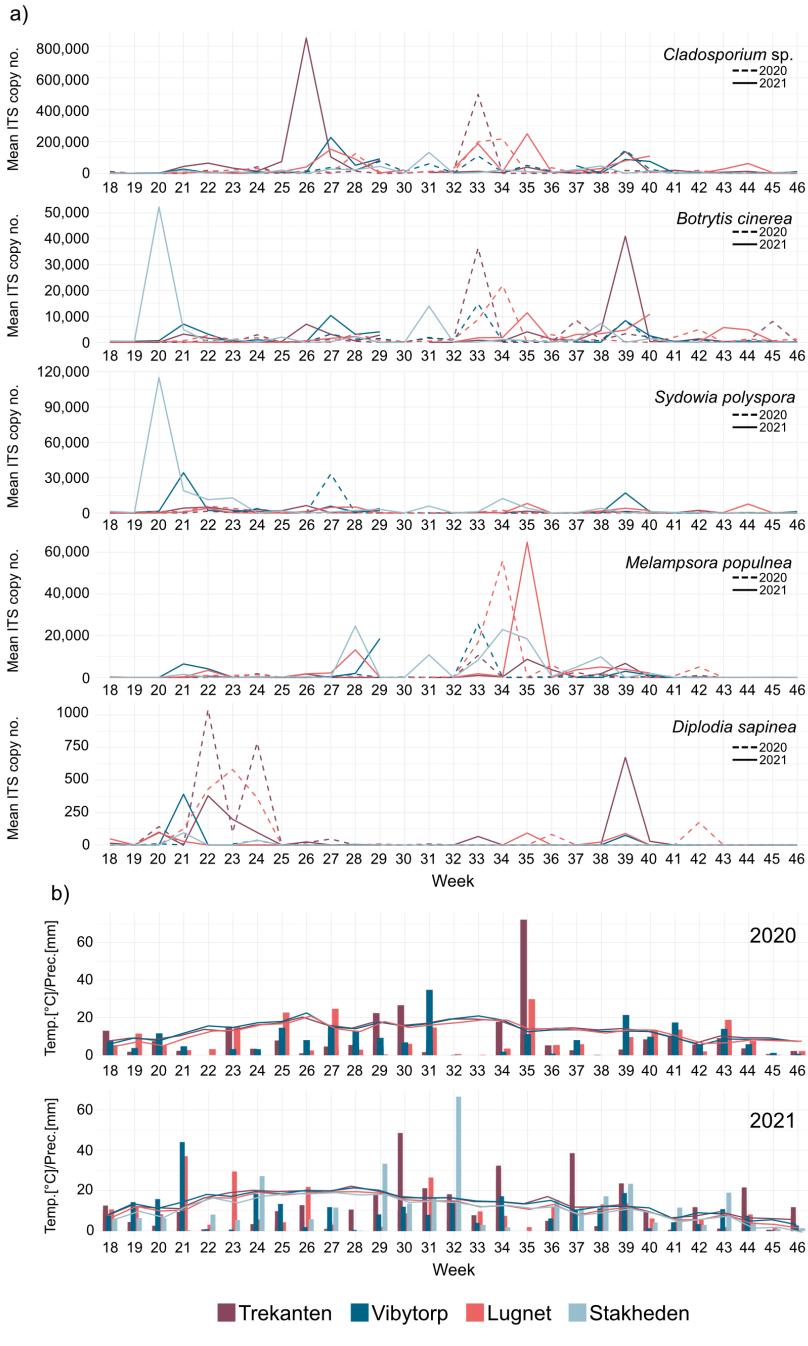

Figure 10. a) Temporal occurrence of nursery pathogens at Trekanten, Vibytorp, Lugnet, and Stakheden forest nurseries, and b) mean temperature (lines) and accumulated weekly precipitation (bars), in 2020 and 2021. Forest nurseries are indicated using different colours, and years are indicated using dashed or solid lines. Figure modified from **paper IV**.

for sampling week (**Figure 10**). The fluctuation of deposited spores probably reflected the dispersal of different spore types. *Melampsora populnea* alternates with *Populus tremula* L. in Northern Europe (Krukela 1973). In early summer, basidiospores are produced from *P. tremula* leaves, which can infect yearly shoots of *P. sylvestris*. Once infected, *P. sylvestris* can spread aeciospores to the alternating host. Urediniospores are then produced, which can re-infect the alternating host. In late summer and autumn, the aeciospores and urediniospores are dispersed under warm and dry weather conditions (Krukela 1973). Thus, the high peaks of deposited spores in late summer and autumn at the nurseries were likely due to the spread of aeciospores and urediniospores, while the low peaks in early summer could indicate the spread of basidiospores which pose a risk of infection of *P. sylvestris* seedlings. The spore deposition was higher in the northern nurseries (Stakheden and Lugnet) than in the southern nurseries (Vibytorp and Trekanten) (**paper IV**), showing spatial differences. These results suggest local variations between the nurseries, e.g., availability of alternating hosts or weather parameters. Furthermore, the spore deposition was positively correlated with temperature and negatively correlated with precipitation (**paper IV**). Thus, differences in weather conditions during the growing season probably impact the spore dispersion of *M. populnea*.

In addition to the more common fungal pathogens, spore deposition of *D. sapinea* was detected in this study (**paper IV**). Overall, *D. sapinea* occurred with low abundance at all nurseries, which could explain the lack of seasonal or regional differences. A recent report detected higher deposited spore loads from spore traps located at sites with ongoing *D. sapinea* infections compared to sites without disease symptoms (Brodde *et al.* 2023). Furthermore, the spore deposition of the considerably large *D. sapinea* spores was suggested to increase closer to more damaged hosts and under wet weather conditions, especially when the host tree had an exposed crown (Brodde *et al.* 2019). Thus, the overall low abundance observed in this study could mean that possible hosts (e.g., *P. sylvestris* trees) in the vicinity of the nurseries were generally healthy, maybe with mild symptoms. Another possibility could have been more severely diseased hosts that were either located too far away or not exposed enough to result in higher abundances. However, the spore deposition fluctuated over the growing season, showing defined peaks in early summer and a few peaks in autumn

(**Figure 10**). Deposited spore loads and spore dispersal of *D. sapinea* were previously associated with higher precipitation (Brodde *et al.* 2023), even though no such correlations were found in the present study.

6. Conclusion and future perspectives

This thesis provides new insight into seedborne, airborne, and foliar fungal communities associated with Swedish forest nurseries. Furthermore, important fungal pathogens potentially affecting seed performance or with the ability to infect and cause disease outbreaks among coniferous tree seedlings were highlighted within the fungal communities.

The main findings are:

- The fungal communities of *P. abies*, *P. sylvestris*, and *Larix* sp. seeds can be distinguished between fungi residing on the seed surface and those found in the seed tissue (**paper I**).

- On the seed surfaces, the fungal communities are highly specified by host tree species and can be differentiated over geographical regions (**paper I**).

- The airborne (**paper IV**) and foliar (**paper II**) fungal communities are clearly shifting following the seedling growing seasons.

- The seedborne (**paper I**), airborne (**paper IV**), and foliar (**paper II**) fungal communities in forest nurseries include a high prevalence of nursery fungal pathogens, e.g., *Cladosporium* sp., *P. herbarum*, *B. cinerea*, and *S. polyspora*.

- *Diplodia sapinea* can cause disease in nursery-grown *P. sylvestris* seedlings (**papers II**, **III**).

- *Diplodia sapinea* was detected as seedborne on commercial *P. sylvestris* and *P. abies* seeds (**paper I**) and as airborne in forest nurseries (**paper IV**), though with a generally low abundance.

Overall, foliar (**paper II**) and airborne (**paper IV**) fungal communities contained high fungal richness and diversity, while seedborne (**paper I**) fungal communities contained noticeably fewer species. This result probably reflects the assembly of fungal communities. The main contribution to seedborne fungal communities likely occurred during cone development, whereas the foliar and airborne fungal communities were mainly influenced by temporal shifts following the seedling growing season. Changes in local conditions at the nurseries (e.g., precipitation, temperature, surrounding vegetation, and nursery practices) likely influenced the observed shifts of the foliar and airborne communities. In addition, the ageing of seedlings probably also affected the composition of foliar fungi (Sieber 2007). The seasonal changes, including shifting climatic conditions and vegetation from early summer to late autumn, likely drove the observed temporal shifts of fungal community composition in all nurseries. Consequently, the forest nurseries could expect patterns of fungal spread to change following the growing season. However, changed climatic conditions, including warmer and drier summers, as well as changed precipitation patterns, will probably impact the fungal spread in the future. Further research involving several growing seasons is necessary to explain possible seasonal shifts. Furthermore, a long-term study could capture changes in fungal spread related to climate change.

This thesis showed a highly abundant and widespread distribution of prevalent nursery fungal pathogens (e.g., *Cladosporium* sp., *B. cinerea*, and *P. herbarum*) in Swedish forest nurseries (**papers I**, **II**, **III**). *Cladosporium* sp. was among the most abundant fungi in each fungal community study, indicating that inoculum can come from seeds as well as from the surrounding environment. Furthermore, this thesis showed that *Cladosporium* sp. was less abundant in the northern nurseries (**paper II**, **paper IV**). Thus, disease incidence might be more common in more southern regions, given the higher frequencies. Higher specificity of species identification of *Cladosporium* sp. needs to be provided to identify the causal

agent of infection, which should be investigated along with the level of disease severity in further studies.

Botrytis cinerea was more abundant among foliar (**paper II**) and airborne (**paper IV**) fungal communities, even though it was detected as seedborne as well (**paper I**). These results suggest that the primary inoculum of *B. cinerea* comes from the surroundings of the nurseries. In contrast, *P. herbarum* was highly abundant among the seedborne (**paper I**) and foliar (**paper II**) fungal communities, whereas it was not detected among the airborne (**paper IV**) fungal communities. Even though reports of disease outbreaks caused by *P. herbarum* have increased lately in Swedish forest nurseries, little is known about the spread and introduction into the forest nurseries. This thesis suggests seeds as a potential source of inoculum, especially concerning the high frequency among foliar fungi and the lack of airborne spread. However, the passive spore traps used to obtain deposited spore loads and the washing of the filter papers might have failed to capture the airborne spread. The possibility of seeds being a source of inoculum should be further investigated as seeds could likely introduce *P. herbarum* into tree seedling production.

After the first observation of *D. sapinea* infections in Sweden by Oliva et al. (2013), the number of reported disease incidences rapidly increased (Brodde 2023). This thesis reports the first confirmed infection of *D. sapinea* in nursery-grown *P. sylvestris* seedlings in Swedish forest nurseries (**papers II**, **III**), which provides evidence for *D. sapinea* as a potential threat to *P. sylvestris* seedling production. Despite showing very low abundance, *D. sapinea* also appeared as seedborne (**paper I**) and airborne (**paper IV**). This suggests a widespread but low abundance of *D. sapinea* in Swedish forest nurseries in accordance with the suggested overall distribution in Sweden (Brodde 2023). Furthermore, *D. sapinea* has an incubation period of about three weeks until pycnidia are visible, and the disease incidence observed in 2019 coincided with the airborne spread observed in 2020 and 2021, even though the studies were conducted in different years. The frequency of *D. sapinea* is suggested to increase with a warmer climate, followed by higher winter temperatures (Fabre *et al.* 2011) and increased drought scenarios (Brodde *et al.* 2023). The abundance and occurrence of *D. sapinea* in forest nurseries could, therefore, increase in the future due to changed climatic conditions.

Economically important nursery pathogens were detected among seedborne, airborne, and foliar fungal communities. Although foliar fungi were obtained from asymptomatic needles (**paper II**), they showed a high prevalence of fungal pathogens, which pose a risk for disease outbreaks under conditions that favour fungal infections. Acting as opportunistic pathogens, disease incidences tend to increase when seedlings experience a stressful environment, e.g., drought, frost, or failure during storage or planting. Changed climatic conditions could increase the frequency of stressful events in the future, where the forest nurseries could face higher disease severity caused by opportunistic pathogens. However, stress-related pathogens can be prevented by keeping tree seedlings healthy and lowering the risk of exposing the seedlings to stressful conditions. Furthermore, forest nurseries can improve preventative control measures by gaining information on the occurrence and spread of nursery pathogens. Following the spore deposition of nursery pathogens (**paper IV**), this thesis provides insight into the airborne spread of common pathogens within forest nurseries. Eventually, this information could be used to predict infection risk. However, further studies on selected pathogens and their dispersal over several seasons are necessary to develop decision-support systems relevant to forest nurseries.

In conclusion, this thesis advances knowledge on seedborne, airborne, and foliar fungal communities in Swedish forest nurseries. The fungal communities harbour a high prevalence of opportunistic nursery fungal pathogens, often recognised as stress-related. These findings underline the importance of managing fungal pathogens by improving disease management strategies in forest nurseries. Preventative control measures, including reduced seedborne inoculum and limiting stressful conditions, can maintain healthy seedlings even in the presence of fungal pathogens.

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Popular science summary

Fungal diseases can result in both economic and environmental problems in forest nurseries. In Sweden, primarily Scots pine (*Pinus sylvestris*) and Norway spruce (*Picea abies*) are densely cultivated in trays consisting of small pots. These cultivation-trays are usually placed in large monocultures, meaning the same tree species is grown over a large area. Additionally, cultivation involves mechanised and high-tech equipment. These conditions can favour a rapid spread of disease-causing fungi among the tree seedlings. To prevent fungal infections, various methods are applied, such as keeping the equipment and greenhouses clean, removing infected plants, or reducing moisture around the seedlings. When an infection occurs and there is a risk of extensive damage, nurseries use chemicals to prevent the fungus from further spreading.

As part of the integrated pest management (IPM) strategy, nurseries should primarily work preventively to minimise the use of chemicals as much as possible. Current preventive practices could be improved if there were better knowledge about the disease-causing fungi occurring in nurseries. This could involve understanding when and where different species of fungi occur. Ultimately, nurseries could reduce their use of chemicals in favour of more environmentally sustainable strategies.

The overall aim of this thesis was therefore to increase knowledge about fungi occurring in Swedish forest nurseries. It was of particular interest to study the occurrence of common disease-causing fungi known to cause serious infections, as well as to investigate the presence of new potential disease-causing fungi. The research focused on studying fungal communities from three areas: commercial seeds of spruce, pine, and larch (*Larix* sp.); healthy pine needles; and fungal spores dispersed in the air around the nurseries.

To investigate where on the seed various fungi were located, a comparative study was conducted between fungal communities from the seed surface and fungal communities inside the seed. This distinction could be significant, for example, in the control of disease-causing fungi, as it is easier to control fungi located on the outside of the seed compared to those inside. The results showed a clear difference between the fungal communities from the seed surface and the seed tissue. Furthermore, the fungal communities from the seed surface differed between tree species and varied depending on the region from which the original seed came. The regional difference means that trade in commercial seeds between different countries can lead to the international spread of fungi.

Furthermore, fungal communities from healthy pine needles were studied to examine which fungi were found on and in the needles during a growing season. Disease-causing fungi can be present on the needles, even if the needles appear healthy. If the seedlings are stressed at any time, for example, during drought or frost, there is a risk that the fungi will infect and damage the seedlings. The results showed that the fungal communities found on pine needles clearly changed during the growing season. Airborne spores can pose a similar risk as from those found on pine needles. Spores from disease-causing fungi can be dispersed in the air at different times during the season and pose a risk when they land on the seedlings. Similar results were obtained from the airborne spores, showing a clear change in fungal communities over time. However, the differences in airborne spores between nurseries and growing seasons were small. The results from the airborne spores indicate that nurseries can expect varying spore dispersal of fungi during the growing season. Some groups of fungi are likely to spread early in the season, while others spread later.

The study on pine needles also included an experiment that examined the effect of microbial products on plant growth and disease control. Biological control can be an alternative to chemical control as part of integrated pest management. Biological control involves the introduction of microorganisms that interact with specific disease-causing fungi through antagonistic activities, i.e., they can inhibit the growth or development of other organisms. The introduction of microorganisms can also strengthen the plant's defence, for example, by making nutrients available, which increases plant growth. This study showed no effect of the microbial products, neither

on seedling growth nor on the proportion of diseased seedlings. The seedlings were generally healthy, even those treated only with water.

Additionally, the fungal communities from commercial seeds, pine needles, and airborne spores all included a large proportion of disease-causing fungi. Among the most common fungal species were disease-causing fungi known to nurseries. Some of these species were *Cladosporium* sp., which causes black mould; *Botrytis cinerea*, which causes grey mould; *Phoma herbarum*, which causes tip blight or dieback; and *Sydowia polyspora*, which causes shoot dieback. Furthermore, this study reported the first confirmed infection of *Diplodia sapinea* in pine seedling in Swedish forest nurseries. *Diplodia sapinea* is a disease-causing fungus that causes Diplodia tip blight in conifers. In North America, it is known particularly to cause problems among jack (*Pinus baksiana*) and red (*P. resinosa*) pine seedlings in forest nurseries. A low amount of *D. sapinea* was also found on seeds, as well as among the spores present in the air. This result suggests that *D. sapinea* occurs in Swedish nurseries as well, though not frequently.

The results of this thesis can contribute to the development of disease control strategies in forest nurseries and provide an increased understanding of fungal communities. At the same time, it highlights the importance of understanding the presence of disease-causing fungi to maintain a production of strong and healthy tree seedlings.

Populärvetenskaplig sammanfattning

Sjukdomar som orsakas av svampinfektioner kan leda till både ekonomiska och miljömässiga problem i skogsplantskolor. I Sverige odlas främst tall (*Pinus sylvestris*) och gran (*Picea abies*) tätt i kassetter bestående av små krukor. Kassetterna placeras vanligtvis i stora monokulturer, d.v.s. samma trädslag odlas över en stor yta. Odlingen involverar dessutom mekaniserad och högteknologisk utrustning. Under dessa förhållanden skapas en gynnsam miljö för skadesvampar som vid en infektion kan spridas snabbt bland plantorna. För att förebygga svampinfektioner tillämpas olika metoder, så som att hålla utrustningen och växthusen rena, ta bort infekterade plantor eller minska fukten runt plantorna. När det väl skett en infektion och det finns en risk för omfattande skada använder plantskolorna kemikalier för att förhindra att skadesvampen spridas ytterligare.

Som en del av strategin för integrerat växtskydds (IPM) ska plantskolorna främst jobba förebyggande för att i bästa mån minska på användandet av kemikalier. Nuvarande förebyggande åtgärder skulle kunna förbättras om det fanns bättre kunskap om skadesvamparna som förekommer i plantskolorna. Det kan till exempel handla om att förstå var och när olika svamparter förekommer. I slutändan skulle plantskolorna kunna minska sin användning av kemikalier till förmån för miljömässigt mer hållbara strategier.

Det övergripande syftet med den här avhandlingen var således att öka kunskapen om svampar som förekommer i svenska skogsplantskolor. Speciellt intressant var att studera förekomst av vanliga skadesvampar som sedan tidigare är kända för att kunna skapa allvarliga infektioner, men även att undersöka förekomsten av nya potentiella skadegörare. Fokus för forskningen låg på att studera svampsamhällen från tre områden: kommersiella frön av gran, tall och lärk (*Larix* sp.); friska tallbarr; samt svampsporer som spreds i luften runt plantskolorna.

För att undersöka var på fröet olika svampar befann sig utfördes en jämförande studie mellan svampsamhällen från fröytan och svampsamhällen inuti fröet. Denna distinktion kan ha betydelse för till exempel bekämpning av skadesvampar då det är lättare att bekämpa svampar som finns utanpå fröet jämfört med de som finns inuti. Resultatet visade en tydlig skillnad mellan svampsamhällena från fröytan och frövävnaden. Svampsamhällena från fröytan var dessutom skilda åt mellan trädslagen och varierade beroende på vilken region det ursprungliga fröet kom ifrån. Den regionala skillnaden betyder att handel med kommersiella frön mellan olika länder kan leda till en internationell spridning av svampar.

Vidare studerades svampsamhällena från friska tallbarr för att undersöka vilka svampar som återfanns på och i barr under en växtsäsong. Skadesvampar kan finnas på barren, även om barren ser friska ut. Om plantorna vid något tillfälle blir stressade, exempelvis vid torka eller frost, finns risken att svamparna infekterar och skadar plantorna. Resultaten från tallbarren visade en tydlig förändring av svampsamhällena under odlingssäsongen. Svampsporer som sprids i luften kan utgöra en liknande risk för infektion som de som kan finnas på tallbarren. Sporer från skadesvampar kan spridas i luften vid olika tidpunkter under säsongen och utgöra en risk när de sen landar på plantorna. Resultaten från de luftburna sporerna var liknande och visade även de en tydlig förändring av svampsamhällen över tid. Däremot var skillnaderna från luftburna sporer små mellan plantskolor och odlingssäsong. Resultaten från de luftburna sporerna indikerar att plantskolorna kan förvänta sig varierande sporspridning av svampar under växtsäsongen. Vissa grupper eller arter av svampar kommer sannolikt att spridas tidigt på säsongen, medan andra sprids senare.

Studien på tallbarr inkluderade också ett försök som undersökte effekten av biologiska produkter på planttillväxt och sjukdomsbekämpning. Biologisk bekämpning kan vara ett alternativ till kemisk bekämpning som en del av det integrerade växtskyddet. Biologisk bekämpning involverar tillförseln av mikroorganismer som interagerar med specifika skadesvampar genom antagonistiska aktiviteter, d.v.s. de kan hämma tillväxten eller utvecklingen av andra organismer. Tillförseln av mikroorganismer kan också stärka plantans försvar, till exempel genom att tillgängliggöra näring, vilket ökar plantans tillväxt. Denna studie visade ingen effekt av de biologiska produkterna, varken på planttillväxt eller andelen sjuka plantor. Plantorna

var generellt friska, även de som enbart behandlats med vatten. Dessutom påverkades inte tallbarrens svampsamhällen av de biologiska produkterna.

Resultaten visade även att svampsamhällen från kommersiella frön, tallbarr och luftburna sporer alla inkluderade en stor andel skadesvampar. Några av de vanligaste skadesvamparna i samtliga svampsamhällen var svamparter som plantskolorna känner till och av erfarenhet vet orsakar svampsjukdomar. Bland de vanligaste svamparterna fanns *Cladosporium* sp., som orsakar svartmögel; *Botrytis cinerea*, som orsakar gråmögel; *Phoma herbarum*, som bland annat orsakar gulnande barr och sämre tillväxt; och *Sydowia polyspora*, som dödar skott. Från den här studien rapporterades dessutom den första bekräftade infektionen av *Diplodia sapinea* hos tallplantor i svenska skogsplantskolor. *Diplodia sapinea* är en skadesvamp som orsakar Diplodiasjuka i barrträd. Skogsplantskolor i Nordamerika har speciellt haft problem orsakade av *D. sapinea* bland rödtall (*Pinus resinosa*) och baksianatall (*P. baksiana*). En låg mängd *D. sapinea* hittades också på frön, samt bland sporerna som kom från luften. Detta resultat tyder på att *D. sapinea* förekommer i plantskolor även i Sverige, men inte frekvent.

Avhandlingens resultat kan bidra till att utveckla strategier för sjukdomsbekämpning i skogsplantskolor samt ge en ökad förståelse för svampsamhällen. Samtidigt belyser den vikten av att förstå förekomsten av skadesvampar för att upprätthålla en produktion av starka och friska plantor.

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II

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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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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.

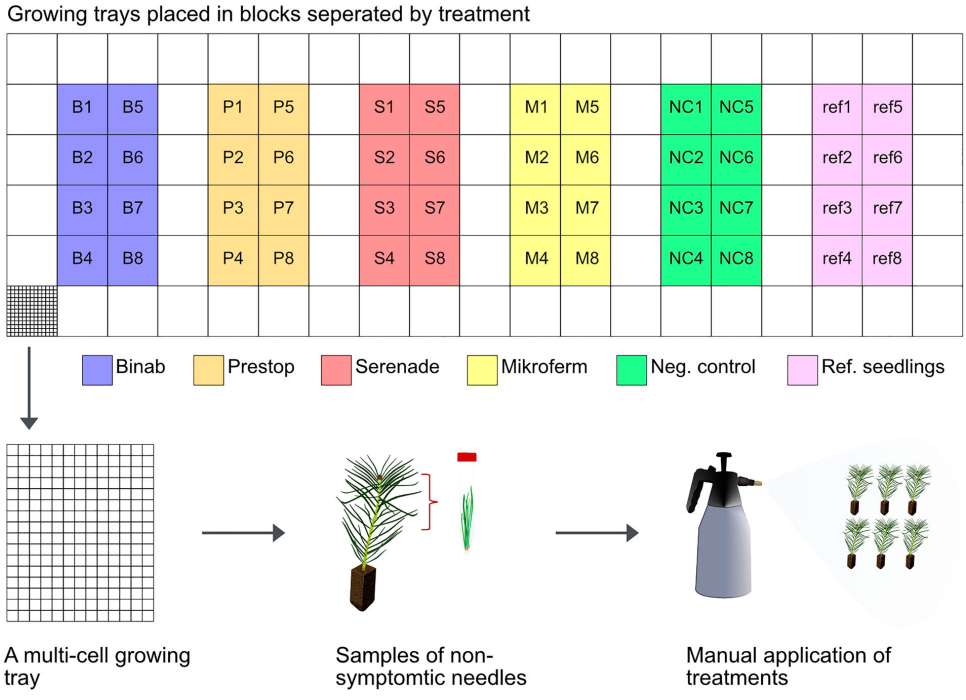

Figure 2. Experimental setup with example from Stakheden nursery. Growing trays of each treatment were placed in blocks with a fixed order and separated by a buffer of non-treated trays of seedlings. Randomised sampling of non-symptomatic needles were followed by manual application of treatments every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

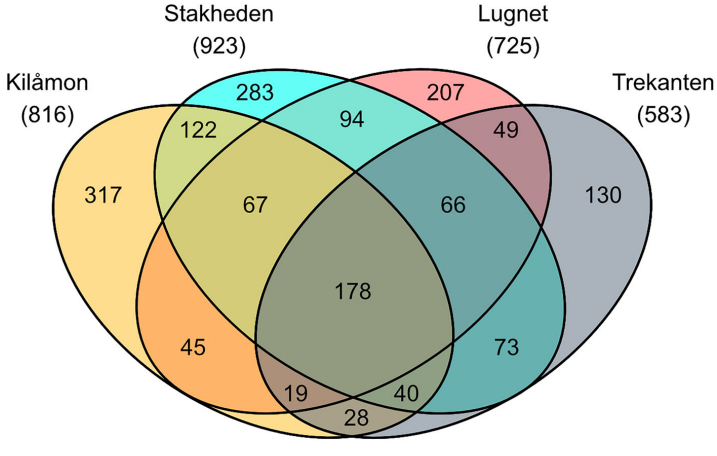

Figure 3. Venndiagram over number of observed fungal OTUs at each nursery (non-rarefied data set). The number of shared and unique fungal OTUs are presented within the ellipses and the total number of fungal OTU from each forest nursery (Kilåmon, Stakheden, Lugnet, Trekanten) is presented within parentheses.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

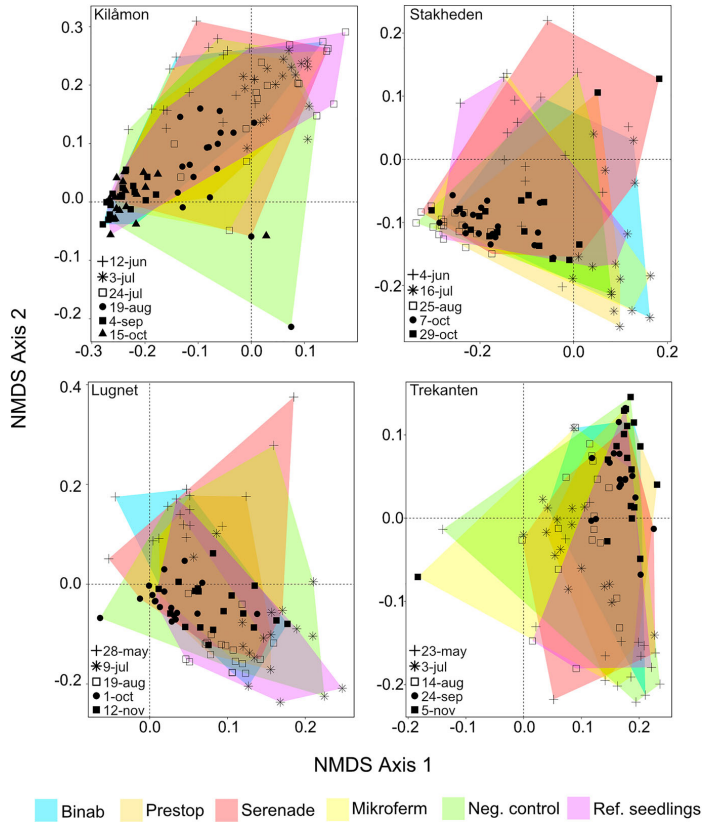

Figure 4. Nonmetric multidimensional scaling (NMDS) of the foliar fungal communities on non-symptomatic *Pinus sylvestris*-needles from four forest nurseries (Kilåmon, Stakheden, Lugnet and Trekanten). Plots are based on Bray-Curtis dissimilarities (no. dimensions = 3, stress value = 0.149). Symbols 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%).

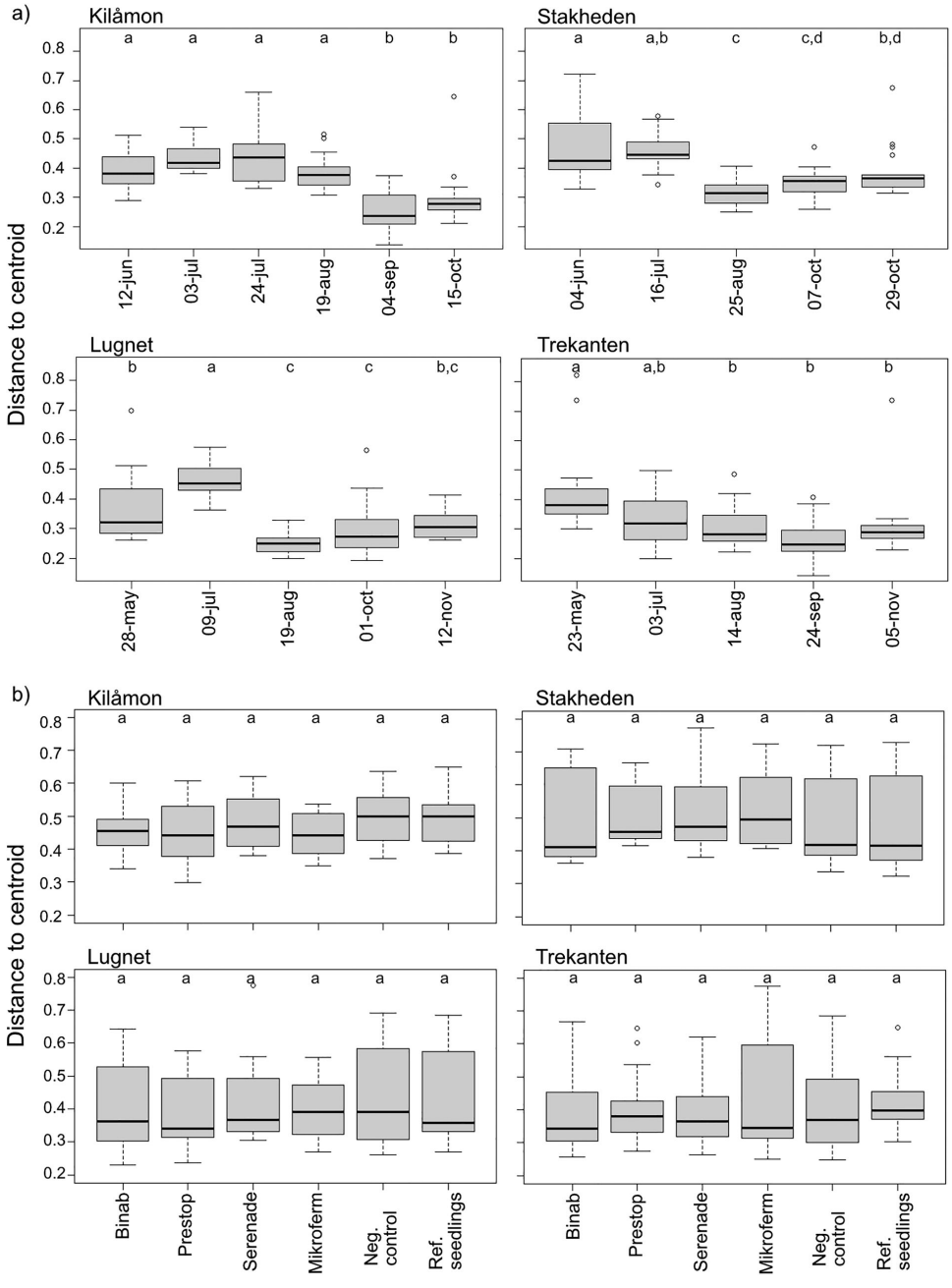

Figure 5. Boxplots presenting the distances to the centroid of foliar fungal communities from samples of non-symptomatic *Pinus sylvestris*-needles from each nursery within (a) sampling time-points and (b) treatments. The distances are based on Bray-Curtis dissimilarity matrices, with permutational analysis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

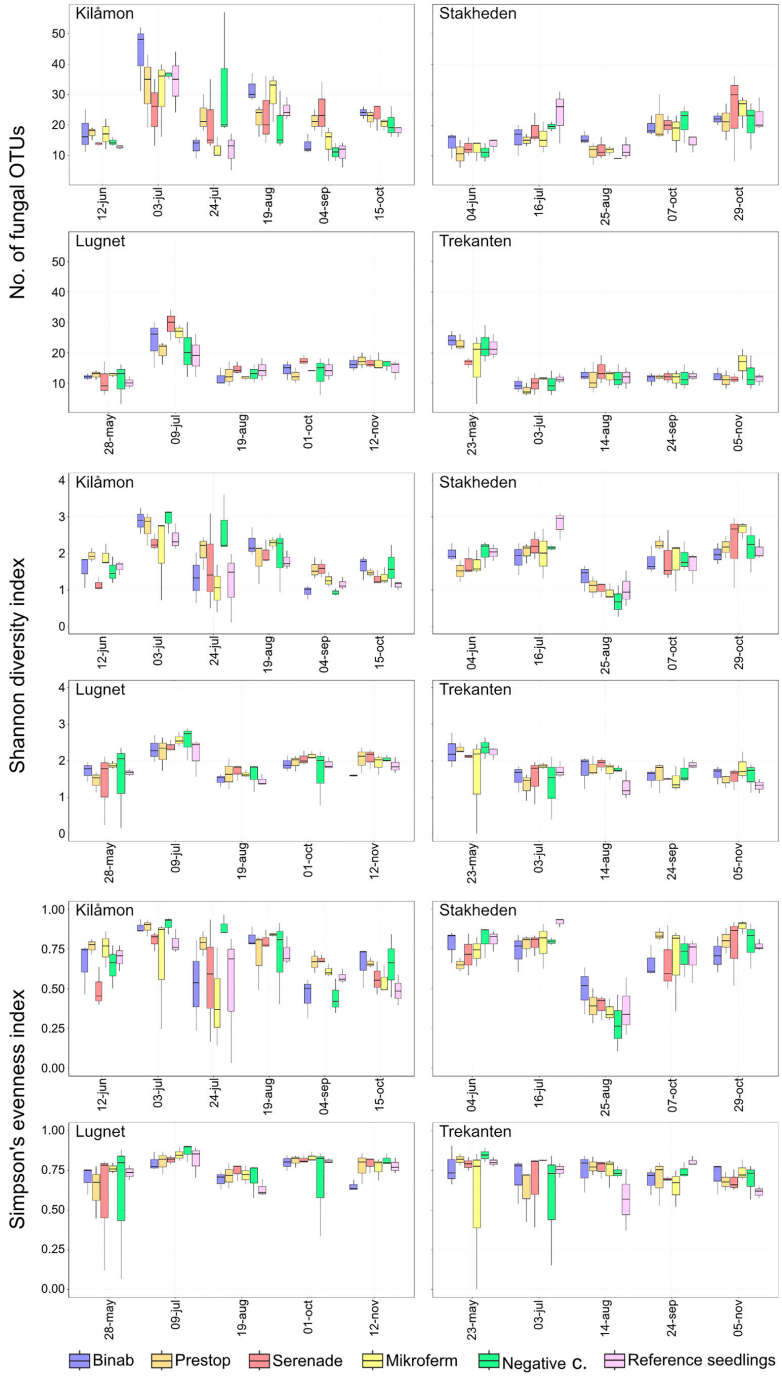

Figure 6. Boxplots presenting (a) Fungal OTU richness, (b) Shannon diversity index, and (c) Simpson's evenness index from non-symptomatic *Pinus sylvestris*-needles separated by sampling time-point, where different colours indicate different treatments. Based on rarefied datasets.

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

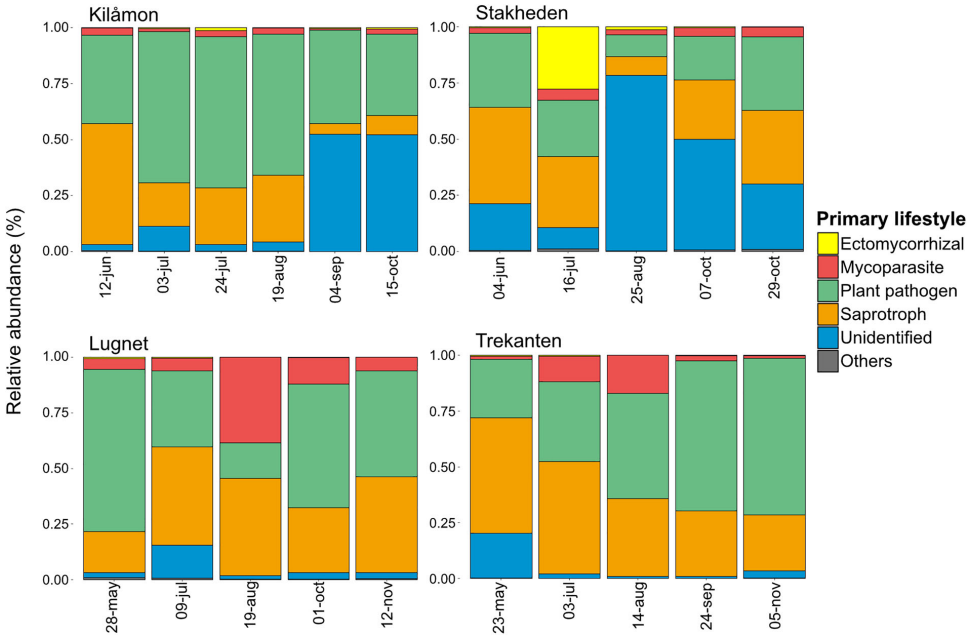

Figure 7. The relative abundance of primary lifestyles for fungal OTUs in non-symptomatic needles of *Pinus sylvestris* that were assigned with a genus (742 OTUs), separated by nursey. Primary lifestyles represented by less than 3% of fungal OTUs in all nurseries, respectively, are grouped into "Others".

(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.

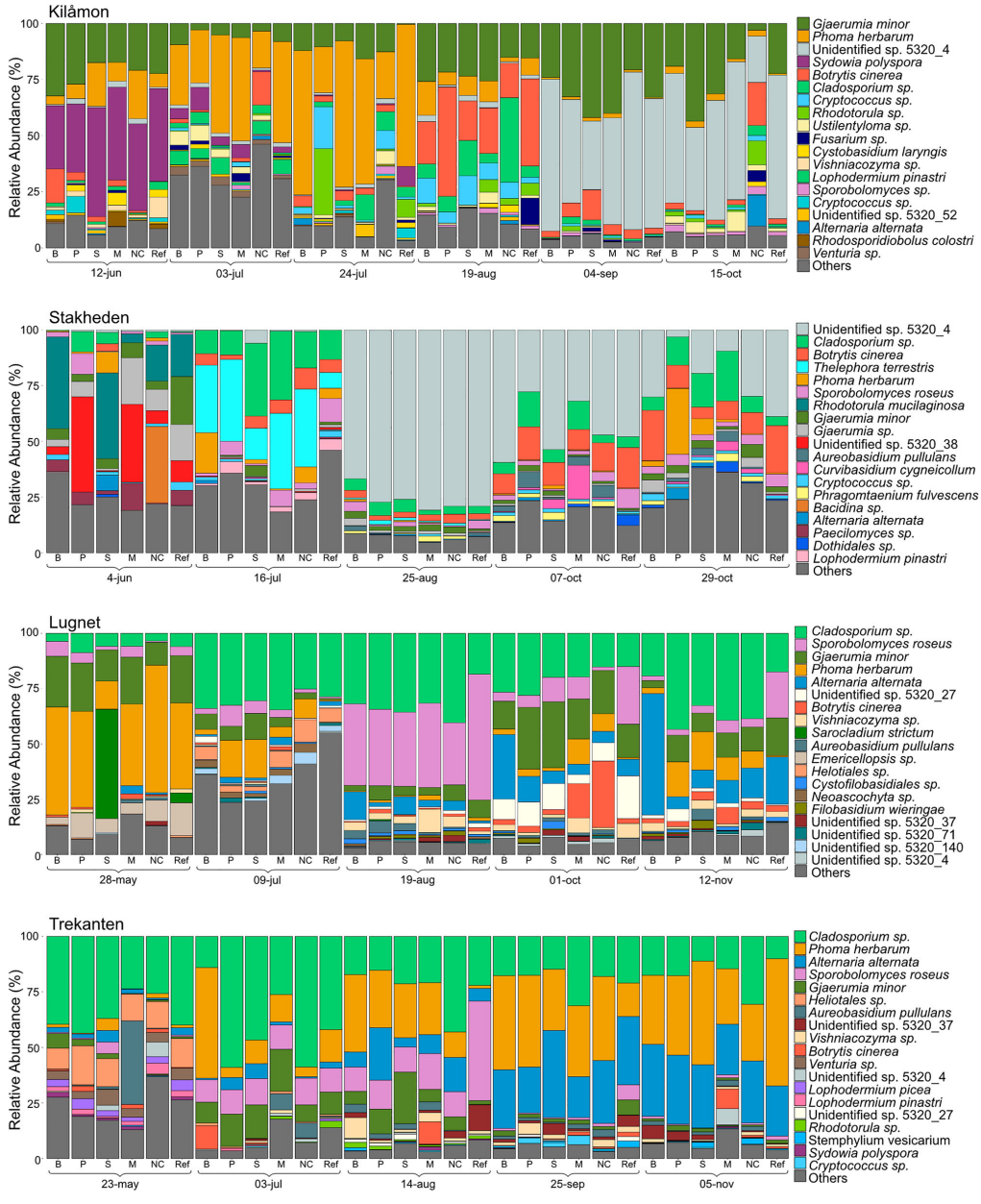

Figure 8. The relative abundance of the 19 most common fungal OTUs in non-symptomatic needles of *Pinus sylvestris*, separated by nursey. Remaining fungal OTUs after the 19 most abundant fungal OTUs are grouped together as "Others". Bars are presented by treatments per time point, where B = Binab, *P* = 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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III

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Diplodia sapinea in Swedish forest nurseries

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Abstract: *Diplodia sapinea* is a common forest pathogen on *Pinus* spp. in a large part of the world. In 2013, disease caused by this pathogen on Scots pine (*Pinus sylvestris*) trees in Sweden was reported for the first time. In this study, we report the first detection of *D. sapinea* on diseased seedlings of *P. sylvestris* from two Swedish forest nurseries. Infected seedlings were collected July–November 2019. *Diplodia sapinea* was identified by morphological characteristics of fungal structures on plant tissues and from culture grown on Hagem agar media, followed by sequencing of fungal ITS rDNA. The result emphasizes the susceptibility of *P. sylvestris* seedlings. More research is needed to better understand the risk for disease spreading within forest nurseries and into the forest through infected plant material.

Keywords: fungal disease; ITS rDNA; pathogen; *Pinus sylvestris*; pine seedlings

In Sweden, ca. 370 million forest tree seedlings are produced annually and almost half of these constitute of *P. sylvestris*, which are primarily cultivated using a container system (www.skogsstyrelsen.se). In this system, intensive management practices (e.g. extensive monocultures, dense cultivation, chemical and mechanical weed and pest control, and shortage of beneficial organisms) may stress seedlings, thereby creating favourable conditions for the establishment and outbreak of fungal diseases.

Diplodia sapinea (Fr.) Fuckel [syn. *Diplodia pinea* (Desm.) Kickx., *Sphaeropsis sapinea* (Fr.) Dyko & Sutton] is a common fungal pathogen on *Pinus* spp. found in all continents (Phillips et al. 2013). Recent observations suggest that distribution of *D. sapinea* has expanded northwards and in 2013 it was reported for the first time in Sweden (Oliva et al. 2013). In 2016, an outbreak was observed in central Sweden, affecting a plantation of *P. sylvestris* the size of ca. 15 ha (Brodde et al. 2019).

Favoured conditions for *D. sapinea* are those that induce host stress, such as drought. Under such conditions, *D. sapinea* is known to cause severe damage on red pine (*Pinus resinosa*) and jack pine (*Pinus banksiana*) seedlings in forest nurseries in North America (Stanosz et al. 2007 and references therein). However, *D. sapinea* has to our knowledge never been reported causing disease on *P. sylvestris* seedlings. In Europe, *P. sylvestris* is one of the principal tree species that is mainly produced in forest nurseries for replantation of harvested forest stands. The lack of knowledge on both *D. sapinea* spread in forest nurseries and effective control measures poses a risk for production of healthy *P. sylvestris* stock. This study reports the first disease incidence by *D. sapinea* in Swedish forest nurseries, and highlights the need for further research in order to understand the potential risk of disease development and spread.

MATERIAL AND METHODS

Infected seedlings of *P. sylvestris* were detected from two forest nurseries in Sweden. Seeds were sown in March 2019 and seedlings were cultivated using an open container system with plastic trays elevated from the ground. During vegetation season, seedlings were regularly monitored for disease infections.

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The first two infected seedlings were collected 9th of July and 6th of August 2019, respectively, from a nursery in central Sweden (59°37.9181' N, 17°30.8624' E). No early stages of disease were observed until confirmed infected seedlings were detected. Surrounding area consisted of a mixed *P. sylvestris* and *Picea abies* forest stand, but also some broadleaved species were found there. Three more infected seedlings were later collected on the 6th of November 2019 from a nursery in southern Sweden (56°42.0931' N, 16°7.4479' E), where no early stages of disease had been observed until confirmed infected seedlings were detected as well. The area around this nursery consisted of mixed broadleaved and coniferous tree species, i.e. *P. sylvestris*, *P. abies*, and *Larix sibirica*. In addition, 20 *P. sylvestris* cones, 10 shoots from mature *P. sylvestris* trees, and 72 asymptomatic *P. sylvestris* seedlings were collected from the nursery in central Sweden and tested for the presence of *D. sapinea*. Similarly, 10 *P. sylvestris* cones, 10 shoots from mature *P. sylvestris* trees and 10 asymptomatic *P. sylvestris* seedlings were tested from the forest nursery in southern Sweden.

Fungal pycnidia on infected seedlings and conidia were analysed and photographed using the Leica dissection microscope (Leica M165 FC, Wetzlar, Germany) and Leica light microscope (Leica DM5500 B, Wetzlar, Germany), respectively. Infected needles were surface sterilized in 70% ethanol for 30 s, placed in 2% sodium hypochlorite for 5 min, and washed three times in sterile distilled water before being placed on Hagem-agar media (Stenlid 1985) in Petri dishes sealed with Parafilm (Bemis Company Inc., USA) in order to isolate fungal cultures. The petri dishes with needles were kept at ca. 21 °C in darkness and inspected daily for the outgrowth of fungal mycelia that was usually observed within three days of incubation. The outgrowing mycelia was subcultured to new Petri dishes and one isolate per seedling was used for species identification by ITS rDNA sequencing (Menkis et al. 2005). Needle and shoot samples from mature trees and seedlings of *P. sylvestris* as well as cone samples were subjected to DNA isolation and tested for the presence of *D. sapinea* using species-specific PCR assay (Smith & Stanosz 2006).

RESULT AND DISCUSSION

Morphological assessment showed that infected *P. sylvestris* seedlings had symptoms characteristic of *D. sapinea* i.e. round and pointy pycnidia on needles and stems (Figure 1A and B). The size of pycnidia was 0.3 ± 0.08 mm in diam. (average ± S.D. of 27 pycnidia). Conidia had an oval shape, were dark brown in colour, were characteristically pigmented and were 30.9 ± 2.1 × 11.5 ± 0.8 µm in size (average ± S.D. of 29 conidia; Figure 1C). Fungal mycelia on Hagem agar media was white at the beginning, but after 7–10 days became dark grey (Figure 1D) and agar media became complete black (Figure 1E). DNA analysis confirmed that all isolates were of *D. sapinea*. Sequences are available from Genbank under accession No. MT457611–M457614.

D. sapinea-infected seedlings from two geographically separated forest nurseries were found in this study. An early study from 1961 reports a *D. sapinea* finding in roots of nursery-grown *P. sylvestris* in Sweden, where Molin et al. (1961) referred to *D. sapinea* as a parasitic root fungus. However, their findings does not correspond to the current view. Furthermore, they did not provide any characterisation of the symptoms or fungal isolates. This suggest that the fungus reported by Molin et al. (1961) was probably another species. This study provides the first evidence that *D. sapinea* can cause disease on *P. sylvestris* seedlings in Swedish forest nurseries. Since no disease symptoms were detected during regular observations, seedlings were most likely infected about three to four weeks before detection. Moreover, *D. sapinea* has an incubation period of about three weeks until pycnidia are formed and maturate, which was visible on infected seedlings (Figure 1B). Diseased seedlings were noticeably smaller than the surrounding uninfected seedlings. This may suggest that seedlings infected by *D. sapinea* might have already been stressed and therefore became more susceptible to infection. In addition, these seedlings had not been exposed to any chemical treatment against fungal diseases which could explain why infection of *D. sapinea* were detected.

A species-specific PCR assay has showed the presence of *D. sapinea* on several cones and shoots of mature *P. sylvestris* trees growing in a radius of ca. 150 m distance from infected seedlings in each forest nursery, indicating that these may be the source of fungal inoculum. Among the 82 asymptomatic seedlings from both forest nurseries, a weak PCR band for *D. sapinea* was detected for a single seedling (1.2% of all seedlings tested) from the forest nursery in southern Sweden, suggesting an early stage of infection or the presence of conidia on

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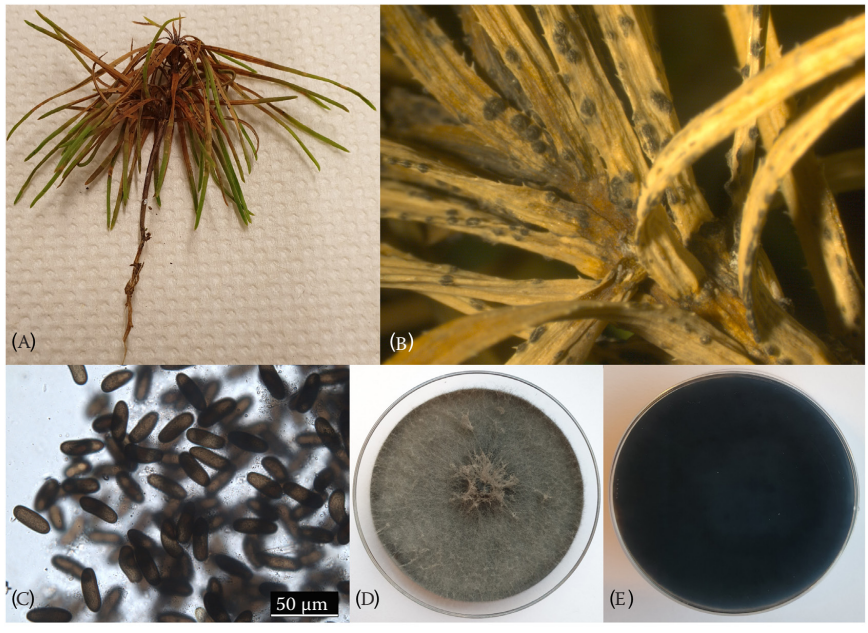

Figure 1. (A) One year-old *Pinus sylvestris* seedling infected by *Diplodia sapinea*, (B) characteristic pycnidia on needles and stem, (C) conidia of *D. sapinea*, 400 × magnification, (D) *D. sapinea* culture on Hagem medium with dark-grey mycelium on the surface and (E) black agar media on the reverse side

the surface. In case this is a latent infection, the rate of such infection would be much lower compared to earlier studies, since up to 20% of asymptomatic *P. banksiana* seedlings were shown to be infected by *D. sapinea* in forest nurseries in North America (Stanosz et al. 2007).

Since chemical pest control is commonly used within forest seedling production, this could explain the low level of latent infections. However, higher rates of latent infections in Swedish forest nurseries could have an impact on the spread of the disease. Changed conditions in the seedling production could pose a potential risk of increasing infection rate of *D. sapinea*, owing to for example drier and/or warmer weather conditions due to climate change or restrictions of the use of chemicals for disease control. Conditions that could stress seedlings, and thus predispose *D. sapinea* infections, should be considered in further studies.

Indeed, studies from North America have demonstrated that infected nursery stock can be responsible for disease outbreak in young pine plantations (Stanosz et al. 2007 and references therein). The result of this study emphasizes that more research is needed to understand the potential risk for disease outbreaks in nursery-grown *P. sylvestris*, the risk of latent infections and control strategies effective against *D. sapinea*.

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This thesis aimed to acquire new knowledge of seedborne, airborne, and foliar fungal communities in Swedish forest nurseries. The results revealed differentiated fungal communities between the seed surface and the tissue, while the airborne and foliar communities shifted over the growing season. All the fungal communities showed a high prevalence of fungal pathogens, and the disease incidence of *Diplodia sapinea* was reported. The findings in this thesis highlight the importance of managing fungal pathogens to maintain healthy tree seedling production.

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