Dieback of *Fraxinus excelsior* in the Baltic Sea Region

Associated Fungi, Their Pathogenicity and Implications for Silviculture

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Dieback of *Fraxinus excelsior* in the Baltic Sea region: associated fungi, their pathogenicity and implications for silviculture.

Abstract

This thesis is comprised of three main studies: (1) the wood-inhabiting fungi found in declining European ash (*Fraxinus excelsior* L.) and their pathogenicity; (2) the relationship between *F. excelsior* phenology, site density and the susceptibility to the dieback; and (3) the potential of natural regeneration of dieback affected ash stands. The studies that are presented here were conducted in Denmark, Lithuania and Sweden.

Combination of different sampling and detection methods revealed a high diversity of fungi in both healthy looking and symptomatic tissues of declining *F. excelsior*. The most frequently detected fungal taxa were *Alternaria alternata, Armillaria cepistipes*, *Aureobasidium pullulans*, *Botryosphaeria stevensii, Cladosporium cladosporioides, Cryptococcus foliicola, Epicoccum nigrum*, *Gibberella avenacea*, *Hymenoscyphus pseudoalbidus*, *Lewia* sp., *Phoma* spp. and *Phomopsis* sp.

In pathogenicity tests nine fungal taxa caused symptomatic discoloration of bark and cambium on *F. excelsior* saplings, though only *H. pseudoalbidus* infected substantial proportion (50-100%) of tested trees.

The seasonal pattern of ash dieback severity, attributed to crown damage of *F. excelsior* trees, significantly increased towards the end of the investigated growth season. Severity of dieback symptoms was more pronounced in the unthinned stands, but otherwise was not related with stand density. However, susceptibility of *F. excelsior* to the disease was found to be dependent on the flushing (bud-bursting) phenology of the trees - late-flushing *F. excelsior* were most severely affected.

Our study demonstrated that vigorous natural regeneration of *F. excelsior* in examined clear-felled sites cannot be expected. Regenerating *F. excelsior* exhibited abundant dieback symptoms. The species composition in sites with long disease history is likely to shift away from *F. excelsior* to early successional pioneer species such as *Alnus incana*, *Betula* spp., and in some instances *Populus tremula*.

Keywords: *Armillaria* spp., *Chalara fraxinea,* dieback, *Fraxinus excelsior, Hymenoscyphus pseudoalbidus,* pathogenicity, regeneration, succession, wood-inhabiting fungi.

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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 Bakys, R., Vasaitis, R., Barklund, P., Thomsen, I. M. & Stenlid, J. (2009). Occurrence and pathogenicity of fungi in necrotic and non-symptomatic shoots of declining common ash (*Fraxinus excelsior*) in Sweden. *European Journal of Forest Research* 128, 51-60.

II Bakys, R., Vasaitis, R., Barklund, P., Ihrmark, K. & Stenlid, J. (2009). Investigations concerning the role of *Chalara fraxinea* in declining *Fraxinus excelsior*. *Plant Pathology* 58, 284-292.

III Bakys, R., Vasiliauskas, A., Ihrmark, K., Stenlid, J., Menkis, A. & Vasaitis, R. (2011) Root rot, associated fungi and their impact on health condition of declining *Fraxinus excelsior* stands in Lithuania. *Scandinavian Journal of Forest Research* 26, 128-135.

IV Bakys, R., Vasaitis, R. & Skovsgaard, J. P. Patterns and severity of crown dieback in young even-aged stands of European ash (*Fraxinus excelsior* L.) in relation to stand density, bud flushing phenotype and season. *Plant Protection Science* (in press).

V Lygis, V., Bakys, R., Gustienė, A., Burokienė, D., Matelis, A. & Vasaitis, R. Forest self-regeneration following clear-felling of dieback-affected *Fraxinus excelsior*: focus on ash (submitted manuscript).

The contribution of Remigijus Bakys to the papers included in this thesis was as follows:

I Responsible for the laboratory work. The fieldwork, data analysis and writing of the paper were conducted jointly with supervisors.

II Contributed to the fieldwork and writing up the paper in collaboration with the supervisors. PCR laboratory work was kindly held by Katarina Ihrmark.

III Responsible for the field- and laboratory work. Analysed the data and wrote the paper in collaboration with supervisors.

IV Responsible for the fieldwork and data analysis. Wrote the manuscript together with co-authors.

V Author did field work, performed data analysis and wrote the manuscript together with co-authors.

Abbreviations

BLAST	basic local alignment search tool
CBS	Centraalbureau Voor Schimmelcultures (Central Bureau of Fungal Cultures)
CTAB	cetyl trimethyl ammonium bromide
DNA	deoxyribonucleic acid
ITS	internal transcribed spacer
PCR	polymerase chain reaction
Rpm	rotations per minute
rRNA	ribosomal ribonucleic acid
SE	standard error
SLU	Sveriges Landbruksuniversitet (Swedish University of Agricultural Sciences)
T-RFLP	terminal restriction fragment length polymorphism

1 Introduction

1.1 Distribution and ecology of *Fraxinus excelsior* in Europe

European ash (*Fraxinus* excelsior L.) is among the highest valuable broadleaved tree species in Scandinavia and Baltic countries, planted widely for landscaping and timber production. This wind-pollinated and wind-dispersed tree species has a very large and continuous distribution. *F. excelsior* is distributed across entire Europe, confined to Atlantic coast in the west and to Volga River in the east, and restricted below 63.4°N in Fennoscandia as the northern limit of its natural range. The main delimiting factors of the species' natural distribution are a lack of tolerance to prolonged drought, winter cold and late spring frosts; on the other hand, *F. excelsior* is adaptive to a wide range of environmental conditions (Dobrowolska *et al*., 2008). The ecology of this tree species exhibits intermediate properties between a pioneer species and a permanent forest component (Pliūra & Heuertz, 2003). *F. excelsior* plays an important role in both primary and secondary succession, and it occupies large areas of all age classes of primary and secondary woodland (Dobrowolska *et al*., 2011). Vegetative regeneration after coppicing is intense, particularly in dense canopies; it is often so prolific that the species becomes invasive (Wagner, 1990; Fraxigen, 2005). Seedlings and saplings of *F. excelsior* are shade-tolerant, though becoming considerably more light-demanding while getting older (Tapper, 1996). This ash species usually occurs in mixed stands with other broadleaf species, while pure stands are less common (Fraxigen, 2005; Dobrowolska *et al*., 2011). In Scandinavia, *F. excelsior* usually thrives in hardwood forests concentrated in the Boreo-nemoral zone and usually grows with *Alnus glutinosa* L., *Ulmus glabra* Huds. (Diekmann, 1994, 1996), *Tilia cordata* Mill. or *Fagus sylvatica* L. (Lawesson, 2000). In Baltic countries the species is usually found in mixed wetland forests alongside with *A. glutinosa*, *Alnus incana* (L.) Moench, *Betula pendula* Roth., *Corylus avelana* L., *Populus tremula* L. and *T. cordata* (Narbutas, 1975; Prieditis, 1999).

Although efficient in natural regeneration, the growth potential, competition ability and adaptivity to environmental changes of *F. excelsior* is strong only if certain ecological requirements are met. This species is mesophilic and highly demanding on soil fertility; it tolerates pH levels as low as 4.5, however prefers soil above 5.5 pH (Pliūra & Heuertz, 2003). *F. excelsio*r is highly tolerant to seasonal water-logging and favours floodplain forests (Fraxigen, 2005). It is a typical species on wet, periodically flooded alluvial soils, as well as on moist slopes and ravines (Diekmann, 1996). Although *F. excelsior* can survive strong water deficit for limited periods of time, on dry soil types this species highly depends on rain water supplies (Dobrowolska *et al*., 2008).

The natural range of *F. excelsior* during centuries has been seriously altered by agricultural activities (Diekmann, 1994). During recent decades, however, the phenomenon of this ash species expansion on abandoned, waterlogged and sloped farmland areas was reported, which presence most likely is related to decrease in rural population (Marigo *et al*., 2000).

1.2 Dieback and decline of forest and trees - concepts and definitions

There are several definitions of forest decline or dieback. These terms are not precise and debate about their definition is ongoing. As discussed by Manion (1991), forest decline can be characterized as the presence of various symptoms such as reduced growth, defoliation, premature fall colouring, dieback of twigs and branches generally beginning in the upper crown and increased prevalence or pathogenicity of decay fungi. Dieback is the death of groups of neighbouring trees rather than isolated trees dying in otherwise green forest ecosystems (Mueller-Dombois, 1987). In forest ecosystems exhibiting decline, progression of decline symptoms are different between trees in the same stand (Ciesla & Donaubauer, 1994). The biotic agents, either bacteria, fungi or insects, are the traditional reasoning behind forest dieback (Mueller-Dombois, 1987). Both definitions “dieback” and “decline” have been used in many studies to describe disease symptoms (Ciesla & Donaubauer, 1994).

Several definitions have been proposed to explain forest and tree decline (Jurskis, 2005): germ theory, climatic impacts, natural succession (decline disease theory), anthropogenic factors or a complex interaction of these factors. The germ theory suggests that the cause of forest dieback or decline is an individual biotic agent. Alien invasive pathogens, such as *Ophiostoma ulmi* (Buisman) Melin & Nannf. (1934) and *Ophiostoma novo-ulmi* Brasier, responsible for Dutch elm disease (Ghelardini, 2007), or *Cryphonectria parasitica* (Murrill) Barr, the causal agent of chestnut blight (Roane *et al*.,

1986) could be good examples supporting the germ theory. On the other hand, outbreaks of native pathogens, associated with forest decline, cannot be explained that easily. Trees and forest ecosystems are usually not susceptible to substantial, existence threatening damage, caused by pests and pathogens (Jurskis, 2005). Thus decline is often directly associated with other major environmental changes, usually caused by humans (environmental pollution, fertilizers, herbicides etc.) or climatic effects (flood, frost, drought, fire etc.), where biotic and abiotic factors are contributing to the stress which exceeds the potential of the trees for adaptation to these changes. Regardless of the exact mechanism, dieback is often a non-linear process. It can emerge at a large scale when changes of environmental conditions exceed a tree species' physiological thresholds of tolerance or trigger outbreaks of insect pests or fungal pathogens (Allen, 2007).

In a broader sense, stand-level dieback can be regarded as natural part of development and succession of local forest ecosystems (Mueller-Dombois, 1983). This led to the "decline disease theory" (Houston, 1981, 1984), extended by the "cohort senescence theory" (Mueller-Dombois, 1988, 1993). Both theories are based on three groups of general factors: (1) predisposing factors (tree genetics and site conditions); (2) triggering factors attributed to periodically recurring environmental stresses; and (3) hastening factors, which become effective after dieback has been triggered by abiotic agents.

In our study, the term "dieback" will be used for the death of *F. excelsior* stands or trees, whilst "decline" will be used in reference to a protracted malfunction of trees, such as reduced tree growth, wilting foliage, gradual defoliation of the crowns, dieback of shoots and twigs or decayed root systems. Dieback will be considered to be part of the decline phenomenon.

1.3 *F. excelsior* dieback in Europe

Ash dieback is an emerging disease causing massive tree mortality and threatening *F. excelsior's* existence at continental scale (Figure 1). Starting in early to mid-1990s, large-scale decline of *F. excelsior* was detected and increasingly observed in Lithuania and Poland (Juodvalkis & Vasiliauskas, 2002; Przybył, 2002a, b; Skuodienė *et al*., 2003; Kowalski & Lukomska, 2005; Lygis *et al*., 2005; Kowalski, 2006). Subsequently, the dieback of *F. excelsior* spread towards south, west and northern Europe, and presently is reported in more than 20 countries (Heydeck *et al*., 2005; Wulf & Schumacher, 2005; Cech, 2006; Kunca, 2006; Halmschlager & Kirisits, 2008; Drenkhan & Hanso, 2009; Engesser *et al*., 2009; Jankovsky & Holdenrieder, 2009; Ioos *et al*.,

2009; Ogris *et al*., 2009; Szabo, 2009; Talgø *et al*., 2009; Barič & Diminič, 2010; Ogris *et al.*, 2010; Rytkönen *et al*., 2010; Chandelier *et al*., 2011; Husson *et al*., 2011; Timmermann, 2011; Anonymous, 2012).

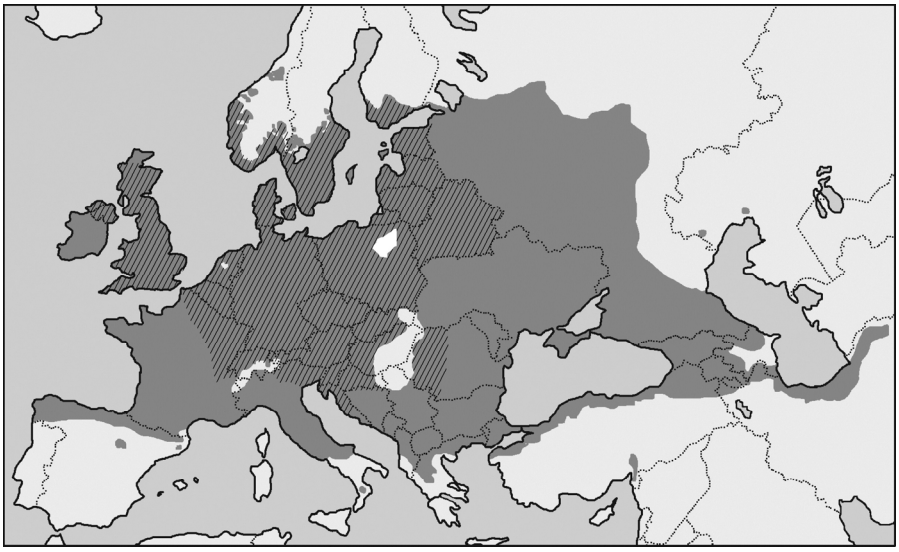

Figure 1. Map of Europe showing *F. excelsior's* natural distribution area (FRAXIGEN, 2005). Lined area indicates reported distribution of the ash dieback in 2012.

In Denmark disease was first recorded in 2003; subsequently, between 2005 and 2007 a rapid spread of the epidemic and massive decline were observed (Thomsen, 2005; Thomsen & Skovsgaard, 2006; Thomsen *et al*., 2007; Skovsgaard *et al*., 2010). Presently, the disease affects populations of *F. excelsior* in all parts of the country (Skovsgaard *et al*., 2010).

Lithuania is a country with one of the longest ash dieback disease histories. The dieback here started around 1995-1996 in north-central part of the country (Juodvalkis & Vasiliauskas, 2002). Due to sanitary clear-fellings the area of *F. excelsior* stands in the country has decreased from 50 800 ha in 1995 (Anonymous, 2001) to 36 300 ha in 2011 (Anonymous, 2011). Currently the disease in the country entered its chronic phase, all *F. excelsior* stands are damaged to various extent and their health condition continues to deteriorate (Riepšas, 2009; Gustienė, 2010).

In Sweden dieback of *F. excelsior* was first observed locally in southern part of the country in 2002; in 2004 the disease was reported widely, ultimately leading to tree death over almost the whole area of species distribution (Barklund, 2005, 2006). Prior to the outbreak of dieback in Sweden *F.*

excelsior has been of commercial importance for timber production; since 2010 this species is listed in the Red Data Book (SLU 2010).

Ash dieback exhibits a wide range of symptoms. Infected trees show wilt of leaves, necroses and cankers on shoots, branches and stems, followed by a gradual dieback of the crown (Figure 2) (Przybył, 2002b, c; Barklund, 2005, 2006, 2007; Kowalski & Lukomska, 2005; Kowalski, 2006; Schumacher *et al*., 2010; Skovsgaard *et al*., 2010).

Figure 2. F. excelsior stand, devastated by dieback.

Other *Fraxinus* species in Europe are also susceptible to the dieback, including *Fraxinus angustifolia* Vahl (Cech & Hoyer-Tomiczek, 2007; Schumacher *et al*., 2007; Kirisits *et al*., 2010), *Fraxinus ornus* L. (Kirisits *et al*., 2009), *Fraxinus americana* L.*, Fraxinus mandshurica* Rupr. and *Fraxinus pennsylvanica* Marshall (Drenkhan & Hanso, 2010).

A range of research attempted to associate ash dieback with miscellaneous environmental disturbances, such as drought, spring frosts, environmental pollution, differences in wood structure or pathogenic microorganisms (Juodvalkis & Vasiliauskas, 2002; Skuodienė *et al*., 2003; Cech, 2005; Cech & Hoyer-Tomiczek, 2007; Schumacher *et al*., 2007; Kirisits *et al*., 2008; Kowalski & Holdenrieder, 2008; Tulik *et al*., 2010). According to present knowledge, the only confirmed biotic agent of ash dieback is the ascomycetous

fungal species *Chalara fraxinea* T. Kowalski sp. nov. (Kowalski, 2006). Recently, *Hymenoscyphus pseudoalbidus* V. Queloz, C.R. Grünig, R. Berndt, T. Kowalski, T.N. Sieber and O. Holdenrieder has been assigned as the teleomorph of *C. fraxinea* (Queloz *et al*., 2010) (Figure 3). The teleomorph is the preferred scientific name of the fungus. The origin of *H. pseudoalbidus*, or the environmental conditions which triggered the pathogenicity of this fungus (if the species is indigenous), are more enigmatic. Geographically, the spread of the dieback exhibited a very pronounced spatial pattern (Timmermann *et al*., 2011); such behaviour is typical for alien invasive microorganisms.

Figure 3. Fruiting body of *H. pseudoalbidus*. Photo: Vaidotas Lygis.

On the other hand, the geographic locality, where ash dieback has been initially observed (Central Lithuania and Nord-Eastern Poland), is very difficult to explain in respect to the theory of *H. pseudoalbidus* alien origin. In Lithuania, forestry companies had no use of imported *F. excelsior* seed and vegetative material, while *F. excelsior* seedlings were grown in local orchards in sufficient quantities (Lithuanian State Forest Service, pers. communication). Before the outbreak of dieback, natural *F. excelsior* stands were left mainly for natural afforestation due to species' prolific and effective regeneration both in clear-cuts (Narbutas, 1975) and in the understory of the stands (Tapper, 1996). Moreover, particular limitations on the import of *F. excelsior* breeding material are implemented due to propagation of autochthonous populations in Lithuania (Lithuanian State Forest Service, pers. communication). The high genetic variability of the *H. pseudoalbidus* populations (Rytkönen *et al*., 2010; Kraj *et al.*, 2012) is also somewhat untypical for a pathogen, which could have been introduced.

1.4 Succession and shift of arborescent species in disturbed forest communities

Succession is defined as the changes observed in an ecological community following a perturbation that opens up a relatively large space (Connell & Slatyer, 1977). Disturbance, such as senile phase of the forest or a disease outbreak caused by a pathogenic organism, always initiate a new cycle of forest ecosystem development. Gaps and openings in forest canopy, resulted by such disturbance, are the driving force in forest cycles (Whitmore, 1989).

Competitive interactions between plants and pathogens or pests, among the physical stresses to plants and competition for resources between plants, are of critical importance to the course of succession (Horn, 1974; Connell & Slatyer, 1977). Pathogens can exert their influence onto forest ecosystems directly through tree mortality, thus affecting forest dynamics at a small (gap formation) or broad (forest development) scale (Castello *et al*., 1995).

As suggested by Campbell & Sloan (1977), when populations decline due to diseases, the final result should be a quasi-stability in the pathogen and forest system through two mechanisms: (1) selection within host species for individuals that are less disease-susceptible; (2) changes in species composition of the forest. For example, the American chestnut (*Castanea dentata* (Marsh.) Borkh.) was eliminated in the forest canopy by chestnut blight in North America in mid-1990s and presumably will be replaced by oak-hickory forests (McCormick & Platt, 1980). Such a successional pattern is possible for *F. excelsior* stands destroyed by ash dieback, although currently no single species has taken the dominant role in forest ecosystems, previously occupied by ash.

Presently, dying or clear-felled *F. excelsior* stands turn into large gaps. Such gaps are very suitable for the establishment of pioneer species with good propagative capacity (Beck & Hooper, 1986; Arthur *et al.*, 1997). In the future, when the canopy of pioneer species enters the mature phase, small gaps will develop, and these are likely to be closed by growth of climax (non-pioneer) species, that became established under canopy (Swaine & Whitmore, 1988; Whitmore, 1989).

2 Aims of the study

The thesis addresses questions about the nature and impacts of fungi that can be found inhabiting declining *F. excelsior*. We aim to identify the fungal pathogens and how they are associated with the recent spread of ash dieback. A further objective of this work was to investigate the successional patterns and assess the potential of *F. excelsior* for natural regeneration in stands with long history of disease, subjected to clear-felling due to sanitary considerations.

The specific objectives were:

- To provide detailed information about fungal communities inhabiting declining *F. excelsior* by combining different sampling and identification methods.
- To identify potential disease-causing fungi by carrying out pathogenicity tests.
- To investigate the relationship between *F. excelsior* flushing (budburst) phenotypes, stand density, time of the season and dieback severity.
- To evaluate forest self-regeneration on the areas initially dominated by *F. excelsior*, which were subsequently deteriorated by the disease, and as a result subjected to sanitary clear-fellings.

3 Materials and Methods

3.1 Study sites, fieldwork and sampling

The fieldwork was conducted in three countries bordering the Baltic Sea - Denmark, Lithuania and Sweden (Figure 4). Investigations of declining *F. excelsior* inhabiting fungal taxa and pathogenicity tests were conducted in Lithuania and Sweden; the assessment of the relationship between disease severity, bud-flushing phenotype, stand density and season was undertaken in Denmark; the evaluation of natural *F. excelsior* regeneration and other tree species succession in clear-felled stands was carried out in Lithuania.

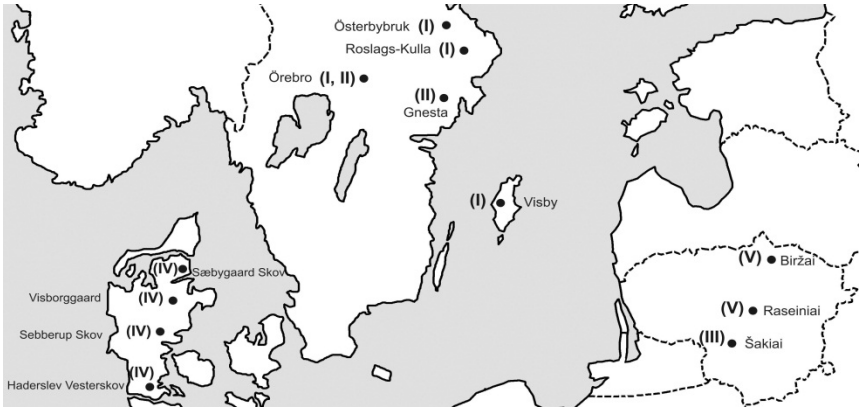

Figure 4. Map of Baltic Sea region showing field work localities. Roman numbers indicates respective study.

Four study sites, described in Paper **I,** are located near Örebro, Österbybruk, Roslags-Kulla and Visby in Sweden, and comprised of 20- to 30-year-old mixed natural *F. excelsior* stands exhibiting ash dieback symptoms. Sampling in Örebro and Visby was conducted in June 2004, in Österbybruk and Roslags-Kulla - in June 2006. In each stand, branches from trees were removed with a

sterile knife and later categorised into the following conditions: (1) visually healthy; (2) showing initial necroses; (3) showing advanced necroses; (4) shoots with dead tops. In total 58, 58, 54 and 61 samples from each respective category were collected during this study.

Conditions of sites in Paper **II** were similar to those described in Paper **I**. Sampling was carried out in two sites in southern-central Sweden near Örebro and Gnesta in September 2005. Both areas consisted of natural mixed 20- to 30-year-old *F. excelsior* stands. All examined stands were showing symptoms of crown decline. Sampling from selected trees included collecting of the branches with dieback symptoms on bark and leaves. In addition, bark samples from stem cankers were collected in Örebro. The collected material was sorted in the laboratory into following symptom categories for subsequent fungal isolations: (1) healthy-looking leaf stalks; (2) necrotic leaf stalks; (3) small bark wounds (less than 5mm); (4) larger bark wounds with necroses (10-15mm); and (5) necrotic bark from stem cankers. The amount of 40, 45, 60, 45 and 30 (220 in total) samples were isolated from each respective symptom category. In addition, samples of necrotic leaves, necrotic leaf stalks and necrotic bark on shoots (32 samples per respective category; 96 samples in total) were subjected to analysis of fungal DNA directly from plant tissues.

The study sites selected for the work described in Paper **III** were located in three natural and managed forest sites in southern Lithuania, Šakiai region. Fieldwork here was carried out in 2003. On each site, *F. excelsior* trees with crown dieback symptoms were classified into four categories: (1) slight crown dieback (5-25 % of crown dead); (2) moderate crown dieback (25-50%); (3) severe crown dieback (50-75%); and (4) complete dieback (75-100%). 33 trees in total from each category were felled and dissected in longitudinal and cross-sections to estimate the length of visible decay in stem from ground level and the extent of decay in stem cross-sections. The extent of visible decay in superficial parts of root systems of the felled trees was measured by digging the root systems up. Sampling was carried out from root systems of 50 *F. excelsior* trees with moderate crown damage. From each tree, one root was excavated and three wood samples were collected per root at distances of 0.5 m, 1 m and 1.5 m from the root collar (150 samples in total).

Incidence of sprouting and sprout health condition was evaluated for 328 *F. excelsior* stumps on three clear-cut sites in order to estimate the potential of self-regeneration of felled stands (**III**). The sites contained *F. excelsior* stands aged between 80 and 120 years, the majority of them dying due to ash dieback prior to felling. On each site, all available *F. excelsior* stumps were examined for sprouting and presence/absence of *Armillaria* decay. Sprouts were examined for presence of the ash dieback symptoms.

The work described in Paper **IV** was carried out in four locations along the east coast of Jutland peninsula, Denmark: (1) in Haderslev Vesterskov; (2) in Sebberup Skov; (3) near Visborggård; and (4) in Saebygaard Skov. All four experimental sites consisted from 5 to 7 plots according to the density of *F. excelsior* stands: (1) 1700-5500 trees/ha (unthinned); (2) 1500 trees/ha; (3) 500 trees/ha; and (4) 100-150 trees/ha. The stands were planted from 1992 to 1995 with two- to four-year-old saplings of *F. excelsior*. In 2007 a total number of 3180 *F. excelsior* trees were assessed for the phenologic phase of bud flushing and phenotypic damage in the crowns. Crown damage, attributed to foliage wilt, dead tops and proportion of dead part of the crowns, was assessed twice, in June and in September 2007.

In Paper **V**, the potential of self-regeneration and succession of arborescent species was evaluated in twenty 1- to 10-year-old clear-cuts, felled between 2000 and 2010. All examined clear-cuts were left to self-regeneration after sanitary clear-fellings. Before harvesting, the sites consisted of mixed stands of *F. excelsior* and Norway spruce (*Picea abies* (L.) H. Karst.), black alder (*A. glutinosa*), grey alder (*A. incana*); silver and downy birch (*B. pendula* and *Betula pubescens* Ehrh.), common aspen (*P. tremula*), small-leaved lime (*T. cordata*) and Eurasian elm (*U. glabra*), established on rich wet soils. Age of *F. excelsior* prior to felling ranged from 35 to 125 years. Composition of regenerating tree species was inspected in 3-m-wide transects, covering 2-3% of the whole clear-cut area. *F. excelsior* self-regeneration potential was assessed by measurement of abundance, height and health condition of the detected ash saplings and sprouts.

3.2 Fungal isolations

For fungal isolations, wood, bark and leaves of declining *F. excelsior* were used. All sample material was surface sterilized before isolation. The samples, obtained in the studies **II** & **III**, were washed under running tap water**.** Subsequent surface sterilization was carried out using open flame **(I** & **III)** or sodium hypochlorite (4% active chlorine) and 96% ethanol **(II)**. Samples collected for the studies in Papers **I** & **III**, were plated onto Hagem agar (Stenlid, 1985); in study **II** samples were plated onto three different types of media - 2% malt agar, 2% water agar and vegetable juice agar. All Petri dishes were incubated for two weeks under room temperature and checked daily for the mycelial growth. Emerging mycelia were subcultured onto the respective agar.

3.3 DNA extraction, amplification and sequencing

All isolated fungal cultures were sorted under the microscope into groups based on their mycelial morphology. Representative cultures of each group were selected for species identification by sequencing of internal transcribed spacer of fungal ribosomal RNA (ITS rRNA). Extraction of DNA, amplification and sequencing followed established methods (Kåren et al., 1997). For extraction of DNA from individual cultures, their mycelia were placed in 2 ml centrifugation tubes with a screw cap and homogenized using FastPrep FP120 tissue homogeniser (Savant Instrument Inc. Holbrook, NY, USA). Then, 0.8 ml of CTAB buffer (3% cetyltrimetylammoniumbromide, 2 mM ethylenediamine tetraacetic acid, 150 mM Tris-HCl, 2.6 M NaCl, pH 8) was added to each tube and these were incubated at 65°C for 1 h. Following incubation, an equal volume of chloroform was added to the tubes, samples were vortexed and centrifuged for 7 min at 13000 rpm. The resulting upper face was transferred to new tubes, DNA was precipitated by adding an equal volume of 2-propanol and pelleted by centrifugation for 20 min at 13000 rpm. Resulting DNA pellets were washed in 200 ml 70% ethanol, dried, dissolved in 50 µl of sterile deionised water, and stored at -20°C. The amplification by PCR of ITS rRNA was done using fungal specific primer ITS 1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes & Bruns, 1993) and universal primer ITS4 (TCCTCCGCTTATTGATATGC) (White *et al*., 1990). Each PCR reaction contained 200 µM deoxyribonucleotide triphosphates, 0.2 µM of each primer, 0.03 U/µl ThermoGreen Taq polymerase with reaction buffer Green, and 2.75 mM final concentration of $MgCl_2$. The thermal cycling was carried out as follows: an initial denaturation step at 95°C for 5 min was followed by 35 amplification cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The thermal cycling was ended by a final extension step at 72°C for 7 min. PCR products were size separated on 1% agarose gels, stained and visualized under UV light. Sequencing was performed using the 8000 Genetic Analysis System with the CEQ DTCS Quick Start Kit (Beckman Coulter). Raw sequence data were analysed using the SeqMan Pro software from DNASTAR package (DNASTAR, Madison, WI, USA).

3.4 Identificaton of fungal sequences

Databases at both GenBank (Altschul *et al*, 1997) and at the Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences were used to determine the identity of ITS rRNA sequences. The latter database contains well documented fungal sequences of pathogenic,

saprotrophic and entophytic fungi the majority of which (in particular of microfungi) have been isolated into pure cultures during previous studies, and morphologically identified at the CBS, Utrecht, the Netherlands. The criteria used for identification were: sequence coverage > 80%; similarity to taxon level 98-100%, similarity to genus level 94-97%.

In Paper **II,** isolates of *H. pseudoalbidus* were also identified morphologically using light microscope and by comparison to a reference culture of this fungus (Kowalski, 2006), kindly provided by Prof. Tadeusz Kowalski.

3.5 Direct sequencing, cloning and T-RFLP

Direct sequencing of fungal DNA from plant tissues is a sensitive method for the detection of potentially all fungal community and is in particular important for detection of slow growing and uncultured species. In Paper **II**, this method was used to assess abundance of *H. pseudoalbidus* in declining *F. excelsior*. On used agar, *H. pseudoalbidus* was a slow growing fungal species, easily overgrown by other faster growing fungi and hence its abundance can be often misrepresented while using mycelial isolation method alone. Methods for direct DNA sequencing from plant tissue followed the protocols previously described by Lindahl *et al*. (2007). DNA extraction, amplification by PCR and sequencing followed the same methods as described above. Then, PCR products were size-separated on 1% agarose gels, visualized under UV and cloned using TOPO TA Cloning Kit with pCR®2.1-TOPO vector and One Shot TOP10 chemically competent *E. coli* (Invitrogen, Carlsbad, CA, USA). The small amounts of bacteria were used directly for PCR, as outlined above, but with primers M13 Forward (GTAAAACGACGGCCAG) and M13 Reverse (CAGGAAACAGCTATGAC).

The cloning approach was combined with T-RFLP (terminal restriction fragment length polymorphism) analysis in order to enable processing of a large set of samples (Dickie *et al*., 2002). In addition, all clones that were sequenced were also subjected to T-RFLP analysis, to enable matching of T-RFLP patterns with sequences. PCR was carried out as above, but the primers were labeled with WellRED fluorescent dyes, ITS1-F with D3-PA and ITS4 with D4-PA. The PCR products were digested with restriction endonucleases. Sample T-RFLP patterns were compared with the reference database constituted by T-RFLP patterns from the clones using the program TRAMP (Dickie *et al*., 2002).

3.6 Testing for the presence of oomycetes

All DNA extractions from the plant tissue (**II**) were also tested for the presence of oomycetes. PCR was carried out using primer P1 (GAAGGATCATTACCACAC) (Geraats, 2003) and universal primer ITS4. Thermal cycling conditions were as described above. In all PCR runs, a sample of *Phytophthora infestans* was included as a positive control. PCR products were sized separated on 1% agarose gels, stained and visualized under UV.

3.7 Pathogenicity tests

We chose 24 fungal taxa from Paper **I**, and 26 fungal taxa described in Paper **III** for pathogenicity tests with one year old *F. excelsior* saplings in a bare-root nursery. The fungi were chosen as they were most consistently isolated from crowns and root systems of declining *F. excelsior.* In addition, one isolate of *Armillaria borealis* Marxm. & Korhonen was also included into the test for Paper **III**; the culture was sourced from the culture collection of the Department of Forest Mycology and Plant Pathology, SLU. The pathogenicity tests in bare-root nursery were carried out against total amount of 580 (including 17 controls) one-year-old *F. excelsior* saplings for two (**I**) and three (**III**) years. In paper **I**, one strain per fungal taxa was tested against *F. excelsior*; each strain was inoculated on 6-7 saplings. In paper **II**, number of strains per fungal taxa ranged between 1 and 8 (6-7 inoculations per strain).

In addition, we added a pathogenicity test for *H. pseudoalbidus* specifically during the study of Paper **II**. For this, eight strains of *H. pseudoalbidus* from our isolations were tested against 96 (including 10 controls) one-year-old *F. excelsior* saplings in the greenhouse for 6 weeks.

For all pathogenicity tests, we used the following protocol: autoclaved wood pieces of *P. tremula* of approximately 1.0x0.2x0.2 cm size were pre-colonized with the respective fungal strain and used as the inoculum. Saplings were cut with sterilised scalpels with the cut being the same size as the inoculum wood pieces. The pre-colonised wood pieces were put onto the wounds with sterile forceps and then sealed with Parafilm™ sealing tape. Scalpel and forceps were sterilized using 70% ethanol and open flame before in between every inoculation. Control saplings received sterile pieces of wood in the same size as the pre-inoculated pieces, using the same inoculation procedure. All saplings were grown in the favourable conditions on rich soil and were periodically watered. At the end of experiment, all *F. excelsior* saplings were cut down at the root collar, transported to the laboratory, washed under the tap water and debarked using sterile scalpels. Any visible necrosis in the cambium was recorded and measured.

4 Results and Discussion

4.1 Fungi associated with declining *F. excelsior*

In total of 109 fungal taxa were found from samples taken from root systems and crowns of declining *F. excelsior*. 50 of them were identified to species, 43 to genus level. A proportion of the fungal taxa (16) remained unidentified.

The most frequently on agar isolated ascomycetes from crowns of declining *F. excelsior* (**I)** were: *Alternaria alternata* (Fr.) Keissl, *Aureobasidium pullulans* (de Bary) G.Arnaud, *Botryosphaeria stevensii* Shoemaker, *Epicoccum nigrum* Link, *Gibberella avenacea* R.J. Cook, *Lewia* sp., *Phomopsis* sp. and *Valsa* sp., totalling a 79.4% of all obtained fungal isolations. Basidomycetes, such as *Phanerochaete sordida* (P. Karst.) J.Erikss. & Ryvarden, *Bjerkandera adusta* (Willd.) P. Karst. or *Coprinus* spp., were seldomly isolated (0.2-0.9%). Through direct sequencing of plant material (**II**) several species very rarely or never found with isolations, were able to be identified, thus providing additional information on the fungal species that are associated with the crowns of declining *F. excelsior*. Molecular methods revealed high frequency of basidiomycetous yeast *Cryptococcus foliicola* F.Y. Bai & Q.M. Wang (recorded in 70% of samples) and pathogenic *H. pseudoalbidus* (61%), followed by *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, (1952), *Phoma exigua* Desm. and *Phoma glomerata* (Corda) Wollenw. & Hochapfel (47-52 %).

Isolations from Paper **III** showed that root systems of declining *F. excelsior* were mainly colonized by *Armillaria cepistipes* (in 24.5% of fungal growth yielded samples) Velen. followed by ascomycetous *Cylindrocarpon* spp., *Pseudeurotium bakeri* C. Booth, *Nectria radicicola* C. Booth and *Scytalidium lignicola* Pesante.(4.1-8.2%) In addition, four other species of wood-decay basidiomycetes were isolated, representing genera *Coprinus*, *Phanerochaete*, and *Pholiota* (2.0-8.2%).

With the exception of *H. pseudoalbidus*, many of the fungi most frequently isolated from the shoots of declining *F. excelsior* have also been isolated from bark, branches and root systems of declining (Przybył 2002b, c; Kowalski & Lukomska, 2005; Lygis *et al*., 2005; Pukacki & Przybył, 2005) as well as in healthy looking *F. excelsior* (Butin & Kowalski, 1986; Griffith & Boddy, 1988, 1990; Kowalski & Kehr, 1992). This suggests that the fungal communities in declining *F. excelsior* are mainly a naturally found community.

4.2 Pathogenicity of fungal taxa detected in declining *F. excelsior*

In the pathogenicity tests the following fungal taxa induced symptomatic necroses in the stems of *F. excelsior* saplings: *A. alternata*, *A. cepistipes*, *E. nigrum*, *H. pseudoalbidus*, *Mycena* sp. 915, *Mycoacia uda* (Fr.) Donk. *Phlebia rufa* (Pers.) M.P. Christ., *Pholiota carbonaria* (Fr.) Singer, *Phomopsis* sp. 57 (**I, II** & **III**). Among these species, only *H. pseudoalbidus* caused necroses on a large proportion of tested saplings (50% in bare-root nursery and 100% in greenhouse). In greenhouse experiment, necroses induced to *F. excelsior* by *H. pseudoalbidus* were highly variable in length (1.1-28.7 cm). The remaining fungal species that were used in the screening, and other potentially pathogenic fungal species, such as *G. avenacea*, *B. stevensii*, *Phoma* spp. or *Botryotinia fuckeliana* (de Bary) Whetzel, are likely to be naturally occurring secondary opportunistic agents. Their pathogenicity is generally limited but could be triggered by environmental factors or prior infestation of *H. pseudoalbidus*. Competitive interaction involving fungi, like *Fusarium* spp., are also known residents in the wounds (Carter, 1971; Carter & Price, 1974; Corke & Hunter, 1979), possibly limiting non-specific-to-host decay fungi from colonizing wood of *F. excelsior*. Such interactions, however, were not investigated in our studies.

4.3 *H. pseudoalbidus* as the causal agent of *F. excelsior* decline

The study **II** found that *H. pseudoalbidus* can frequently be isolated from various parts of declining *F. excelsior* trees: necrotic leaves, healthy-looking and necrotic leaf stalks, necrotic shoot bark, and wounds on shoots and stems. While *H. pseudoalbidus* was frequently and consistently isolated in all of the symptom categories, sampling approaches demonstrated a broad capacity of this fungus to colonize a wide spectrum of physically and physiologically different niches. Although this fungus was prevalent in symptomatic samples

of declining *F. excelsior*, symptomless samples also were often infected. *H. pseudoalbidus* was isolated from 93% of stem cankers, 91% of necrotic leaf stalks, 27-28% of bark wounds and 30% of visually healthy leaf stalks. Molecular techniques appeared to be more powerful in confirming the presence of *H. pseudoalbidus* in declining *F. excelsior* than pure-culture isolations and showed that the highest abundance of this pathogen was in leaf stalks (97%), followed by shoot bark (41%) and leaves (39%). A pronounced abundance of this pathogen in leaves and leafstalks is in accordance with the hypothesis that ascospores are the primary source of infection and this presumably takes place mainly through leaf petioles (Kirisits & Cech, 2009; Kirisits *et al*., 2009; Kraj *et al*., 2012). While samples used for pure culture isolations were collected either from healthy-looking tissue or at the zone of advancing necroses, high frequency of *H. pseudoalbidus* shows that fungus actively spreads through the tissue of *F. excelsior* and even dominates over other microbial species at the early stages of symptom development. The high abundance of this fungus in declining *F. excelsior* found in this study is in good accordance with results from pathogenicity tests (**II**), where each of the eight tested strains of *H. pseudoalbidus* induced cambium necrosis in all inoculated *F. excelsior* saplings. Frequent isolations of *H. pseudoalbidus* and its consistent detection by molecular methods in the sample groups of various dieback symptoms, together with the results of pathogenicity tests clearly demonstrate the involvement of this fungus in the ash dieback. Furthermore, the present study demonstrates that different isolates of *H. pseudoalbidus* might differ in aggressiveness (attributed to the length of induced necroses). In order to investigate this, pathogenicity tests on clonal material of *F. excelsior* were needed; this was not conducted in this work.

Pathogenicity tests also showed that susceptibility to *H. pseudoalbidus* differed between inoculated *F. excelsior* trees. A natural genetic variation in susceptibility to ash dieback has been confirmed in recent studies and observations (Olrik *et al*., 2007; McKinney *et al*., 2011; Stener, 2012). Thus, the practical selection for trees with higher resistance to the pathogen is a possibility for afforestation and conservation programs in the future.

4.4 *Armillaria* spp. as an opportunistic damaging agent of *F. excelsior*

Associations between *Armillaria* spp. and declining broadleaves in the Baltic Sea region were reported previously. Lygis *et al.* (2005) showed that in Lithuania root rot in declining *F. excelsior* stands was prevailingly caused by *A. cepistipes*. *A. cepistipes* was also found to be associated with root systems of

declining *Quercus robur* L. stands in Norway (Keča & Solheim, 2010). In Denmark, however, *Armillaria gallica* Marxm. & Romagn. - a very close relative, was the only *Armillaria* species reported from root systems of declining *F. excelsior* (Skovsgaard *et al*., 2010).

Armillaria rot was previously shown to reduce density and height of vegetative regeneration from the stumps (Wolken *et al*., 2009). The study, described in Paper **III**, has also demonstrated that the extent of root and butt rot in declining *F. excelsior* positively correlates with the severity of crown dieback. On the other hand, our results indicate that root rot, although seemingly enhancing *Fraxinus* decline, is a secondary factor in the pathological process. In particular, this conclusion is supported by the observed presence of visually healthy roots in trees with already dead crowns. In addition, assessment of *F. excelsior* coppicing in the clear-cuts explicitly demonstrated an airborne origin of ash decline infection also on the stumps which were heavily decayed by *Armillaria* spp., because decay was absent from the cut bases of the sprouts. Nevertheless, decay in the stumps and root systems of felled *F. excelsior* had clear negative effect on sprouting: of 77 non-decayed stumps, sprouting was recorded on 45 (58.4%), while among 251 decayed stumps, sprouting was recorded on 82 (32.7%) The results of Paper **III** are therefore consistent with two related studies, which also concluded that *Armillaria* spp. have only secondary role in decline of *F. excelsior*. These observations were based on other parameters such as size and age of *A. cepistipes* individuals in declining *F. excelsior* stands (Lygis *et al*., 2005), or associations between symptoms of *F. excelsior* crown dieback and symptoms caused by other pathogens (Skovsgaard *et al*., 2010). Besides, stress to the trees induced by defoliation, can trigger accumulation of sugar reserves in root systems (Parker & Houston, 1971; Wargo *et al*., 1972) and may therefore reduce the activity of fungal-lysing bark enzymes, thereby creating more relevant conditions for infections by *Armillaria* spp. (Wargo, 1976).

Although *A. cepistipes* is generally known to have pronounced saprophytic capabilities (Wahlström, 1992), it is an opportunistic pathogen able to attack stressed trees (Entry *et al.*, 1986). In our study (**III**), the stress was likely associated with crown decline caused by *H. pseudoalbidus* - a pathogenic fungus known to have a decisive role in *F. excelsior* decline (Kowalski & Holdenrieder, 2008, 2009; Paper **II**). Similarly to *A. cepistipes*, *A. gallica* was also known to be unable to attack vigorous trees. However, it was actively involved in tree decline following their defoliation (Keča & Karadžič, 2006; Marcais & Breda, 2006). Conducted inoculation tests (**III**) confirmed the opportunistic pathogenic behaviour of *Armillaria* spp. in the process of *F. excelsior* decline. As a result, *A. cepistipes* was able to produce only very

confined discolorations in sapwood of vigorously growing *F. excelsior* and this was on three out of eleven inoculations (27.3%).

In our study, only a single genotype of *A. cepistipes* was detected in the infected roots of a particular tree. This is consistent with the results by Lygis *et al*. (2005), where in declining ash stands single genotypes of this fungus commonly encompassed several trees of *F. excelsior.*

4.5 Decline of *F. excelsior* in relation to stand density, flushing phenology and time of the season

The study described in Paper **IV** investigated the extent and temporal pattern of crown damage, attributed to *H. pseudoalbidus*, in stands of *F. excelsior* in relation to bud flushing phenotype, stand density and time of the season. The investigation revealed no clear evidence that stand density influenced the development of dieback in young, even-aged stands of *F. excelsior*. Disease severity (average crown damage) averaged between 23.1-30.1% in June and 39.4-53.4% in September, and was most pronounced in unthinned *F. excelsior* stands (1700-5500 trees/ha), but otherwise was found unrelated to the density of remaining stands (100-1500 trees/ha). It is known that factors, such as increased shading, moisture and proximity of plant tissues, and limited nutrient availability particularly in dense, single-species stands may induce susceptibility in plants to fungal infections (Niemelä, 1992; Garcia-Guzman & Dirzo, 2001). Since *F. excelsior* in general is a light demanding tree species, it must be thinned at regular basis in order to maintain high vigour of the trees (Dobrowolska *et al*., 2011). Subsequently, dense stands were predisposed to *H. pseudoalbidus* infections due to tree to tree competition and loss of their vigour, resulting in more severe dieback of these stands as compared to thinned areas. Furthermore, the seasonal pattern of disease severity was similar in all examined stands disregarding stand density, and the extent of tree crown damage increased significantly towards the end of the growth season.

The development of *F. excelsior* dieback, as in many other plant diseases, probably depends on the host's seasonal morphogenesis, including the time of bud flushing and leaf emergence. As *H. pseudoalbidus* sporulates in June (Kirisits & Cech, 2009), it can be hypothesized that *F. excelsior* trees of late-flushing phenotypes are more susceptible to infection by *H. pseudoalbidus*. Such leaf age-related susceptibility to biotic stress, when susceptibility of plant leaves decreases with increasing leaf age, was previously described (Koch & Mew, 1991; Barth *et al*., 2004).

Our results presented in Paper **IV** demonstrated that bud flushing phenology exhibited a strong correlation with dieback susceptibility, whereas the

proportion of crown damage was much higher on dormant (55.3% in June, 70.2% in September) rather than on flushing (26.9% and 48.5%, respectively) trees. This repeatedly indicates that late-flushing phenotypes of *F. excelsior* are more susceptible to *H. pseudoalbidus* infection than early flushing ones. It is quite unlikely that the severity of the disease can significantly influence the timing of bud flushing, because in our study a substantial number of heavily damaged trees were flushing in May. This is consistent with similar study where strong correlation between flushing phenology and the health of *F. excelsior* was also reported (Pliūra & Baliuckas, 2007). Additionally, in a study on the relationship between genetic variation in *F. excelsior* and its susceptibility to *H. pseudoalbidus*, trees with late flushing and late leaf shedding were also more susceptible to the disease (McKinney *et al*., 2011).

4.6 Succession and species shift in dieback affected and clear-felled stands of *F. excelsior*

In Paper **V,** all examined clear-cuts exhibited favourable conditions for natural regeneration of *F. excelsior* stands and this could be because of the following reasons: (1) *F. excelsior* constituted a large proportion (40-100%) in stand species composition prior to dieback (2) site conditions in the clear-cuts favoured rapid establishment and growth of *F. excelsior* seedlings; and (3) sprouting of *F. excelsior* stumps enhanced regeneration. However, after the felling clear-cuts were predominately invaded by early successional tree species, well adapted for colonizing openings, particularly *Betula* spp. (on average 4582±1166SE seedlings/ha; or 32.9% of all regeneration), *A. incana* (4518±1142SE; 32.4%) and *P. tremula* (1559±653SE; 11.2%). These species, however, either are of relatively low economic value, or were not dominating stand component in pre-disturbed forest ecosystems and therefore are not desirable in regeneration of former *F. excelsior* stands.

The incidence of *F. excelsior* regeneration in the inspected clear-cuts was low and ranged between 0 and 1933 trees/ha (on average 599±124SE trees/ha; 4.3% of all regeneration), and was slightly higher on the oldest sites (on average 1106±828SE trees/ha, 8.2%). In contrast, depending on the site conditions, the understory layer of *F. excelsior* saplings in stands before disease outbreak often comprised 30000-105000 ash trees/ha (Narbutas, 1975).

Among observed regenerating *F. excelsior*, 53.9% were diseased (showing dieback symptoms), 16.8% - dead and 29.3% were asymptomatic. Such poor health condition of regenerating ash trees and their slower growth than of *A. incana, Betula* spp. and *P. tremula* indicates that situation with *F. excelsior* stands is likely to become even worse in the nearest future.

In the past, stands of *F. excelsior* generated large quantities of seedlings (Narbutas, 1975) and this diminished the importance of stump sprouts for its natural regeneration. Presently the situation has changed and prolific stump sprouting appears to be an important source of natural regeneration in the stands affected by *F. excelsior* dieback. *H. pseudoalbidus* infections, however, had clear negative impact to the health conditions of the *F. excelsior* sprouts (Figure 5) - there were 80.4% of sprouts dieback-affected as compared to 56.4% of diseased *F. excelsior* seedlings, emerging from the soil. In clear-cuts of different age there were significant differences in abundances of *F. excelsior* emerging from soil and those sprouting from stumps: in 1-2 year-old clear-cuts, trees emerging from the stump sprouts comprised 36.2% of the whole ash regeneration, in 3-4 year-old - 21.7%, in 6-7 year-old - 4.4%, and in 8-10 year-old - 0%. This indicates that the sprouts cannot survive the constant infections of *H. pseudoalbidus* and that root systems are losing their vigour. In addition, the presence of decay in the stumps of felled *F. excelsior* had a pronounced negative impact on coppicing as only 12.3% of decayed stumps were sprouting, while percentage of sprouted non-decayed stumps was 38.4%.

Figure 5. Dieback-affected *F. excelsior* sprout.

Our results therefore suggest that abundant natural regeneration of *F. excelsior* cannot be expected in the areas with long disease history. If regeneration is

delayed for too long, dieback affected stands and clear-cuts may not develop favourable conditions necessary for successful regeneration of *F. excelsior*. Diseased root systems may not coppice vigorously and undesirable tree species will outcompete remaining ash for available resources, especially light. Under high disease pressure, available silvicultural measures (clearings, thinnings or additional planting) are not economically realistic to avert ash decline. With decreasing numbers of the seedlings and constant attacks by *H. pseudoalbidus*, it is likely that *F. excelsior* will gradually disappear from its otherwise natural habitats. In Lithuania (Lithuanian Forest State Service, pers. communication) and Denmark (Kjaer *et al*., 2011) ash dieback problem is so severe that *F. excelsior* is no longer used for planting. Consequently, early-successional tree species will likely lead to a shift in species composition of regenerating former *F. excelsior* stands, or stand-forming species of a higher timber value (*A. glutinosa*, *Q. robur*, *U. glabra*, and *P. abies* in particular) will be promoted by respective silvicultural measures.

5 Concluding remarks

The combination of different sampling strategies and detection methods revealed rich fungal communities inhabiting symptomatic and non-symptomatic shoots, leaves and roots of declining *F. excelsior* (**I**, **II** & **III**). The most consistently isolated fungi were isolated from symptomatic categories of samples. Investigated fungal communities were in accordance with similar studies on *F. excelsior*, therefore suggesting presence of mainly natural wood-inhabiting fungi in declining *F. excelsior*. Pathogenicity tests with isolated fungi demonstrated occasional and opportunistic pathogenicity of most species, but only *H. pseudoalbidus* consistently yielded dieback symptoms.

Frequent isolation and consistent detection by molecular methods demonstrated frequent abundance of *H. pseudoalbidus* in symptomatic as well as in sound-looking parts of the crowns of declining *F. excelsior* (**II**). Together with results of pathogenicity tests, our study confirmed ultimate involvement of this ascomycete in dieback of *F. excelsior*.

Susceptibility of *F. excelsior* saplings to *H. pseudoalbidus* differed sharply in conducted pathogenicity tests (**II**). Moreover, higher susceptibility of late-flushing phenotypes of *F. excelsior* to ash dieback was revealed in Paper **IV**. These data suggest the presence of certain resistance to disease of particular *F. excelsior* individuals, determined either by genetically controlled host resistance or ecology of the pathogen. Such resistance, screened by selection, might be rewarding in the future for *F. excelsior* conservation and/or afforestation.

The studies on natural *F. excelsior* regeneration and succession of tree species in dieback-devastated areas showed low density and poor health conditions of regenerating ash due to constant infections of *H. pseudoalbidus* (**V**). Once dominated by *F. excelsior*, investigated forest ecosystems are

shifting towards fast-growing tree species, such as *A. incana*, *Betula* spp. and *P. tremula*.

6 Future prospects

Numbers of unidentified fungal taxa in crowns and root systems of declining *F. excelsior* demonstrated that additional effort is required to reveal more detailed information about fungal communities in declining *F. excelsior*. To determine the interactions between *H. pseudoalbidus* and other wood-inhabiting fungi the comparative studies in both areas, i.e. devastated and still unaffected by ash decline, could be of great interest.

The observed patterns of disease severity (**IV**) are probably associated with ecological features, life cycle and infection biology of the pathogen that still remain largely unknown. Elucidating the biology of *H. pseudoalbidus* should be prioritized in the near future.

Inoculation experiments revealed that *F. excelsior* significantly differs in susceptibility to *H. pseudoalbidus* infections. Presently, selection by screening of *F. excelsior* individuals, resistant to dieback perhaps is the only measure reasonable for conservation of *F. excelsior*, at both species and stand levels. Due to intention of many countries to propagate local seed and vegetative material for afforestation, screening of local disease resistant *F. excelsior* is of great importance.

The origin of *H. pseudoalbidus* is crucial for understanding the process and prediction of the future of ash decline. Spatial distribution pattern and high pathogenicity of *H. pseudoalbidus* is characteristic for an alien invasive species. On the other hand, unusual for invasive species high intra-population genetic diversity and hypothesis about possible mutation of native *H. pseudoalbidus* to highly pathogenic strains of this fungus (Queloz *et al*., 2010) suggest the possibility of the autochtonous origin of this fungus. Thus, additional investigation towards the origin of *H. pseudoalbidus* could be a logical step.

According to present knowledge, ash decline has started in Central Europe and then spread in all directions, particularly to Western Europe. Therefore, the

investigation of *F. excelsior* populations in the most eastern ash distribution area would be of high interest and priority.

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