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Interactions between invasive pathogens and resident mycobiome in the foliage of trees

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Department of Botany, Institute of Ecology and Earth Sciences,
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LIST OF ORIGINAL PUBLICATIONS

This thesis is the summary of the following papers, which are referred to in the thesis by their Roman numerals I–IV. The author of the thesis is highlighted in bold type. All published papers are reprinted here with permission from the copyright owners.

I **Agan, A**., Drenkhan, R., Adamson, K., Tedersoo, L., Solheim, H., Børja, I., Matsiakh, I., Timmermann, V., Nagy, NE., Hietala, A.M. 2020. The relationship between fungal diversity and invasibility of a foliar niche – the case of ash dieback. *Journal of Fungi* 6(3), 150.

II Hietala, A.M., **Agan, A**., Nagy, N.M., Børja, I., Timmermann, V., Drenkhan, R., Solheim, H. 2022. The native *Hymenoscyphus albidus* and the invasive *Hymenoscyphus fraxineus* are similar in their necrotrophic growth phase in ash leaves. *Frontiers in Microbiology* 13, 892051.

III **Agan, A**., Tedersoo, L., Hanso, M., Drenkhan, R. 2022. Traces of *Hymenoscyphus fraxineus* in North-Eastern Europe extend further back to history than expected. *Plant Disease* July 2022, https://doi.org/10.1094/PDIS-04-22-0807-RE.

IV **Agan, A**., Solheim, H., Adamson, K., Hietala, A.M., Tedersoo, L., Drenkhan, R. 2021. Seasonal dynamics of fungi associated with healthy and diseased *Pinus sylvestris* needles in northern Europe. *Microorganisms* 9, 1757.

Author's contribution to the studies (* denotes a moderate contribution, ** a high contribution, *** a leading role).

	I	II	III	IV
Original idea	**	**	***	***
Study design	***	**	***	***
Data collection	***	**	***	***
Data analysis	***	***	***	***
Manuscript preparation	***	**	***	***

INTRODUCTION

Invasive pathogens in the tree foliage

Currently one third of the land area on Earth is covered by forests (Gilani and Innes 2020). This land area is home to approximately 3 trillion trees (Crowther *et al*., 2015). The total leaf area of all those trees combined is estimated at 1,017,260,200 km^2. This area represents an immense habitat for microorganisms (Vorholt 2012). Foliage microorganisms, including fungi, can affect host physiology and performance, giving way to an understanding that the evolution and ecology of plants can only be fully understood in a holobiont – host and its associated microorganisms – context (Agler *et al*., 2016). The function and structure of these habitats can be significantly altered by different invasive plant pathogens (Loo 2009). A plant pathogen is considered invasive when it has been introduced into regions where it has been previously absent and where it behaves as an agent of disease and possesses a threat to the biological diversity of naturally occurring plants (Santini *et al*., 2013).

Regarding the interaction of invasive alien plant pathogens with the native fungal community, the 'Diversity Resistance' hypothesis states that stable communities with a high overall species richness should be highly competitive and more resilient to invasion (Levine and D'Antonio 1999). The fundamental assumption of this hypothesis is that niche space in diverse natural communities acts as a limiting factor, and that such communities are structured by interspecific competition (Levine and D'Antonio 1999; Laforest-Lapointe *et al*., 2017). For example, Sieber (2007) has postulated that endophytes are able to accelerate needle senescence in conifers as soon as the density of colonization exceeds a certain threshold. This threshold can be breached specifically in situations when adverse conditions such as lack of light, nutrients, water, or low temperatures occur. Infection rates can be considerably higher in these extreme conditions compared to normal situations resulting in larger population density of pathogens and premature needle cast. Several observational and/or experimental studies suggest that the presence of niche specialists and generalists in local communities and their ratio to colonists in recipient ecosystems influence the outcome of species invasions (Blackburn *et al*., 2011). There are also additional factors that can influence the local establishment of and ensure the invasive phase of an introduced species that is typically enabled by high propagule pressure (Stachowicz and Tilmann 2005). The similarity of recipient microbial community structure at different locations (Paini *et al*., 2016), viability of small introduced populations (Fauvergue *et al*., 2012), and/or existence of specific ecological drivers such as mean annual temperature and human population density are examples of attributes that affect the outcome of pathogen introduction (Dawson *et al*., 2017).

The interaction between invasive alien fungal plant pathogens and native fungal community influences the outcome of colonizations, but it can also affect

the resident mycobiomes. Therefore it is important to have a more precise understanding of when certain invasive pathogens have arrived in new environments. This way we can trace back pathogen existence and consider possible influences on natural mycobiomes of forest trees. This in turn can also help us to plan counteractive measures.

Invasive plant pathogens often go unregistered during the early stages of local establishment as the infection pressure may not be high enough at this stage for these organisms to cause obvious symptoms. With rapid development of HTS (high throughput sequencing) technologies in the recent decade, it has become feasible to use mycological and botanical herbaria for retrospective investigation about their time of arrival. In recent years there have been a number of studies focusing on different herbaria to obtain such information concerning different lichen and fungal species (Drenkhan *et al*., 2016; Heberling and Burke 2019; Gueidan *et al*., 2019; Gross *et al*., 2021; Gueidan and Li 2022; Runnel *et al*., 2022).

Fungal pathogens and endophytes in trees in general

Increasing global trade of plants and plant material and the ongoing climate change have accelerated the movement of alien fungal pathogens across continents. As fungi can spread along with imported seeds, plants, soil of potted plants or different plant-based materials, such as timber, they have potential to travel vast distances (Santini *et al*., 2018). Fungi that are imported to new environments can have pathogenic potential particularly on native plants that are related to the original host species but lack any recent co-evolution with the invader. The evolutionary naive host has insufficient constitutive or induced defence mechanisms against these invasive alien species. At the same time, some fungal pathogens have become more aggressive and increased in abundance in already established habitats. Disease outbreaks by several indigenous or long-known alien fungal pathogens such as *Dothistroma septosporum* (Dorogin) M. Morelet have increased significantly during the last decades (Hanso 1994; Hanso and Drenkhan 2008, 2012). These epidemics, even pandemics in some case, can cause devastating ecological and economical losses. Classic examples of pandemics caused by invasive fungal pathogens include the Dutch elm disease caused by *Ophiostoma ulmi* and *O novo-ulmi* Brasier, and chestnut blight caused by *Cryphonectria parasitica* (Murrill) Barr. Both diseases initiated in the beginning of the 20th century and decimated billions of elm and chestnut trees, respectively, in Europe and North America (Rigling and Prospero 2007). Additional examples include pitch canker of *Pinus* species, caused by *Fusarium circinatum* Nirenberg and O'Donnell and ash dieback caused by *Hymenoscyphus fraxineus* (Drenkhan *et al*., 2014, 2020).

Ash dieback

Dieback of European ash (*Fraxinus excelsior* L.) represents one of the most recent epidemics of a continental scale in Europe. The disease, caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz and Hosoya (Baral *et al*., 2014) (syn. *H. pseudoalbidus* V. Queloz, C.R. Grünig, R. Berndt, T. Kowalski, T.N. Sieber and O. Holdenrieder (Queloz *et al*., 2011), Helotiales, Ascomycetes), was discovered in Poland in the early 1990s (Przybył 2002). Since that time it spread rapidly across the distribution range of European ash (McKinney *et al*., 2014; Solheim and Hietala 2017). Several studies have concluded that the spread rate of *H. fraxineus,* a fungus that has high capacity to produce airborne ascospores, is around 50–75 km per year (Gross *et al*., 2014; Solheim and Hietala 2017). Its main host in Europe, the European ash, is growing in natural mixed forests, parks, and as single trees in cities across a wide range of environmental conditions (Marigo *et al*., 2000); besides economic importance owing to its valuable wood, it is considered an ecologically important tree species in western, central, northern, and eastern parts of the continent (Pautasso *et al*., 2013). For example, Mitchell *et al*. (2014) concluded that out of 953 species associated with European ash, 69 are considered as "highly associated" with its host. Species that depend on ash are particularly threatened by the decline of this tree species. During 30 years of disease, the crown defoliation of *Fraxinus* species across Europe has almost doubled and the overall survival probability of ash populations has reached a critical threshold (George *et al*., 2022).

The main uses of *F. excelsior* timber include interior design, flooring, and furniture manufacturing (Sibul 2007). In Estonia, *F. excelsior* is of little economic importance, as it only accounted for 0.4% of growing stock in 2018 and it rarely produces pure stands (Estonian Statistical forest inventory 2018).

In addition to European ash, *H. fraxineus* is also able to colonize and cause symptoms on the native narrow leaved ash *F. angustifolia* Vahl. However, this tree species seems to be symptomless in some parts of southern Europe possibly due to warm and dry climate in this part of the continent (Kirisits *et al*., 2010; Drenkhan *et al*., 2014a; Nielsen *et al*., 2017). It is also noteworthy that third European native *Fraxinus ornus* L. seems to be quite resistant to the disease (Ibrahim *et al*., 2017). In addition to these native European ash species, *H. fraxineus* is also able to colonize leaves and to certain extent, cause symptoms to the North American ash species *F. nigra* Marsh., *F. pennsylvanica* Marsh., *F. americana* L., and to the Asian ash species *F. mandshurica* Rupr., *F. chinensis* Roxb. and *F. sogdiana* Bunge growing in European parks and arboreta (Kirisits *et al*., 2009; Drenkhan and Hanso 2010; Drenkhan *et al*., 2015; Drenkhan *et al*., 2017; Nielsen *et al*., 2017). Trees infected with *H. fraxineus* die eventually, although the progress of the disease seems to be slower in older trees (Kowalski and Holdenrieder 2009; Rosenvald *et al*., 2015). Importantly, field observations have shown that approximately 1–5% of the European ash trees possess some degree of tolerance against this fungus (McKinney *et al*., 2014), the mechanisms of which remain largely unknown and debated. In the light of this knowledge,

H. fraxineus is listed as one of the most serious forest disease agents (Hyde *et al*., 2018), accompanied with huge economical losses in some parts of Europe. For example, in the Great Britain alone, it is estimated that total economic cost of ash dieback extends to £15 billion (Hill *et al*., 2019).

H. fraxineus does not only cause losses in genetic diversity of different ash species. It has been predicted that ash dieback also represents an insidious threat to co-occurring associated communities on ash trees, for example lichens (Jönsson and Thor 2012). Furthermore, it has played a significant role in the decline of a closely related fungal species that also uses leaf vein system as a sporulation substrate during the saprotrophic phase in leaf debris, the European indigenous *Hymenoscyphus albidus* (Roberge ex Desm.) W. Phillips. *H. albidus* is regarded as a relatively rare species based on a low number of herbarium deposits (Baral and Bemmann 2014; Drenkhan *et al*., 2016). As *H. albidus* has never been isolated from living tissue (neither leaf nor petiole) and is known only from the saprotrophic phase (Baral and Bemmann 2014), its life cycle has been a subject of speculation. Baral and Bemmann (2014) concluded that the pseudosclerotial plates involved in securing the saprotrophic phase are relatively small in *H. albidus*, compared with those of *H. fraxineus,* which typically extend throughout the entire petiole and rachis system. Combined with an obviously high saprobic competence, both *Hymenoscyphus* species harbour an extensive repertoire of cell wall active enzymes and appear better equipped for saprobic feeding than the other necrotrophic members of Helotiales with characterized genomes (Stenlid *et al*., 2017). In inoculation trials utilizing tissue wounding, *H. albidus* inoculation induced very short lesions in rachis of *F. excelsior* and *Fraxinus pennsylvanica* in comparison to those induced by *H. fraxineus* (Kowalski *et al*., 2015). Similarly, *H. albidus* inoculations on stem wounds of *F. excelsior*, *F. pennsylvanica* and *F. mandshurica* have induced only minor lesions compared to the much wider lesions caused by *H. fraxineus* (Kowalski *et al*., 2015; Gross and Holdenrieder 2015; Gross and Sieber 2016).

The ash dieback agent *Hymenoscphus fraxineus* probably originates from East Asia, where it is associated mainly with leaves of the native Asian ash species, *F. mandshurica* and *F. chinensis* (Hosoya *et al*., 1993; Zhao *et al*., 2012; Zheng *et al*., 2013; Gross *et al*., 2014; Cleary *et al*., 2016; Drenkhan *et al*., 2014, 2017). It is also noted that in its natural habitat, the Russian Far-East, it causes only minor shoot dieback symptoms (Drenkhan *et al*., 2017). Even though the exact time of *H. fraxineus* arrival to Europe is still a matter of debate and there is also a possibility that the virulent European strain of *H. fraxineus* evolved in Europe, a genome-wide study has placed the time of introduction approximately 14–32 years before the massive invasion across Europe began (Sønstebø *et al*., 2017). This is in agreement with the long dormant phase of other pathogenic fungi following establishment, e.g. *Dothistroma septosporum* and *Lecanosticta acicola* (Adamson *et al*., 2018; Laas *et al*., 2022)

Fungal endophytes in conifer needles

Like in deciduous tree species, the fungal diversity and composition in the crowns of coniferous tree species are mostly influenced by biotic and abiotic factors, tree health condition, vegetation (the presence of other tree species; ground vegetation), latitude, climate and microclimate, needle age, position of needles in tree crown, etc. (Terhonen *et al*., 2011; Millberg *et al*., 2016; Taudière *et al*., 2018). Endophytes, in addition to having a role in the natural defence of coniferous tree species, can also play a significant role in premature needle senescence after the density of colonization exceeds a certain threshold (Sieber 2007).

Furthermore, as needles stay on the tree considerably longer than leaves, there are certain fungi that are more prevalent on young first-year needles. While most needle endophytes can infect needles of all age classes, with the susceptibility of the needles and the frequency of colonization being considered to increase with needle age, the spruce-infecting *Chrysomyxa* spp. (Gaeumann *et al*., 1959) and the larch needle cast fungus *Meria laricis* infect needles of their host trees only during the first four weeks after needle emergence. In addition, there are examples of first-year needles of Sitka spruce (*Picea sitchensis* (Bong.) Carr.) and Eastern white pine (*Pinus strobus* L.) having an extremely low amount of endophytes from the genera *Lophodermium* and *Hormonema* in comparison to older needles (Magan *et al*., 1994; Deckert and Peterson 2000).

Previous studies using either fungal culturing or Roche 454 sequencing of the ITS2 region have shown that sampling time and geographical location can be the most common drivers of fungal diversity and richness of Scots pine (*Pinus sylvestris* L.) needles (Terhonen *et al*., 2011; Millberg *et al*., 2016). Overall fungal species diversity of *P. sylvestris* needles showed an increase along the north to south gradient in Fennoscandia (Terhonen *et al*., 2011; Millberg *et al*., 2016) and from spring to autumn (Terhonen *et al*., 2011). Taudière *et al*. (2018) concluded that richness of endophytic fungal communities in Corsican black pine (*Pinus nigra* J.F Arnold) was similar across sites and tree cohorts or needle location within the canopy (shade needles compared to the light needles) but differed significantly among forest patches and trees of different age. Moreover, Johnson and Whitney (1992) showed that colonization of black spruce (*Picea mariana* (Mill.) Britton, Sterns and Poggenburg) needles by endophytic fungi increased from 4% in the current-year-needles to 90% in 3-year-old needles.

Invasive conifer diseases: Dothistroma needle blight

Conifers are affected by different pathogens that can cause considerable economic losses due to climate change (Bednářová *et al*., 2013). In Estonia and northern Europe in general, Scots pine is one of the most economically important tree species. As the most common tree species in Estonia, it grows on a wide range of soils and forest site types, and its timber is mostly used in building and furniture manufacturing. Scots pine dominated forests cover 31% of Estonian land

(Statistical forest inventory 2021). There are several important foliar fungal pathogens on Scots pine in Northern Europe, for example *Dothistroma septosporum* and *Lophodermium seditiosum* Chevall. (Hanso and Drenkhan 2008, 2012).

Dothistroma needle blight (DNB) is a serious foliar disease of pine caused by two species: *D. septosporum* and *D. pini. D. septosporum* is more widely distributed across entire Europe, while *D. pini* is considered to occur only in southern and central parts of the continent (Drenkhan *et al*., 2016). DNB agents are distributed across the world and infect ca. 110 different species of Pinaceae. It appears that species of genus *Pinus* are most susceptible (Drenkhan *et al*., 2016). During the last decade, several host jumps of *D. septosporum* have been recorded, for example to the non-native white fir (*Abies concolor* (Gordon) Lindley ex Hildebrand) in Estonia (Drenkhan *et al*., 2014b) and to non-native cedars (*Cedrus* spp.) in the UK (Mullet and Fraser 2016). *D. septosporum* was first described in North-West Russia already in 1911 (Doroguine 1911; Barnes *et al*., 2014). It has had several outbreaks in the northern hemisphere since the 1990-s (Bradshaw 2004; Drenkhan *et al*., 2016). In Estonia, the pathogen was first discovered from needles of the non-native Austrian pine (*Pinus nigra*) in 2006. One year later it had already spread to the native *P. sylvestris* (Hanso and Drenkhan 2008), and by the end of 2008 the fungus had spread all over the country (Hanso and Drenkhan 2008). Of other northern European countries, Norway recorded the first finding of *D. septosporum* in the northern part of the country in 2009 (Solheim and Vuorinen 2011). Some of the collected samples originate within the Arctic circle and are therefore considered as the northernmost records of *Dothistroma* species in the world.

Long-existing needle diseases: Lophodermium needle cast

Of other species inhabiting pine needles, *Lophodermium* species are very abundant (Reignoux *et al*., 2014). *Lophodermium* Chevall. is a genus in the family Rhytismataceae (Rhytismatales, Ascomycota) which has been relatively well studied. Species within this genus are known to be ecologically diverse (Reignoux *et al*., 2014) and have been the most commonly isolated species from different conifers (i.e., *Pinus*, *Abies* and *Picea*; Stone *et al*., 2000). Pine needles are infected by the species of this genus in late summer and early autumn, infected needles are then shed in the following spring before shoot flush (Hanso 1963; Diwani and Millar 1987; Hanso and Drenkhan 2012).

Even though more than 20 *Lophodermium* species can inhabit coniferous trees and shrubs, only one of them, *L. seditiosum* Minter, Staley and Millar is considered a major pathogen (Minter and Millar 1980). Among the other *Lophodermium* species inhabiting pine needles, *L. seditiosum* was previously considered a collective species referred to as *Lophodermium pinastri sensu lato* (historically *L. pinastri* (Schrad.) Chev). First records of this species in Estonia are from year 1856 (Dietrich 1856). Thereafter, *Lophodermium* needle cast has been known in

Scots pine nurseries and young plantations as a foliar disease, mostly killing young seedlings during epidemic years (Hanso 1963; Ericsson *et al*., 1980; Hanso and Drenkhan 2012). Moreover, the growth of younger trees can be considerably hindered by this pathogen due to defoliation of the two youngest needle age classes which are also considered the most productive (Drenkhan *et al*., 2006). *Lophodermium* needle cast, caused by *L. seditiosum* may damage young Scots pine trees until 22–23 years of age (Hanso and Drenkhan 2012). It is still a matter of debate how the disease outbreaks caused by invasive and indigenous pathogens, are affected by forest management, climate change, global trade, or a combination of all these factors (Woods *et al*., 2005; Drenkhan *et al*., 2016).

Motivations and objectives of the thesis

In general, this thesis addresses the relationships between fungal diversity and fungal pathogens in tree foliage. In spite of rapidly developing HTS technologies, a lot is still unknown about how specific traits of pathogen and fungal community facilitate the ecological success of fungal pathogens in the foliage of deciduous and coniferous trees. In this work I clarify these aspects by focusing on *Hymenoscyphus fraxineus* and *Dothistroma septosporum,* and employing PacBio third-generation sequencing accompanied with fungal-specific primer pair ITS1catta (Tedersoo and Anslan 2019; Paper **I**) and ITS4ngs (Tedersoo *et al*., 2014) for community profiling of fungi associated with the foliage of the corresponding host tree species. To gain insights about the effects *H. fraxineus* has on epiphytic and endophytic fungal communities in the foliage of its host, I washed the leaflets of European ash collected from two different sampling sites (one in Estonia and the other in Norway) in Tween 20 solution and left others unwashed, leaflets of rowan (*Sorbus aucuparia* L.) acted as a non-host control for *H. fraxineus*.

Hymenoscyphus albidus, an ash leaf decomposer native to Europe, is becoming increasingly rare (Baral and Bemmann 2014; Drenkhan *et al*., 2016). *H. albidus* has never been isolated from living leaf tissue and its life cycle is still questionable. With this orientation basis, I also shed light into autecology of *Hymenoscyphus albidus* and the role of this fungus in living tissues of European ash. For this purpose, fungal community profiling was carried out for living leaves of European ash at a stand free of shoot dieback, but showing leaf symptoms similar to those caused by *H. fraxineus*. Until now, the information about *H. albidus* has been relatively fragmented and concentrated on its saprotrophic phase in leaf debris (Baral and Bemman 2014). For comparison, I analysed the effect of the globally spread pine needle pathogen *D. septosporum* on the fungal diversity of Scots pine needles on healthy and diseased trees.

Ascocarp records of invasive *H. fraxineus* in Estonia date back to 1997 and apparently represent the oldest records of the species in Europe (Drenkhan *et al*., 2016). I searched for evidence of earlier (i.e., before 1997) establishments of *H. fraxineus* from three different botanical herbaria in Estonia. The aims were to

A) estimate the arrival time of this pathogen to Estonia and Northern Europe, and B) to find evidence of any previous existence of *H. albidus* in Estonia.

The insights gained in this thesis could further our understanding about host-mycobiome-pathogen interaction and help us pinpoint the peak sporulation periods and related abiotic conditions of pathogens in question (*H. fraxineus*, *D. septosporum*). This in turn can help us improve our monitoring and control strategies of these pathogens.

To address these objectives, I formulated the following research hypotheses, the corresponding articles are referred to by Roman numerals:

1) Invasive and indigenous fungal pathogens of foliage seriously affect the fungal species richness and composition of coniferous and deciduous tree species **(I; II; IV)**.

2) The fungal foliage pathogens in *Fraxinus excelsior* and *Pinus sylvestris* have different sporulation peaks in one calendar year and these peaks are the most important drivers of fungal richness within the foliage of *F. excelsior* and *P. sylvestris* **(I; IV)**.

3) While long considered as a saprotroph, *Hymenoscyphus albidus* is able to cause necrotic lesions on leaflets of European ash weakened by autumn senescence in areas without ash dieback agent *Hymenoscyphus fraxineus* **(II)**.

4) The invasive pathogen *Hymenoscyphus fraxineus*, was introduced into Europe significantly earlier than previously thought **(III)**.

MATERIAL AND METHODS

Study sites and sampling

To determine the effect fungal pathogens have on the species richness and composition in the foliages of deciduous and coniferous tree species, samples were taken in 2014 (**I; II; IV**), 2015 (**IV**) and 2016 (**II**) from six study sites in two northern countries: Norway and Estonia. These countries were chosen under the Estonian Norwegian cooperation project EMP162. In Norway the study sites were situated as follows: Ås (N59.67888, E10.77527; 100 m a.s.l.; **I; II**), Gransherrad (N59.69167, E9.04215; 188 m a.s.l.; **IV**), Engerdal (N61.74605, E11.97542; 544 m a.s.l.; **IV**). The Estonian sites were situated in Vedu (N58.48511, E26.75700, 100 m a.s.l.; **I**), Konguta (N58.22816, E26.15588; 35 m a.s.l; **IV)** and Haabsaare (N57.75912, E26.50384; 95 m a.s.l; **IV**). To determine the exact role of *H. albidus* in the green leaves of *F. excelsior*, sampling was carried out in two sampling areas in Norway, Stjørdal (N63.44634, E10.98547, **II**) and Ås (N59.67888, E10.77527; 100 m a.s.l.; **I; II**). In order to trace the historical presence of *H. fraxineus* in Estonia, samples were taken in 2015 from three different botanical herbaria: herbarium of the Tallinn Botanic Garden (TALL; **III)**, herbarium of dendrology in the Estonian University of Life Sciences (EULS; **III**) and herbarium of the Botanical Garden of the University of Tartu (TU; **III**).

At both sites (**I**), leaves were collected at 1–4-week intervals across the season from selected and marked trees within each of the following groups: 1) two ash trees showing obvious signs of *H. fraxineus* infection in their shoots, 2) two ash trees without any shoot symptoms, and 3) two rowan trees for control. Rowan was chosen as a control because it often occurs along with *Fraxinus excelsior* and possesses similar compound leaves, but is not a host for *H. fraxineus*. One compound leaf of each ash and rowan tree was sampled per time point. Samples from three sampling times were subjected to DNA sequencing, totalling 72 (24+24+24) samples per site, altogether 144 samples.

In each sampling scheme – for normalization purpose – the samples were weighed prior to processing. To remove propagules residing on the tissue surface and to compare the epiphytic and endophytic fungal communities, one leaflet-sample was washed in Tween 20 detergent (one drop of Tween per 500 ml of distilled water) for one hour under shaking (50 rpm), prior to pulverizing and DNA extraction, and the other leaflet-sample from the same leaflet pair was processed without washing.

Sampling was done in 2014 and 2015 at four different sites in Estonia (Haabsaare, Konguta; **IV**) and Norway (Gransherrad, Engerdal; **IV)**. In each site four Scots pine trees, two of them with DNB symptoms and two without DNB symptoms were sampled. From the same sample trees (marked in forest), the needles were collected separately from a random shoot of the third branch whorl from the top of the tree canopy and from a random shoot on the third alive (green) branch

whorl from bottom of the tree canopy. This was done to check any qualitative or quantitative differences in fungal communities of these definite parts of canopies. From each tree, three random needle pairs within each needle age class (1–3) were sampled. These were cut into 0.5–1 cm pieces, placed into 2 ml Eppendorf tubes, and stored in –20 °C for future analyses.

All the herbarium specimens of different *Fraxinus* species **(III)** were first assessed visually for *H. fraxineus* -like symptoms that include wilting of entire leaflets or necrotic areas on otherwise healthy-looking leaflets. One-cm^2 subsamples were taken from necrotic areas of leaflets. In the case of entirely green leaflets (i.e., that had no distinct ash dieback symptoms), a random area that included both leaflet side veins and blade tissue was selected and 1-cm^2 subsample was also taken randomly from the leaflet **(III).** Different specimens within the herbarium were separated from each other by sheets of paper and samples of ash with signs of infection were separated by other samples within the herbarium. The instruments used (scalpel, tweezers) were sterilized in ethanol and flamed between each sampling.

Molecular analysis

The DNA of samples collected and analysed in Estonia was extracted using GeneJET Genomic DNA purification kit (Thermo Fischer Scientific, Lithuania; **I; II; III; IV**) according to Drenkhan *et al*. (2017). In samples collected and analysed in Norway (**I; II**), the extraction was carried out using Qiagen DNeasy Plant Mini Kit according to manufacturer's instructions (Qiagen, Hilden, Germany). Primers ITS4ngs (Tedersoo *et al*., 2014) and ITS1catta (Tedersoo and Anslan 2019) were used (**I; II; III; IV**) to amplify fungal DNA. In all four papers, the PCR products were sequenced using PacBio third generation sequencing in the University of Oslo in Norway. The primer ITS1catta was originally designed for article **I** in order to differentiate *H. albidus* from *H. fraxineus* while excluding amplification of plant DNA and to avoid the long intron in the 3' end of the rRNA 18S gene of *Hymenoscyphus* species, but it has also been used successfully in other studies included in this thesis (**II; III; IV**). The reverse primer ITS4ngs was equipped with 10–12 base multiplex identifier (MID) index that differed from any other of the used 107 indices by at least four bases.

Conventional PCR (**I; II; III; IV**) was carried out with two replicates for each sample in 25 µl reaction volume containing 0.5 µl of forward and reverse primer, and 5 µl of HOT FIREPol Blend Master Mix Ready to Load (Solis BioDyne, Tartu, Estonia). Amplification was performed as follows: 15 min at 95 °C, followed by 25 cycles of 30 s at 95 °C, 30 s at 55 °C, 1 min at 72 °C, and a final step at 72 °C for 10 min. The PCR reactions were checked for the presence of a product on 1% agarose gels. In case of no visible band, we repeated the amplification by increasing the number of cycles up to 35. The PCR products were purified using GeneJet DNA purification kit (Thermo Fischer, Vilnius, Lithuania) following the manufacturer's instructions. The amplicons were pooled into separate

sequencing libraries for each sampling site on equimolar basis. Library preparation followed the protocols established for the RSII instrument of PacBio third-generation sequencing platform (Pacific Biosciences, Inc. Menlo Park, CA, USA). The libraries were loaded to SMRT cells using the diffusion method. Sequencing was performed using P6-C4 chemistry for 10 hours following Tedersoo *et al*. (2018).

For quantitative PCR of *H. fraxineus* (**I; II; III**), we used the primers and probes designed and tested for specificity by Ioos *et al*. (2009). For detection of *H. albidus* (**II**; **III**), we used the primer probe set designed and tested for species specificity by Husson et al. (2011), with the modification of using JOE as the reporter dye instead of YY. For Estonian samples (**I; III**), qPCR was carried out in 20 µl reaction, the reaction mix included 1 µl of fluorescent tag and 4 µl of x HOT FIREPol Blend Master Mix Ready to Load (Solis BioDyne, Tartu, Estonia). Amplification was performed according to Ioos et al. (2009), with some modifications related to the PCR mixture: an initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and primer binding in 60 °C for 55 seconds using Rotor-Gene Q MDx qPCR machine. The extension step followed the protocol by Ioos *et al*. (2009). For the Norwegian samples (**I; II)**, Takyon™ Low Rox Probe MasterMix dTTP Blue (Eurogentech, Seraing, Belgium) was used according to manufacturer instructions with Applied Biosystems ViiA 7 qPCR machine and the above-described cycling parameters, except that 65 °C was used at the annealing and extension phases. Standard curves for DNA quantity were constructed with the PCR conditions used in each country, based on DNA extracted from pure cultures of *H. fraxineus* and *H. albidus*. The obtained Ct values were plotted against log-transformed template DNA amounts to prepare a standard curve to quantify pathogen DNA by interpolation in leaflet samples.

Bioinformatics analysis

Bioinformatics was carried out by using various programs implemented in Pipecraft 1.0 (Anslan *et al*., 2017; **I; II; III; IV**). Using mothur (Schloss *et al*., 2009), reads <100 bp were removed and longer sequences were demultiplexed allowing 1-base differences to index and 2-base differences to primer (**I; II; III; IV**). Using UCHIME (Edgar *et al*., 2011), de novo chimera filtering was performed. The full-length Internal Transcribed Spacer (ITS) region was extracted from the rRNA genes using ITSx (Bengtsson-Palme et al., 2013). Using CD-HIT (Fu *et al*., 2012), sequences were clustered into Operational Taxonomic Units (OTUs) based on 99% sequence similarity (**I; II; III; IV**). As clustering may merge *H. fraxineus* and *H. albidus* sequences under one OTU, we added one *H. albidus* sequence manually in order to evaluate this possibility (**I; II; III**). The remaining OTUs were taxonomically identified based on representative sequences against the UNITE v. 7 database (Kõljalg *et al*., 2013). OTUs were considered as members of fungi if their representative sequences matched best fungal taxa at e-value

<e–50. Representative sequences that had >99% sequence similarity to reference sequences were assigned to species hypotheses (SHs) based on UNITE (Kõljalg *et al*., 2013). Higher level classification of Fungi was based on the e-value and sequence similarity criteria of Põlme *et al*. (2020; **I; II; III; IV**).

Statistical analysis

OTU richness was calculated for each sample, using PAST3 (Hammer *et al*., 2001) for rarefaction to check if the number of samples was sufficient to capture most of the species diversity (**I; II; III**). The statistical calculations were done in package lme4 as implemented in R version 4.0.3 (Bates *et al*., 2014), where sampling site was added as a random factor and square root of total number of sequences per sample served as a covariate (**I; II**). A possible effect of tree health, date of sampling, and treatment on the abundance of *H. fraxineus* was tested using a linear mixed model (**I**). Linear mixed model was also used to evaluate a possible effect of site, tree health, needle age, needle location in the canopy, and date of sampling on the relative abundance of *D. septosporum*, *Lophodermium* spp. and overall species richness. The species richness and percentages of species were log-transformed prior to analyses (**IV)**.

Calculations of differences in log-transformed qPCR estimates of *H. fraxineus* and *H. albidus* DNA level (**I; II; III**) between ash phenotype, tree species, treatment and sampling date were done in Excel using ANOVA with Tukey HSD, differences with P value ≤ 0.05 were considered significant. Extrapolation of total fungal biomass using qPCR and read percentage data for *H. fraxineus* was performed according to Cross *et al*. (2017; **I**). We also compared PacBio sequence read percentages of detected species between unwashed and washed leaflets (**I**) using ANOVA with Tukey HSD. Differences between ash phenotype, treatment and site were considered significant with P value ≤ 0.1.

To test the differences in fungal communities in relation to the experimental factors and their interactions (**I; II; III; IV**), we used PERMANOVA+ (Anderson *et al*., 2008). OTU abundance matrix was square-root transformed to reduce the effect of dominant species. Bray-Curtis dissimilarity (Bray and Curtis 1957) was used as a distance measure. Fungal community structure was visualized using PCoA **(I; II; IV)** and CAP **(III)** as implemented in Primer v6 (Clarke and Gorley 2006). We also performed a probabilistic species co-occurrence analysis across all samples to detect any species that showed negative or positive association with *H. fraxineus* (**I**) or *D. septosporum* (**IV)** using the R function co-occur (Griffith *et al*., 2016). These analyses were performed separately for each site (**I; IV**) and tree species present at each site (**I**).

Table 1. An overview of research articles

	I	II	III	IV
Sites	Vedu (EE), Ås (NO)	Ås (NO), Stjørdal (EE)	Different herbaria in Estonia	Gransherrad (NO); Engerdal (NO); Haabsaare (EE); Konguta (EE)
Studied taxa	*Fraxinus excelsior;* *Hymenoscyphus fraxineus;* *Sorbus aucuparia*	*Fraxinus excelsior;* *Hymenoscyphus albidus;* *Hymenoscyphus fraxineus*	*Fraxinus excelsior;* *Fraxinus pennsylvanica;* *Fraxinus mandshurica;* *Hymenoscyphus fraxineus*	*Pinus sylvestris;* *Dothistroma septosporum;* *Lophodermium seditiosum;* *Lophodermium conigenum*
Sampling years	2014	2014; 2016	2014	2014–2015
Studied sampling sites	Naturally regenerated forest; forest covered former agricultural land	Naturally regenerated forest	Herbarium specimens	Naturally/artificially regenerated forest
Response variables	Species richness; fungal biomass; species composition; relative abundance of species	Fungal biomass; species composition; relative abundance of species	Relative abundance of species; species composition	Species richness; species composition; relative abundance of species
Explanatory variables	Sampling site; tree species; tree health; sampling time; leaf treatment (washed/unwashed)	Sampling site; presence of symptoms on leaflets of *Fraxinus excelsior*	Sampling year, sampling site, herbarium	Sampling site; tree health; canopy location; sampling time; symptoms of *Dothistroma septosporum*
Statistical analysis	GLM; ANOVA; PerMANOVA; Species co-occurrence	ANOVA; PerMANOVA	PerMANOVA	GLM; PerMANOVA; Species co-occurrence

RESULTS AND DISCUSSION

In partial agreement with hypothesis 1 and 2, overall natural fungal species richness of European ash leaflets showed a significant decline in September, concomitant with a significant increase of invasive pathogen *H. fraxineus* in sequence read proportion and DNA amount estimates determined by qPCR in both unwashed and washed ash leaflets (Figure 1). At the same time, no significant decline in overall species richness across sampling season on unwashed and washed rowan (used as a non-host control tree species in Paper **I**) leaflets sampled from the same sites was observed. Several studies, based on fungal culturing, carried out in stands free of ash dieback indicated that on European ash species diversity of natural mycobiomes increases from May to October (Reiher 2011; Scholtysik *et al*., 2013). The observed different trajectory in paper **I** indicates that *H. fraxineus* disturbs the natural succession of ash leaf mycobiome in autumn, a time when endophytes with weak parasitic activity resume growth as a response to weakening of leaflet defence mechanisms due to senescence. As native ash leaf-associated fungi showed low sporulation levels during the peak sporulation of *H. fraxineus* (Cross *et al*., 2017), we can assume that these fungi spread early in the growing season and their propagule numbers remain below the carrying capacity of leaves until autumn senescence. The most crucial part for *H. fraxineus* to challenge the resident fungal community seems to be the strong mid-season switch from the saprobic phase to the parasitic period, giving it an advantage when it comes to substrate capture and interference competition mediated by allelochemicals (Halecker *et al*. 2014). In this regard it is expected that *H. fraxineus* also had a significant effect on fungal species composition, according to permutational ANOVA (3.7% of variation explained; $P<0.001$), although the presence of *H. fraxineus* was not the most important factor. Other significant factors that influenced fungal species (paper **I)** were sampling site (country), explaining 14.5% of the variation in fungal composition ($P<0.001$), sampling time (4.9%; $P<0.001$), washing treatment (2.1% $P<0.005$) and tree species (1.8%; $P<0.05$). There were also distinct differences in fungal species composition in areas where *H. fraxineus* was present (Vedu and Norderås; Paper **II**) and an area where *H. albidus,* instead of *H. fraxineus* was dominant (Stjørdal). Sampling site explained 32.4% of the variation in the dataset in paper **II**. Differences in average overall fungal species richness between unwashed and washed ash and rowan leaflets across the sampling season are visualized in Figure 1.

We found no support to the hypothesis 1 that the pine needle pathogen *D. septosporum* affects fungal species richness of Scots pine foliage (IV). Several population genetic analyses have indicated that *D. septosporum* originates from Europe and has co-evolved with Scots pine longer than previously expected (Drenkhan *et al*., 2013; Adamson *et al*., 2018; Mullett *et al*., 2021). This may also explain the results of observations that DNB seems to cause minor symptoms on Scots pine in northern Europe and seems to affect only needles in

the lower part of tree canopies (Drenkhan *et al*., 2013; Adamson *et al*., 2018). Generally, the induction of only minor symptoms on a host is characteristic to host-pathogen interactions that have been co-evolving for a considerable amount of time (Harrington and Wingfield 1998; Ennos 2001).

In this regard, it seems plausible that *D. septosporum* has no significant effect on overall fungal species richness of Scots pine needles. The constructed full model across two countries and four sampling sites showed that the most significant predictor of general fungal species richness in Scots pine needles was sampling site ($F_{1.183} = 37.7$; $R^2_{adj} = 0.727$; $p < 0.05$), as fungal species richness in needles of Scots pine was significantly higher in Estonian sites than in Norwegian sites. Considering season, fungal species richness in needles of *P. sylvestris* was higher in autumn than in spring ($F_{2.183} = 17.1$; $R^2_{adj} = 0.150$; $p < 0.05$; Figure 1). Additionally, the sum of effective temperatures had a significant positive relation with overall species richness in needles ($F_{1.183} = 11.0$; $R^2_{adj} = 0.607$; $p < 0.01$).

Interestingly, high sequence read percentage of *Lophodermium conigenum* ($F_{1.183} = 16.7$; $R^2_{adj} = 0.102$; $p < 0.001$) was accompanied with lower general species richness but only in needles sampled in spring. The results of several other metabarcoding studies focusing on pine needles have also recorded a high abundance of *L. conigenum* (Taudière *et al*., 2018; Lazarević and Menkis 2020). Thereby Lazarević and Menkis (2020) concluded that *L. conigenum* was mostly prevalent on trees with good overall health in moderate growing conditions and had very low prevalence in places where beetle attacks and necrotic lesions (agent unknown) on needles occurred. *L. conigenum* was also the most isolated fungus on symptomless needles of Scots pine in Finland (Terhonen *et al*., 2011). In contrast, Millberg *et al*. (2016) concluded that *L. conigenum* was more abundant on symptomatic needles of Scots pine in northern Sweden. The exact role of *L. conigenum* in pine phylloplane remains to be clarified, but it may be antagonistic against both *L. seditiosum* and *L. pinastri* s. str (Minter 1981). Different *Lophodermium* species are antagonistic towards one another and some data alludes to the fact that antagonistic feedback from pine needle endophytes increases the fruiting body production of *L. seditiosum* (Hanso 1994). As species richness was significantly different in Estonia and Norway ($P<0.05$), we also constructed two country-specific models to understand the difference. In Estonia, the most important and only significant predictor of species richness was sampling season ($F_{2.83} = 8.42$; $R^2_{adj} = 0.657$; $p < 0.05$), whereas the significant predictors were sampling season ($F_{2.114} = 2.45$; $R^2_{adj} = 0.305$; $p < 0.05$) and needle age class ($F_{2.114} = 24.4$; $R^2_{adj} = 0.201$; $p < 0.001$) in Norway. *L. seditiosum* epidemics occur irregularly and depend on weather conditions (Martinsson 1979). We can confirm that the years of sampling (2014 and 2015) were not epidemic years of *L. seditiosum* (pers. Comm. by R. Drenkhan). Hanso and Drenkhan (2012) concluded that the most favourable years to *L. seditiosum* epidemics are the ones where high level of precipitation is recorded during growing season (May – August). In Estonia, the year 2014 was one of the warmest and driest years among the last 50 years (Yearbook of Estonian Meteorology 2014), thus unlikely to trigger

L. seditiosum epidemic. Further studies that include sampling of needles in epidemic years of *L. seditiosum* are needed to profile the fungal community dynamics of *P. sylvestris* needles.

In agreement with hypothesis 1, fungal species composition in foliage of coniferous tree species, in addition to deciduous *F. excelsior,* was influenced by pathogens as the mycobiome of Scots pine needles was significantly (p=0.004) different also in pine trees with visible *D. septosporum* symptoms (**IV**). Other factors that influenced the fungal species composition in paper **IV** were as follows: sampling site (4.9 % of variation was explained, p=0.002), needle age, needle location in canopy (3.8 and 3.8 % respectively). On symptomatic trees the most prevalent OTUs were *Lophodermium conigenum*, Phacidiaceae sp. And *Dothistroma septosporum* with relative abundances of 16.5, 15.5 and 5.6 percent, respectively. In healthy-looking trees *Lophodermium conigenum*, *Sydowia polyspora* and *Capnodiales* sp prevailed, with relative abundances of 27.7%, 8.1% and 6.7%, respectively. A Swedish study (Millberg *et al*., 2016) on Scots pine showed distinct differences in fungal species composition between visibly healthy and symptomatic trees, although the authors did not specify the causative agent of the symptoms. There also remains a possibility that both fungal species composition and tree receptiveness to pathogens can be influenced by the genetic background of the tree itself, a possibility that needs to be addressed in further studies. The results of PERMANOVA analysis across two papers (**I; IV**) are visualized in PCoA and CAP plots on figures 2 and 3.

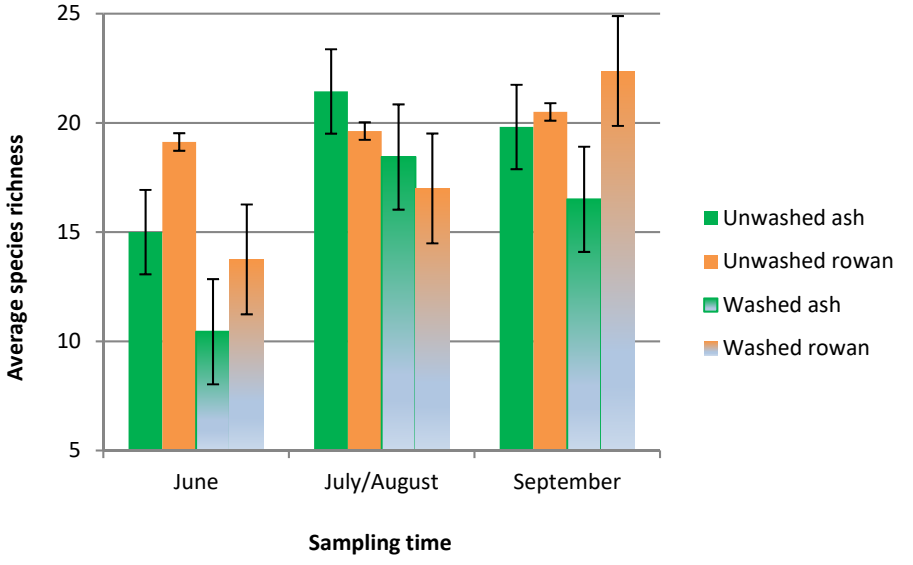
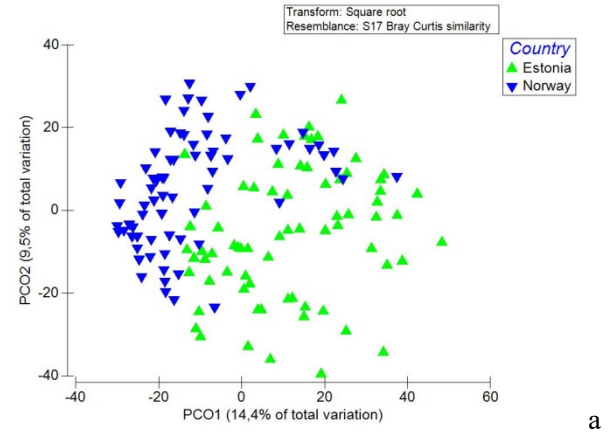
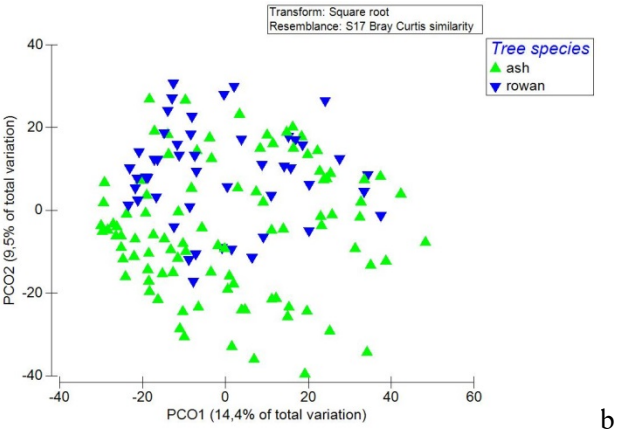


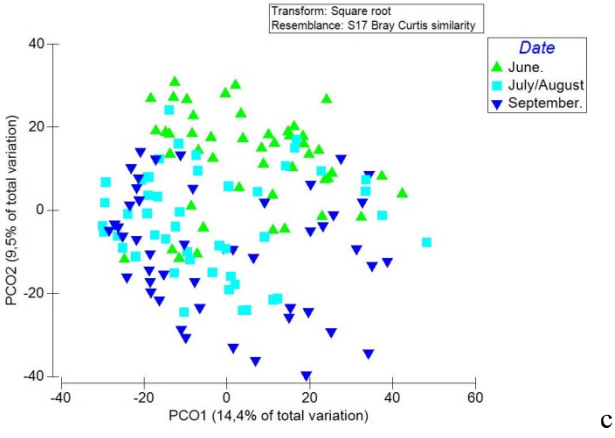
Figure 1. Average fungal species richness (species per sample) from PacBio data in unwashed and washed leaflets of ash and rowan across the sampling period. Data from Estonia and Norway pooled (N=144; Paper **I**). Whiskers show standard error.



a



b



c

Figure 2. Principal coordinates analysis, presenting trends in fungal community structure for the different sampling sites (countries) (a), tree species (ash and rowan; b) and sampling times (c), Paper **I**.

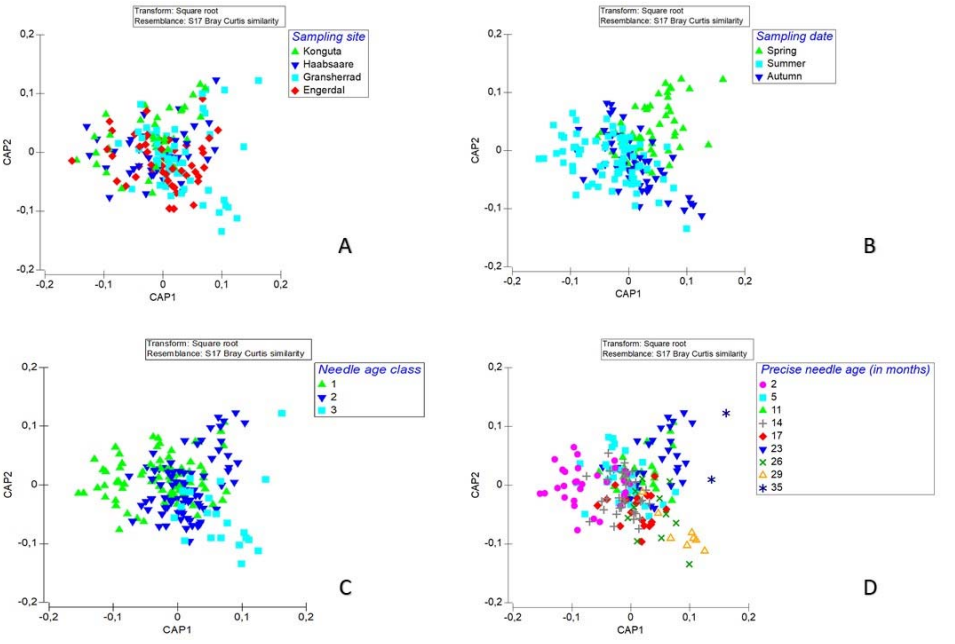


Figure 3. Canonical analysis of principal coordinates (CAP) of differences in fungal community structure on Scots pine needles between the four investigated sampling sites in northern Europe (two in Estonia, two in Norway); (a) three sampling dates, (b) three needle-age classes, (c) needle age in months (d), Paper **IV**.

In agreement with hypothesis 2, fungal pathogens in leaflets of European ash and needles of Scots pine had different seasonal peaks in their abundance. *H. fraxineus* showed a steady increase in relative abundance from spring to early autumn (**I**), whereas pine needle pathogen *D. septosporum* was highly abundant in summer and showed a distinct decrease in autumn (**IV**). These results show clearly that these two pathogens have distinct seasonal strategies in occupying the host tissues. While *H. fraxineus* uses a massive content of propagules to overcome the defence mechanisms of the leaflet at the end of the growing season (September; **I**), *D. septosporum* forms a web of hyphae on the needle surfaces already during summer, but without causing any distinct symptoms, possibly competing with other fungal species for space on the needle surface (**IV**). The identification of seasonal peaks are also important to plan control strategies of those two pathogens. While *H. fraxineus* is highly abundant on leaves in autumn it is important to start the monitoring of the number of fruiting bodies and propagule amounts in June. The monitoring of *D. septosporum* should start in spring coupled with prophylactic treatment of fungicides in nurseries, to assess and minimize the possible serious outbreak of the fungus.

When concentrating on other fungal species in paper **I** it became evident that the leaflet niche of both ash and control tree species rowan was dominated mostly

by epiphytic propagules of *Vishniacozyma* yeasts, the dimorphic fungus *Aureobasidion pullulans* de Bary., C*ladosporium ramotenellum* and *H. fraxineus.* Endophytic thalli mostly comprised of biotrophs (*Phyllactinia* and *Taphrina* species) and a necrotroph *Venturia fraxini* (**I**). The availability of nutrients, organic and inorganic molecules leaching from plant leaves, such as sugars, organic acids, amino acids, methanol, and various salts are the main factors shaping the size and structure of microbial and fungal communities. These factors also shape the dynamics of different fungal species inhabiting the foliage of forest tees (Tukey 1971; McGrath and Andrews 2006). The abundance of the above-mentioned nutrients varies with plant species, leaf age, growing conditions and season (Fonseca and Ignacio 2006). It is also noteworthy, that the community structure of epiphytes depends on the specific carbon utilization profiles of fungal species, for example *Vishniacozyma* species that were extremely abundant on both *F. excelsior* and *S. aucuparia* in Paper **I** show variation in their ability to assimilate specific carbon sources such as starch (Wang and Lin 2011). Both mechanical wounding and pathogen infection are known to facilitate the leaching of such nutrients from leaves, the affected plant leaves supporting higher yeast populations compared with healthy leaves (Nix e*t al*., 2009). In addition, the overall species richness also showed seasonality in the foliage of both tree species (Paper **I**). Species that showed a clear seasonal pattern had distinct differences in the time of their abundance peaks (**I**). The relative abundance of all ascomycetes on Scots pine needles showed a decrease from spring to autumn. Relative abundance of ascomycetes was 68.1% in spring, 50.4% in summer and to 50.1% in autumn. The niche of Scots pine needles was mostly dominated by filamentous endophytes from the species *Lophodermium conigenum* and *Sydowia polyspora,* filamentous endophyte *Lophodermium pinastri*, and also the needle pathogen *Dothistroma septosporum* (Paper **IV;** supplementary figure 1). The more common species showed seasonal differences in their relative abundance. In contrast to visual assessment of *D. septosporum* symptoms on Scots pine needles, the relative abundance of *D. septosporum* increased from 4.3% in spring to 11.0% in summer and declined in autumn (until 3.7%). *D. septosporum* is able to form an extensive web of hyphae on the needle surfaces that can persist epiphytically for several weeks, possibly using up the nutrients, including starch leaking from within the needles that are usually used by yeast species. Moreover, the available amount of starch leaking to the surface of *Pinus* sp. needles can be up to two times lower than the amount of starch available on leaf surfaces of *F. excelsior* (Kainulainen *et al*., 1998; Niinemets 1999). All these factors constituted in lower percentage of yeast species on the needle surfaces compared to leaf surfaces in Papers **I** and **IV**.

In contrast to *D. septosporum*, the relative abundance of *L. conigenum* was highest in spring (37.1%) and declined in summer (11.4%) and autumn (7.3%), being most abundant in older needles in spring in both countries (i.e. 2–3 year old needles in Norway, 23%;, 2 year old needles in Estonia, 23%), these showing significantly higher ($P<0.05$) relative abundance compared to young needles in spring (1 year old; 1.8% in Norway, 4.0% in Estonia) and old needles in summer

or autumn (2 and 3 year old needles; Paper **IV,** supplementary figure S4). This, in tandem with the higher percentage of *L. conigenum* on senescing needles compared to *D. septosporum*, suggests that *L. conigenum* is a typical endophyte as its colonization level increases along with needle age and senescence. The fruiting bodies of *L. conigenum* are formed on shed litter in spring; therefore, we can presume that feedback from saprobic sporulation contributed to the spring peak in the abundance of this fungus.

The needle pathogen *Lophodermium seditiosum* was detected only in the Estonian samples, at the low abundance of 0.3% of all sequences, which is best explained by the fact that the weather conditions in the sampling years 2014/2015 were not favourable for pathogen outbreaks (Hanso and Drenkhan 2012). In the northern hemisphere, *L. seditiosum* has been found in both symptomatic and asymptomatic needles (Millberg *et al*., 2016), showing that latent infections are also possible. *L. seditiosum* has also been considered a weak competitor against the endophytic mycobiome of pine needles, including other *Lophodermium* species (Hanso 1994).

Consistent with hypothesis 3, our findings show that *Hymenoscyphus albidus*, which generally is considered to be a saprotroph only, is able to cause necrotic lesions on leaflets of European ash weakened by autumn senescence in areas where *H. fraxineus* in not present. Both HTS data and specific qPCR assay showed a significantly higher *H. albidus* relative abundance and DNA estimates in lesion areas than in green leaf areas ($P<0.05$; **II**) in leaflet samples of European ash collected from Stjørdal. The absence of *H. fraxineus* at the time of sampling in Stjørdal was confirmed by HTS data and *H. fraxineus* specific qPCR assay carried out with DNA from leaflet samples (**II**). While the association of *H. fraxineus* to leaflet necrosis is well known through inoculation studies (Nielsen *et al*., 2017), the lifestyle of *H. albidus* has been a matter of debate. Baral and Bemmann (2014) speculated that the ascospores of *H. albidus* can infect living leaf tissues in a similar manner to *H. fraxineus*. This was questioned by Kowalski *et al*., (2015), who responded by stating that *H. albidus* has never been isolated from living leaf tissues, which in their opinion casts doubts to the possibility of *H. albidus* having an endophytic lifestyle. In paper **II,** we were able to record *H. albidus* infecting green leaf tissues for the first time, while the leaves were still attached to the tree. Even though we did not attempt to fill Koch postulates with the necrotic symptoms associated with *H. albidus*, leaf rachis inoculation studies with *H. albidus* have induced short necrotic lesions (Kowalski *et al*., 2015). As the first necrotic leaf veins associated with *H. albidus* were observed in late September and early October during two sampling seasons, it is reasonable to presume that *H. albidus* is able to cause necrosis on leaflets that are already weakened by autumn senescence, a feature that is also observed in the life cycle of *H. fraxineus* in its native range in Asia (Drenkhan *et al*., 2017b). Thus, both *Hymenoscyphus* species have prolonged endophytic phase in ash leaves in their respective native range but can enter necrotrophic growth

phase in weakened host tissues. This suggests a link between the colonization mode of these two fungi and physiological condition of their hosts. Strikingly, it seems that the two *Hymenoscyphus* species have major differences in their fecundity. *H. albidus* has features of a *k*-selected species that tend to have relatively stable populations, fluctuating near the carrying capacity of the environment. The *k*-selected species are known to have a small number of offspring that all have a high possibility to survive until adulthood. This was also supported by the fact that in the Stjørdal site it took meticulous searching to find the fruiting bodies of *H. albidus* (pers. comm. by Ari M. Hietala). The lineage of *H. fraxineus* invasive in Europe seems to be *r*-selected. *R*-selected species are characterized by high growth rates, typically exploiting less crowded ecological niches, and producing abundant offspring. *H. albidus* and *H. fraxineus* have possibly been subjected to different environmental selection pressures in their native ranges. The adaptation to a host that is phylogenetically closely related to European ash, a tree species with high occurrence frequency in Europe, and the presence of environmental conditions favorable to *H. fraxineus* life cycle completion in most years, may enable build-up of high infection pressure and challenge of leaf defense responses already during the growing season.

In agreement with hypothesis 4, we found evidence of *H. fraxineus* being present earlier in northern Europe than previously thought (III). As we were able to successfully determine and identify *H. fraxineus* and *H. albidus* from mycological herbaria (Drenkhan *et al.*, 2016), we turned our focus on botanical herbaria in Estonia. From there we were able to determine *H. fraxineus* in two specimens in the herbarium of the Tallinn Botanic Garden (TALL). We initially identified 9 sequences of *H. fraxineus* using HTS from the leaves of *Fraxinus chinensis* Roxb., collected from Tallinn Botanical Garden, northern Estonia in 1992 (Figure 4a) that originated from a tree grown from a seed collected from Beijing in 1985. Another *H. fraxineus*-positive herbarium sample of *F. chinensis* subsp. *rhynchophylla* was also collected from Tallinn botanical garden in 1978 (Figure 4b) and identified by Sanger sequencing and qPCR. This particular tree was grown from a seed collected from Shamora, Russian Far East. Using species- and strain-specific qPCR assay we were able to determine that both of the herbarium specimens harboured the European strain of *H. fraxineus*. These sampled trees were possibly infected by other near-by growing trees.

As other authors have also questioned the possibility of transferral of a viable pathogen via ash seeds from more remote areas (Marčiulynienė *et al*., 2018), there remains a possibility that the European strain of *H. fraxineus* emerged in Europe as a result of the hybridization of two different Far-East Asian strains thought to be the founders of a European population of the pathogen (Sønstebø *et al*., 2017). In the light of these results, it is crucial to minimize the possibility of new introductions of more virulent strains of *H. fraxineus* from East Asia. It has to be taken into account that the first visible symptoms of ash dieback in Estonia were not seen until the first part of 2000s, making the latency period,

during which the pathogen builds a high enough inoculum to cause symptoms, as long as two decades. Paper **I** and several other studies (Zheng and Zhuang 2014; Cleary *et al*., 2016; Drenkhan *et al*., 2017b) have illustrated that *H. fraxineus* can be present on leaves of trees that have no visible symptoms. The long latency period and the fact that *H. fraxineus* can also be present on symptomless leaves show clearly how dangerous invasive plant pathogens can establish themselves in new environments and persist there for several decades before they become problematic, and only then is their presence first observed.

a **b**

Figure 4. *H. fraxineus* positive herbarium specimens collected from Tallinn botanical garden in 7.07.1992 (a) and 28.07.1978 (b), respectively (paper **III**). The red arrow shows a lesion from which the sample was taken.

CONCLUSIONS

The following main conclusions and working hypotheses can be derived from this thesis:

- *Hymenoscyphus fraxineus* has a negative effect on fungal species richness of European ash leaflets late in the growing season; this indicates that in addition to effects on its host, invasive fungal pathogens may also have a significant effect on indigenous fungal communities inhabiting the same niche (**I**).
- The relative abundance of the invasive pathogen *H. fraxineus* on European ash increased from spring to autumn (highest in September). This means that the highest influence of the pathogen to the mycobiome of leaflets is expressed in the second half of the growing season when the leaflet infection also occurs.
- The highly similar leaflet niche of European ash and rowan was dominated by *Vishniacozyma* yeasts, the dimorphic fungus *Aureobasidion pullulans, Cladosporium ramotenellum*, endophytic thalli of biotrophs (*Phyllactinia* and *Taphrina species*) and the indigenous necrotroph *Venturia fraxini* (**I**). The overall fungal richness of European ash and rowan had distinctly different seasonal trajectories.
- Comparison of mycobiomes between healthy and symptomatic European ash leaflets revealed no significant differences in relative abundance of *H. fraxineus,* but saprotrophic species, e.g., *A. pullulans,* were more prevalent in leaflets of the symptomatic European ash trees (**I**). No species showed significantly higher read percentage on healthy ash trees than diseased ash trees.
- Similar to *H. fraxineus* that has the ability to cause leaf necrosis in its native range in Russian Far East, *H. albidus* (considered a fully saprotrophic fungus until now) shows an ability to cause necrosis on leaflets of *F. excelsior* weakened by autumn senescence in northern Europe; thus *H. albidus* cannot be considered only as a saprotroph (**II**).
- *H. albidus* and *H. fraxineus* differ in fecundity and offspring quality, suggesting that *H. albidus* is a *k*-selected species, while the lineage of *H. fraxineus* invasive in Europe behaves as an *r*-selected species (**II**).
- *H. fraxineus* was present in introduced ash species in European parks and arboreta at least 14 years before the first reports of dieback in Poland and Lithuania in 1992 (**III**), suggesting an earlier introduction of this pathogen into European nature.
- In Scots pine foliage, the endophytic *Lophodermium conigenum,* but not pathogens *L. seditiosum* or *D. septosporum,* had a significant negative effect on fungal species richness, although only in older (2–3 years old) needles in spring (**IV**). The low abundance of *L. seditiosum* suggests that sampling years were not epidemic years for the pathogen and its maximum impact on the needle mycobiome remains to be studied. Fungal species composition was significantly different in *D. septosporum* symptomatic and healthy needles of Scots pine.
- *L. conigenum* was highly abundant on needles of *Pinus sylvestris*, regardless of the sampling site, but seemed to prefer older, 2–3 year old needles.

SUMMARY

During the last decades, several tree species have suffered under the pressure of indigenous pathogens or invasive alien fungal pathogens whose establishment into northern European nature has probably been mediated by climate change and human activity. Epidemics, such as that of ash dieback and many others, are clear examples of how invasive alien pathogens can cause huge economic losses. In addition to economic losses, diversity decrease of native plants that have no established natural defence mechanisms against invasive species due to lack of co-evolution is also apparent. This diversity decrease of native plants can result in a genetic bottleneck which in turn endangers their future adaptability. Although some invasive and many native pathogens are well known and documented for their impact on the host trees, little is still known about their effect on the resident fungal communities competing for the same niche. This thesis sheds light to these knowledge gaps in relation to *Hymenoscyphus fraxineus* and *Dothistroma septosporum* in the foliage of *Fraxinus excelsior* and *Pinus sylvestris,* respectively.

The ash dieback agent *H. fraxineus* was discovered in Poland and Lithuania in the early 1990s, by now it has spread all over Europe. While its main host is the European indigenous *Fraxinus excelsior*, it is also able to infect several other ash species growing in Europe (both exotic and native). In addition to the huge economic losses, its negative effect includes also the loss of co-occurring species communities like lichens. *H. fraxineus* has also played a significant role in decline of the native fungus *Hymenoscyphus albidus,* a species that also uses leaf vein system as a sporulation substrate during the saprotrophic phase in leaf debris. Even before ash dieback *H. albidus* was considered a relatively rare species as there is only a small number of known herbarium deposits.

Out of different coniferous tree species growing naturally in Northern Europe, Scots pine has the largest problems related to pathogenic fungi. The damages caused by needle pathogens such as *Dothistroma septosporum* have increased during the last decade, possibly due to climate change. This thesis addresses diversity of fungi in the foliage of *Pinus sylvestris* and *Fraxinus excelsior* and tries to elucidate the effects different pathogens have on endophytic and epiphytic fungal communities of the affected organs of these respective tree species. Also the thesis opens details of ash dieback agent arrival time to northern Europe.

To consider effects pathogens have on natural mycobiome of tree foliage, we sampled European ash leaflets and needles of Scots pine from different sites in two North European countries – Estonia and Norway (**I; IV**). European rowan (*Sorbus aucuparia*) was included as a control species in paper **I**. Little is known about the role of *H. albidus* in green leaflets of *Fraxinus excelsior*. We tried to clarify this role by sampling leaflets of *F. excelsior* that manifested similar symptoms as those associated with *H. fraxineus* in a stand where *H. fraxineus* was not present at the time of sampling in 2016 (**II**). A total of 109 herbarium specimens across three herbaria in Estonia were sampled to clarify the arrival time of *H. fraxineus* into northern Europe (**III**). Main hypothesis derived from

these four separate studies were as follows: 1) Invasive and indigenous fungal pathogens of foliage seriously affect the fungal species richness and composition of coniferous and deciduous tree species (**I; II; IV**); 2) The fungal foliage pathogens in *Fraxinus excelsior* and *Pinus sylvestris* have different sporulation peaks in one calendar year and these peaks are the most important drivers of fungal richness within the foliage of *F. excelsior* and *P. sylvestris* (**I; IV**); 3) While long considered as a saprotroph, *Hymenoscyphus albidus* is able to cause necrotic lesions on leaflets of European ash weakened by autumn senescence in areas without ash dieback agent *Hymenoscyphus fraxineus* (**II**); 4) The invasive pathogen *Hymenoscyphus fraxineus*, was introduced into Europe significantly earlier than previously thought (**III**).

The results indicated that *H. fraxineus* reduces overall fungal species richness of both endophytic and epiphytic fungi on ash leaflets. However, this reduction of species richness was evident only late in the growing season when the number of *H. fraxineus* propagules on leaf tissues and in the air is high. In contrast to previous studies, we were able to record for the first time that *H. albidus* is able to cause necrosis on ash leaflets while they are still attached to the tree, showing that *H. albidus,* just like *H. fraxineus,* can cause necrosis on leaflets weakened by autumn senescence in its natural habitat where *H. fraxineus* is absent. Thus, *H. albidus* cannot be considered only as a harmless saprotroph of ash leaf litter. Moreover, we documented the earliest records of *H. fraxineus* infection of *F. chinensis* subsp. *rhynchophylla* and *F. chinensis* on two herbarium specimens collected from Tallinn Botanical Garden in years 1978 and 1992. The former sample predates the first observations of ash dieback in Europe by 14 years (Poland and Lithuania 1992). We also cannot exclude the possibility that *H. fraxineus* arrived into Northern Europe even earlier, the build-up phase of its infection pressure having gone unnoticed.

Scots pine needle pathogens *Dothistroma septosporum* and *Lophodermium seditiosum* had no significant effect on the fungal species richness in pine needles. As the sampling year 2014 was not an epidemic year of *L. seditiosum*, its observed low abundance was even expected. The overall fungal species richness of Scots pine was mostly influenced by sampling site, the sum of effective temperatures and the relative abundance of *Lophodermium conigenum*. *L. conigenum* is a common native pine needle endophyte that was extremely abundant in 2–3-year-old needles in spring and showed a rapid decline in summer and autumn. The fungal species composition in Scots pine needles in trees with *D. septosporum* symptoms was significantly different compared to visibly healthy trees. Other factors that influenced the fungal species composition were as follows: sampling site, needle age and needle location in canopy. Further studies are needed to evaluate the role of *L. conigenum* in *P. sylvestris* phylloplane.

We can conclude that needle fungal pathogens have no significant effect on fungal richness in Scots pine needles and that naturally occurring endophytes prevail in this niche. Possibly, *D. septosporum* is a weak competitor and the dynamics of species richness in pine needles is influenced mainly by other indigenous fungal species, climatic factors, and genotype of the tree. Also, as

previous studies suggest, *D. septosporum* originates from Europe and has co-evolved with Scots pine for a considerable amount of time and its effect on the mycobiome of its foliage is minimal.

The results of this thesis show that invasive pathogens can be present in new environments, even decades earlier before any visible symptoms occur. Trade of plant material from countries where these pathogens are native involves a risk of introduction of more virulent strains. Selection pressures in the introduced range may also lead to positive selection of more virulent strains.

SUMMARY IN ESTONIAN

Invasiivsete patogeenide ja loodusliku mükobioomi vastastikmõju puude lehestikus

Kliimamuutused ja globaalne kaubandus mõjutavad oluliselt dendropatogeenide levikut ja seeläbi maailma metsade tervist. Viimaste aastakümnete jooksul on sagenenud nii invasiivsete, aga ka varem tuntud seenpatogeenide põhjustatud haiguspuhangute esinemine. Jalakasurma ja saaresurma ning mitmete teiste seenhaiguste puhangute mõju bioloogilisele mitmekesisusele ja inimese majandustegevusele on märkimisväärne. Invasiivsete seenhaiguste nagu saaresurm (tekitaja *Hymenoscyphus fraxineus*) ja jalakasurm (tekitaja *Ophiostoma novo-ulmi*) ohtlikkus tuleneb sellest, et peremeestaimedel puuduvad väljakujunenud kaitsemehhanismid haigustekitajatega toimetulemiseks, mistõttu võib invasiivsete patogeenide kahju ökosüsteemide kestvusele ja selle kaudu ka majandusele kujuneda laiaulatuslikuks. Näiteid selle kohta on tänapäevaks kuhjunud mitmeid.

Uut ohtlikku epideemiat Euroopas – saarikute ulatuslikku hukkumist täheldati Poolas ja Leedus juba 1990. aastate alguses. Nende põhjuseks oleva saaresurma tekitajat *H. fraxineus* kirjeldatigi esimesena just Poolas, 2006. aastal. Nüüdseks on see patogeen levinud peaaegu üle terve Euroopa, olles muuhulgas ka loodusliku, s.t. varem tuntud saare lehti saprotroofina lagundava seene *Hymenoscyphus albidus* arvukuse languse või koguni väljasuremise peamiseks põhjustajaks. Seepärast on *H. albidus* Euroopa looduses muutunud nüüdseks juba haruldaseks.

Lisaks lehtpuudele on invasiivseid seenpatogeene tuvastatud ka mitmetel okaspuuliikidel. Põhja-Euroopa looduses kasvavatest okaspuuliikidest on invasiivseid seenpatogeene tuvastatud kõige rohkem majanduslikult olulisel harilikul männil. Ühed olulisemad okkapatogeenid harilikul männil tänapäeval on *Dothistroma septosporum*, varasematel aegadel *Lophodermium seditiosum*. Neist esimese esinemine on viimastel aastakümnetel oluliselt suurenenud eelkõige kliimamuutuste, täpsemalt – kliima soojenemise tõttu.

Kuigi kõikide eelpoolnimetatud patogeenide esinemise ja leviku mitmeid aspekte on juba põhjalikult uuritud, on endiselt vähe informatsiooni nende mõjust peremeestaimede lehtedes ja okastes esinevatele looduslikele seenekooslustele. Käesolevas doktoritöös analüüsitaksegi seente liigilist mitmekesisust nii hariliku saare lehtedes kui ka hariliku männi okastes, et selgitada, kuidas erinevad (invasiivsed ja põlised) patogeenid mõjutavad puuliikide lehtedele ja okastele omaseid seenekooslusi. Samuti selgitatakse kahe meile saabunud ehk olulisima invasiivse seenpatogeeni – *Hymenoscyphus fraxineus* ja *Dothistroma septosporum* täpsemaid esinemise kõrghetki lehtedes ning seda, kuidas need võisid mõjutada vastavatele biotoopidele muidu omaseid seenekooslusi. Seepärast analüüsiti ühe näitena ka Euroopale looduslikult omase mikroseene *H. albidus* esinemist kui üht mudelliiki saarelehtedes, ja seda võrdlevalt saaresurma tekitaja poolt asustatud (nii Eestis kui ka Lõuna-Norras) ja asustamata (Kesk-Norras) aladel. Ühtlasi aitab käesolevas töös patogeenide hulga kvalitatiivne hindamine taime kudedes paremini planeerida vastavate konkreetsete patogeenide seire ja tõrje strateegiat. Invasiivsete

patogeenide levikuviiside paremaks mõistmiseks otsitakse ja analüüsitakse töös ka varaseimaid jälgi saaresurma tekitaja *H. fraxineus* kohta Eestis, kasutades selleks erinevate saareliikide herbaareksemplare kolmes erinevas Eesti herbaariumis. Lisaks täpsustatakse Euroopa loodusliku saarelehtede lagundaja *H. albidus*e rolli hariliku saare rohelistes eluslehtedes, millist siiani on peetud ohutuks saare lehematerjali lagundajaks.

Doktoritöö peamised hüpoteesid on järgnevad: 1) Invasiivsed ning kohalikud lehe- ja okkapatogeenid mõjutavad oluliselt puude lehestikus olevate seente liigilist mitmekesisust ja -koosseisu (**I;II;IV**). 2) Patogeenide esinemisrohkuse haripunkt hariliku saare lehtedes ning hariliku männi okastes on sesooniti erinev ning see mõjutab enim lehestikuseente liigirikkust (**I;IV**). 3) Saaresurma tekitajast *H. fraxineus* asustamata alal on ka saprotroofiks peetav looduslik mikroseen *Hymenoscyphus albidus* võimeline kahjustama hariliku saare lehti (**II**). 4) Invasiivne patogeen *H. fraxineus* introdutseeriti Euroopasse oluliselt varem kui oli seni dokumenteeritud (**III**).

Materjal doktoritöös esitatud eesmärkide täitmiseks pärineb Eesti-Norra koostööprojektist EMP162 ja sisaldab autori poolt neljalt erinevalt proovialalt Norrast ning kolmelt proovialalt Eestist, kuid niisamuti saare herbaareksemplare kolmes erinevas Eesti herbaariumis (Tartu ülikooli loodusmuuseumi herbaarium, Tallina botaanikaaia herbaarium, Eesti Maaülikooli dendroloogiline herbaarium). Neli doktoritöösse kaasatud artiklit rajanes kokku 471 proovi analüüsil. Neist artikkel **I** analüüsis 144 proovi, artikkel **II** 28, artikkel **III** 109 ja artikkel **IV** 190 proovi. Proovidest eraldati DNA ning need sekveneeriti mass-sekveneerimise platvormil PacBio, milleks kasutati universaalseid seente praimereid ITS1catta ning ITS4ngs.

Doktoritöö tulemused näitasid, et *H. fraxineus* mõjutab negatiivselt nii epifüütsete kui ka endofüütsete seente liigirikkust hariliku saare (*Fraxinus excelsior*) lehtedes, kuid ei mõjuta seente liigirikkust saarega samas puistus kasvava ning kontrolliks võetud hariliku pihlaka (*Sorbus aucuparia*) lehtedes (**I**). *H. fraxineus*'e negatiivne mõju lehtede seenekooslustele on nähtav siiski vaid kasvuperioodi lõppfaasis – sügisel, kui õhus ja lehepindadel on patogeeni askospooride arv kõrgeim ja lehtede eneste kaitsevõime on nende vananemise tõttu vähenenud. Seetõttu on ka *H. fraxineus* saartele kõige ohtlikum just kasvuperioodi lõpus, mil seene eosed on võimelised nakatama veel puudele kinnitunud lehti, kuid nende kaudu võrseid ja seejärel kogu puud, kusjuures lehed on vaid stardiplatvormiks puude nakatumisele ja tõelisele kahjule, mis avaldub puude võrsetes ja okstes.

Laialt levinud hariliku männi okkapatogeenidel *D. septosporum* ja *L. seditiosum* ei olnud olulist mõju seente üldisele liigirikkusele hariliku männi okastes (**IV**). Samas peab märkima, et peamine analüüsiaasta 2014 ei olnud *L. seditiosumi* puhanguaasta, mispärast see ei pruugi väljendada nimetatud patogeeni tegelikku võimalikku mõju okkaseente kooslustele. Hariliku männi okastes elavate seente liigirikkuse olulisemateks mõjutajateks osutusid pigem proovivõtukoht, efektiivsete õhutemperatuuride summa ja männiokastes esineva loodusliku endofüüdi *Lophoderimum conigenum* suhteline arvukus. *L. conigenum* oli arvukaim kahe- ja kolmeaastastes männiokastes, kusjuures just kevadsesoonil

esines seent rohkem kui suvel ja sügisel (**IV**). Oluliseks osutunud *L. conigenum*'i tegelik roll männiokaste mükobioomis vajab edasisi mitmekülgseid uuringuid.

Saadud tulemustest võime siiski järeldada, et kuigi seenekooslused visuaalsete *D. septosporum*i sümptomitega puude ja visuaalselt tervete puude vahel erinesid, on okkapatogeenide mõju looduslikule männiokaste seente biootale minimaalne ning pigem domineerivad selles koosluses looduslikud endofüütsed seened. Invasiivne *D. septosporum* on aga tõenäoliselt seni veel suhteliselt nõrk konkurent ning liigirikkust hariliku männi okastes mõjutavad teised looduslikult esinevad seeneliigid, klimaatilised tegurid ja puu genotüüp. Siiski, eelnevad tööd viitavad asjaolule, et *D. septosporum* on Euroopas esinenud olulisemalt kauem kui seni arvatud ning seetõttu ka hariliku männiga koos arenenud. See võib olla üheks faktoriks, mis selgitab patogeeni *D. septosporum* vähest mõju hariliku männi okaste mükobioomile.

Erinevalt varasematest teadmistest õnnestus meil selle töö käigus esmakordselt tõestada, et saprotroofseks peetav *H. albidus* on siiski võimeline tekitama nekrootilisi laike, s.t. kahjustama hariliku saare rohelisi lehti ja just aladel, kus saaresurma haigustekitaja *H. fraxineus* ei esine. Samuti selgus, et saaresurma tekitajaga *H. fraxineus* asustatud aladel puuduvad igasugused jäljed *H. albidus*'e kohta (**II**). See tulemus viitab siiski ka *H. albidus*'e mõningale sarnasusele saaresurma tekitaja *H. fraxineus*'iga, kes tekitab oma looduslikus levialas Venemaa Kaug-Idas saarelehtedele niisamuti vaid nekrootilisi laike, just tingimustes mil lehtede füsioloogilise aktiivsuse vähenedes sügise arenedes on nende kaitsevõime langenud. Doktoritööst selgus, et Põhja-Euroopas on *H. albidus* võimeline tekitama sügisestel lehtedel samasuguseid kahjustusi, mis näitab, et *H. albidus*'t ei saa pidada ainult saprotroofseks saarelehtede lagundajaks, vaid tegemist on pigem nõrga patogeeniga.

Invasiivsete patogeenide vs. kohalike seente levikustrateegia selgitamisel (täpsemalt: *H. fraxineus* vs. *H. albidus*) tuvastati töö ühe niisamuti olulise tulemusena herbaareksemplaride põhjal, invasiivne saaresurma tekitaja *H. fraxineus* kahel introdutseeritud saareliigil *Fraxinus chinensis* subsp. *rhynchophylla* ja *F. chinensis*, kusjuures mõlemad eksemplarid olid kogutud Tallinna botaanikaaiast vastavalt 1978. ja 1992. aastal. See viitab vähemalt 14-aastat varasemale nimetatud haigustekitaja saabumisele Euroopasse, võrreldes tema poolt tekitatud oluliste kahjude alguse ajaga. Välistada ei saa sedagi, et *H. fraxineus* saabus Euroopasse varemgi, kuid vajas siin kohanemiseks veelgi pikemat aega ja võimalik, et kliimamuutusest tulenenud soodsamat keskkonda.

Doktoritöö näitab kokkuvõttes, kuidas invasiivsed patogeenid mõjutavad juba olemasolevaid puude lehtede seenekooslusi ja seda ühe aspektina ka olukorras, kus lehtede kaitsevõime puude lehestikus on langenud füsioloogilise aktiivsuse vähenemise tõttu sügisel. Doktoritöös kajastuva artikli **II** tulemustest selgub, et Euroopas looduslikuks peetav mikroseen *H. albidus* esineb Kesk-Norras vaid saaresurma tekitajast vabadel aladel, kusjuures Eestist nimetatud liiki pole õnnestunud leida. Ühtlasi leiti, et mõlema nimetatud seene, kusjuures üks neist invasiivne, teine põlisasukaks arvatav, elustrateegiad on erinevad. *H. albidus* on k-strateeg, milliseid iseloomustab vähene järglaste arv isendi kohta põlvkonnas.

K-strateegid asustavad paljude seostega, väljakujunenud ökonišše. *H. fraxineus*e suur järglaste arv põlvkonna kohta viitab aga sellele, et selle seeneliigi puhul on tegemist r-strateegiga, kes asustab reeglina vaid vähemtäitunud ökonišše. Selle töö tulemused kinnitavad ühtlasi selgelt, et invasiivsed patogeenid võivad uutes elupaikades kohal olla isegi aastakümneid varem kui algavad kahjustused puudel ning ilmnevad nähtavad sümptomid. Juba Eestisse saabunud invasiivsete patogeenide ohtlikkuse vähendamiseks on vajalik piirata nende uute, potentsiaalselt ohtlikumategi seenetüvede impordi võimalusi välisriikidest. Samas ei saa aga välistada ka virulentsemate seenetüvede tekkimise võimalust kohapeal, s.o. Ida-Euroopas. Mõlema võimaluse minimeerimiseks on oluline pidev seire, s.o. võimalike ohtlike seenpatogeenide varajane avastamine, mis teeks võimalikuks nende õigeaegse tõrje rakendamise.

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