



Load of the ash dieback pathogen *hymenoscyphus fraxineus* differs in soil

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Abstract

The ascomycete *Hymenoscyphus fraxineus* causes the devastating ash dieback disease of European ash (*Fraxinus excelsior* L.). Spore traps are often used to measure the amount of ascospores in the environment, but the pathogen-load of the soil in ash stands has not been recorded so far. This is of particular interest with regard to the occurrence of ash stem necrosis, a decisive factor for the severe course of the disease. In order to gain a more differentiated insight into the pathogen-load in ash stands, we analysed soil samples from four ash tree sites in southern Germany, covering a clone plantation, two seed orchards and a forest. The pathogen-load was determined using a quantitative TaqMan real-time PCR assay for ten to twenty plots per stand. Results obtained by the species-specific assay highlighted that the pathogen-load is heterogeneously distributed in the ash stands. *H. fraxineus* DNA targets were detected in 17% of the soil samples. The pathogen-load differed according to soil depth, with the highest pathogen abundance in the top 5 cm, followed by 5–10 cm and finally 10–15 cm. Pathogen-load and thereby infection pressure were found to be highly variable for the individual trees in one stand. Overall, the study discovered detectable levels of *H. fraxineus* in the soil of all four study sites, which supports the hypothesis that *H. fraxineus* can be found in the soil of ash stands. The qPCR approach was found to be an effective method for monitoring the load of *H. fraxineus* in soil and for demonstrating the successful application of the method on the sample type of custom-made spore traps. Results suggest the implication of site-specific pathogen-load determination in future *H. fraxineus*-monitoring and selection of less susceptible ash trees for breeding and seed production.

Keywords Ash dieback · Pathogen-load · Spore traps · Real-time PCR

Introduction

Since the early 2000s, the invasive fungal pathogen *H. fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (anamorph: *Chalara fraxinea* T. Kowalski) and the associated disease, ash dieback, have been observed in Germany (Schumacher et al. 2007). The appearance of the pathogen resulted in severe dieback symptoms and declining ash (*Fraxinus* spp.) populations (Enderle et al. 2017). The outbreak of this epidemic in Europe was first reported in Poland during the early 1990s (Kowalski 2006), followed by a rapid spread, and

subsequently reports all over central Europe (Timmermann et al. 2011). The origin of the pathogen is in east Asia where it is associated with *Fraxinus mandshurica* Rupr. (Zhao et al. 2013). Due to the lower genetic variation in the European *H. fraxineus* compared to Asian populations, it is suggested that the introduction of the pathogen is limited to a few incidents (Drenkhan et al. 2014; Gross et al. 2012a; McMullan et al. 2018)

Common ash (*F. excelsior*) and narrow-leaved ash (*F. angustifolia*) are the species most affected by *H. fraxineus* infection (Gross and Holdenrieder 2013). Besides the restriction of susceptibility of different *Fraxinus* spp., the ash dieback disease (ADB) affects ash trees of all ages and stand types including mixed-, monodominant- and urban forests. Nevertheless, there is a positive relationship between ash density and disease severity, due to inoculum availability influenced by mating opportunity (Laubray et al. 2023).

The life cycle of both, the naturalised *H. fraxineus* and its suppressed sister species, *Hymenoscyphus albidus*, relies on

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saprotrophic growth on ash leaf petioles (Gross et al. 2012b; Kowalski and Holdenrieder 2009). Sharing this similar ecological niche showed that competition between these two closely related ascomycetes can lead to the extinction of *H. albidus* (McKinney et al. 2012). The rapid spread of *H. fraxineus* within Europe can be credited to airborne ascospores, which serve as the primary mode of pathogen dissemination (Grosdidier et al. 2018; Gross et al. 2012b). A typical release period for central Europe extends from April to October culminating in August (Dvořák et al. 2016). After spore release, infections are highly weather dependent with high humidity favouring infections (Haňáčková et al. 2017; Mansfield et al. 2018, 2019). *H. fraxineus* has been detected in diverse parts of ash trees, including leaves (Gross et al. 2012b), shoots (Haňáčková et al. 2017), stems (Husson et al. 2012), collars (Kräutler et al. 2015) and roots (Baxter et al. 2023; Schumacher et al. 2010). Infection symptoms include wilting of leaves and the formation of lesions, which tend to enter a state of permanent rest, particularly during the winter months (Bengtsson et al. 2014; Kräutler et al. 2015). Besides typical crown dieback symptoms, stem collar necrosis and rot are detrimental to the ash's health and safety within the forest system (Enderle et al. 2017). Nevertheless, stem collar necrosis is rather associated with fungal communities than singular infection of *H. fraxineus* (Peters et al. 2023).

H. fraxineus and its cryptic sister species *H. albidus* are hardly distinguishable by morphological features; therefore, molecular detection based on species-specific markers is mandatory (Queloz et al. 2011). Diagnostic of *H. fraxineus* is based on isolation with subsequent in vitro cultivation (Peters et al. 2023), molecular diagnostic via PCR (Johansson et al. 2010), real-time PCR (Chandelier et al. 2010; Ios et al. 2009) and high-throughput sequencing approaches (Chandelier et al. 2021). Various molecular analyses and sample types can be used to observe pathogen distribution and spread patterns, as well as pathogen-load (Chandelier et al. 2014; Dvořák et al. 2016). Different sampling methods like active or passive spore traps (Chandelier et al. 2014; Dvořák et al. 2016; Grosdidier et al. 2018; Laubray et al. 2023) with subsequent molecular detection have been used to elucidate the spatial and temporal pathogen distribution at different levels. However, spore traps can be bulky, cost-intensive, high in maintenance and are therefore often used scarcely or self-build solutions are made (Chandelier et al. 2021). ADB disease severity is highly influenced by host density, neighbouring trees, climate and topography (Cracknell et al. 2023; Dvořák et al. 2016; Enderle et al. 2013; Marçais et al. 2016), and forests are subject to a diversity of vertical and horizontal microclimates (DeFrenne et al. 2021). Symptom formation of ADB is observed at different grades of severity in crowns within one stand (Enderle et al. 2018), thus influencing microclimates mostly through crown architecture and ash

density (Grosdidier et al. 2020). Since climate is a highly influential variable in disease formation and ascospore release (Cracknell et al. 2023; Dvořák et al. 2016), the spatial differences observed in microclimates and topography could influence the distribution of *H. fraxineus* pathogen-load. Geographical patterns of pathogen abundance can be obtained by multiple spore traps per plot (Gadoury and MacHardy 1986; Laubray et al. 2023) or the use of natural spore deposits such as soil (Chamberlain and Chadwick 1972). Since hyphae, ascospores and conidia of *H. fraxineus* can withstand harsh conditions for extended periods (Gross and Holdenrieder 2013), spatial detection and quantification of pathogen within soil can be achieved. The pathogenic-load of *H. fraxineus* in the environment has been subject of multiple studies investigating ascospore dispersal patterns, its occurrence and correlation with weather-related variables (Aguayo et al. 2018; Chandelier et al. 2010; Dvořák et al. 2023), while of interest for pathogen deposition patterns and the occurrence of ash stem necrosis, the pathogen-load in soil has not yet been examined. This is particularly interesting due to the observed spatial variability in stem necrosis incidence (Enderle et al. 2013) and its exacerbating effect on the decline of ash tree health (Chandelier et al. 2016). Thus, we hypothesize that *H. fraxineus* is present in the soil, and that its abundance exhibits spatial variability, similar to the variability observed for stem necrosis. The objective is to evaluate and provide a workflow from DNA extraction to detection via qPCR for *H. fraxineus* in different soil types and ash stands. Additionally, the workflow was tested on two custom-made spore trap samples to demonstrate its versatility on different sample material.

Hence, the aims of this study were (I) to establish a protocol for the detection, and absolute quantification of *H. fraxineus* in the topsoil and custom-made spore traps, (II) to get insights into the spatial distribution of *H. fraxineus* across soil layers and (III) to describe the pathogen-load on soil level across various ash stands.

Materials & methods

Study sites

A total of four sampling sites in southern Germany were investigated, to cover three different site types and soil types (Table 1). The plantations in Schorndorf, Emmendingen and Grabenstätt consist mainly of ash (*F. excelsior*) in monoculture surrounded by mixed forest and trees planted with a distance of 7 m, 10 m and 1.5 m, respectively. By contrast, the plot located near Kaisheim is included in the extensive monitoring plots and comprises of a mixed forest containing 55% ash (*F. excelsior*), 26% hornbeam (*Carpinus betulus*) and several species of oak trees (*Quercus* spp.) (Langer et al. 2022).

Table 1 Study site names with respective locations in decimal degrees, federal state, site type, tree spacing and soil type. Tree spacing and year of planting do not apply to the mixed forest stand due to the natural regeneration

Study site	Location	State	Site type	Tree spacing	Year of plantation	Soil type
Schorndorf	48.777189101057644, 9.42642187849277	Baden-Württemberg	Seed orchard	7 m	1992	Medium silty sand
Emmendingen	48.110646730650274, 7.872328899612421	Baden-Württemberg	Seed orchard	10 m	1995	Sandy silt
Grabenstätt	47.84140371837484, 12.512144652560108	Bavaria	Clone plantation	1.5 m	2014	Highly silty sand
Kaisheim	48.807060477719205, 10.794439704946669	Bavaria	Mixed forest	N.A	N.A	Highly silty sand

Soil samples

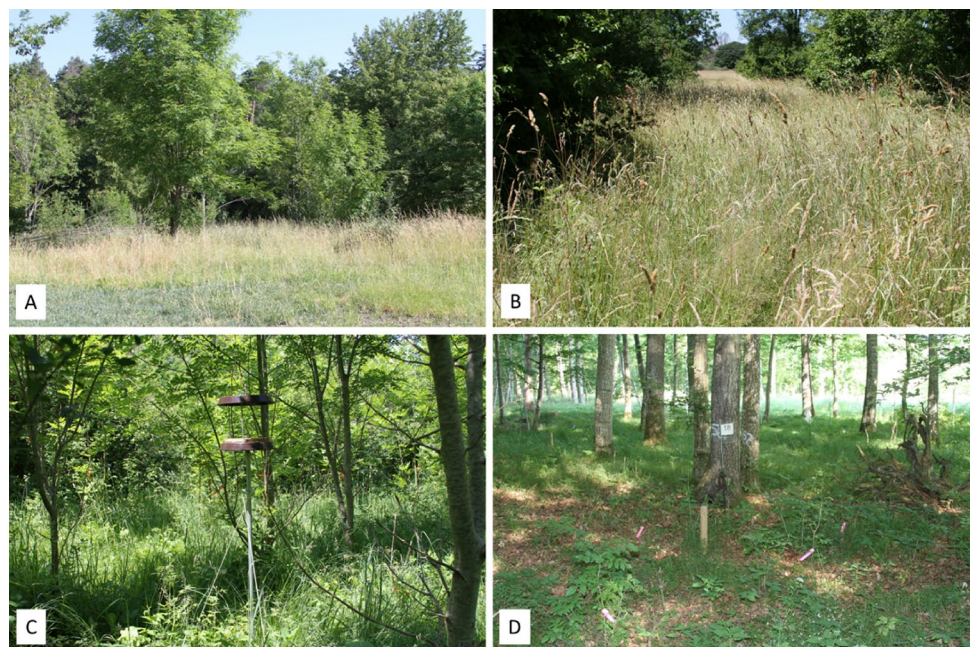
The soil type was determined as a pool sample for each sample site, according to Köhn (1928). Soil samples for molecular detection were collected at study sites in southern Germany where ash trees (*F. excelsior*) exhibited varying degrees of symptoms of ash dieback, as determined by visual observation, according to Peters et al. (2021). Sampling at each site was restricted to plots, with 100 m² in size and 20 plots per study site for the seed plantations and the mixed forest, and 10 plots for the smaller clone plantation. Soil samples were collected randomly within each plot using an Egner's cane soil sampler. In July 2023, 20 soil samples were collected from plot 3 at the Schorndorf site at three depths: 0–5 cm, 5–10 cm and 10–15 cm. Each sample was analysed individually as part of the soil layer study. A pathogen-load study was conducted at all

four sites, with a sample pool of four subsamples per plot taken at a depth of 0–5 cm in order to assess the general pathogen-load in June 2022. The soil was therefore sampled on two occasions: once to study the soil layer at the Schorndorf site (2023) and once to study the pathogen-load at all sites (2022).

Spore trap samples

Passive spore traps were prepared and implemented as previously described (Aguayo et al. 2018; Eisen et al. 2022) with paper filters (Whatman® No1, 15 cm in diameter) and Vaseline® and incubated for 7 days at a height of 160 cm (Fig. 1C). Two spore traps were incubated for a duration of seven days at the Kaisheim site (Plot 1, Fig. 3D), with incubation starting on 22 June 2022 and 29 June 2022.

Fig. 1 Study sites near Schorndorf **A**, Emmendingen **B**, Grabenstätt **C** and Kaisheim **D**. Flora of four different sites with high grasses and no natural ash rejuvenation **A**, **B**, **C** and low to no grass growth with natural ash rejuvenation **D**. Passive spore trap in Grabenstätt **C** with paper filter. All pictures were taken in June 2023



DNA extraction

DNA extraction was performed with the FastDNA™ SPIN Kit for Soil (MP Biomedicals Germany GmbH, Eschwege, Germany) using the manufacturer's latest protocol (May 2021) and 500 mg of soil as input amount. Extractions for amplification controls consisted of 500 µl of *Hymenoscyphus fraxineus* liquid culture for positive control; 500 mg of soil sample from Serbia, Belgrade (coordinates: 44.86936020312691, 20.24776516559037), with no adjacent ash trees in surrounding area was used as control for DNA quality positive for the ammonium oxidizing bacteria (AOB) template but negative for *H. fraxineus* template. Spore traps were prepared with a one-hole puncher (6 mm diameter), and fourteen obtained paper discs were used for DNA extraction with the previously described FastDNA™ SPIN Kit for Soil. The concentration of DNA in soil samples was determined using the Qubit™ fluorometric device (Invitrogen, Waltham, USA), employing the broad range dsDNA quantification assay kit with 2 µl of input DNA from every sample, followed by dilution to 10 ng/µl.

Absolute quantification and detection limit

A duplex real-time PCR assay was employed to identify the pathogen *H. fraxineus* (Chandelier et al. 2011), with the additional objective of detecting AOB (Hermansson and Lindgren 2001; Kowalchuk et al. 1997) as a proxy for DNA quality, particularly in the context of inhibitors. Positive detection of the AOB marker ensures sufficient quantity and quality of DNA within tested samples. Experiments were performed on a StepOnePlus™ real-time PCR Cycler (Applied Biosystems, Foster City, USA) using

primer and probe combinations Cf-F/R/S (final concentration 0.4 µM) and CTO189fC/RT1r/TMP1 (final concentration of 0.33 µM for primers and 0.26 µM for probe) (Table 2), 12.5 µl 2×Luna Universal qPCR Master Mix, 40 ng of total DNA, 0.15 units of thermolabile uracil-N-glycosylase (UNG) (Roboklon GmbH, Berlin, Germany) and nuclease-free water to a final volume of 15 µl per reaction. Assay parameters started with UNG digestion at 37 °C for 2 min, initial denaturation and UNG deactivation at 95 °C for 10 min, followed by 40 cycles of a two-step protocol of 95 °C for 15 s and 60 °C for 1 min, with fluorescence data acquisition after each cycle.

Absolute quantification was performed using synthetic dsDNA (gBlocks™, Integrated DNA Technologies Germany GmbH, Munich, Germany) designed to represent the partial integrated transcribed spacer (ITS) sequence (Table 3) (Chandelier et al. 2010; Ios et al. 2009) of *H. fraxineus*. The copy number of the standard was calculated using the manufacturer's protocol (<https://eu.idtdna.com/pages/education/decoded/article/tips-for-working-with-gblocks-gene-fragments>, accessed March 2023) to obtain a dilution series of 7 concentrations with increasing copy numbers from 1×10^0 copy to 1×10^6 copies (Table 3). Samples were considered positive if all three technical replicates displayed a positive signal for *H. fraxineus* and the internal control concurrently. The concentrations of *H. fraxineus* detected in the samples were expressed as the number of copies of the target sequence in 40 ng of template DNA.

The limit of detection was assessed using all seven concentrations and absolute quantification was performed omitting the lowest concentration of 1×10^0 . Values of quantification, standard curve, and quantitative cycle

Table 2 Primers and probes used in this study, nucleotide sequence, target organisms and references. Ammonium oxidising bacteria are abbreviated as AOB

Oligonucleotide designation	Amplicon size [bp]	Nucleotide sequence (5'-3')	Oligonucleotide length [bp]	Target organisms	References
Forward	456	AGC TGG GGA AAC CTG ACT G	19	<i>H. fraxineus</i>	(Johansson et al. 2010)
Reverse		ACA CCG CAA GGA CCC TAT C	19	<i>H. fraxineus</i>	(Johansson et al. 2010)
Cf-F	81	CCC TTG TGT ATA TTA TAT TGT TGC TTT AGC	31	<i>H. fraxineus</i>	(Chandelier et al. 2010)
Cf-R		GGG TCC TCT AGC AGG CAC AGT	23	<i>H. fraxineus</i>	(Chandelier et al. 2010)
Cf-S		6-FAM-TCT GGG CGT CGG CCT CGG-BHQ-1	19	<i>H. fraxineus</i>	(Chandelier et al. 2010)
CTO 189fC	116	GGA GGA AAG TAG GGG ATC G	20	AOB	(Kowalchuk et al. 1997)
RT1r	(for <i>Nitrosomonas</i> sp. strain PLL12, Acc. No. OP146089.1)	CGT CCT CTC AGA CCA RCT ACT G	22	AOB	(Hermansson and Lindgren 2001)
TMP1		HEX-CAA CTA GCT AAT CAG RCA TCR GCC GCT C-BHQ1	28	AOB	(Hermansson and Lindgren 2001)

Table 3 The sequence of double-stranded synthetic gBlock™ fragment was designed to represent the amplicon sequence in the described assay by Chandelier et al. 2010 and Ioos et al. 2009 to detect *H. fraxineus*

Target	Nucleotide sequence (5'-3')	Length [bp]	Reference
ITS	CCTCACGGGTAGAAACCCACCCCTGTGTATATTATATTGTTGCTTTAGCAGGTCGCCCTCTG-GCGTTCGGCTCGGCTGACTGTGCCTGCTAGAGGACCCTAAATTTGAAATACAGTGTCGTC	125	this study

threshold were obtained using StepOne™ Software V2.3 (Applied Biosystems, Foster City, USA).

PCR detection of *H. fraxineus*

A species-specific end-point PCR approach for the detection and sequencing of a *H. fraxineus* DNA-Marker has been performed using a primer pair designed by Johansson et al. (2010) (final concentration 0.2 μM) with 12.5 μl Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs GmbH, Frankfurt/Main, Germany), 40 ng of source template and water to a final volume of 25 μl. Assay parameters were applied as described in Johansson et al. (2010). PCR products were separated at 80 V for 90 min on a 1% agarose gel, stained with GelRed (Genaxxon bioscience GmbH, Ulm, Germany) and visualised with a UV transilluminator. PERFECT™ Plus 2kb DNA marker (Roboklon, Berlin, Germany) was used as a length marker. All oligonucleotides used in this study were manufactured by Metabion (Planegg, Germany). Sanger (dideoxy) sequencing of the PCR products was performed by Macrogen Europe (Amsterdam, Netherlands) using the PCR primers.

Results

The duplex qPCR assay was evaluated using a standard curve generated with gBlocks™ in increasing concentrations. Findings revealed an efficiency of 96.52% and a detection limit of 10 copies per reaction at a C_q value of 33 (Fig. 2b). The overall standard deviation was less than 0.73 for all detected concentrations observed over three technical replicates (Table 4). To estimate the detection limit in copies per gram of soil, the qPCR result of sample GR2 was used (Table S1). Since 40 ng of sample GR2 carried 10 copies of the *H. fraxineus* target, and 500 mg of soil from GR2 extraction yielded 15.4 μg of total metagenomic DNA (30.8 μg/g soil), the detection limit of 0.25 copies/ng (10 copies/40 ng) corresponds to 7700 copies per g of soil (30,800 ng/g soil × 0.25 copies/ng). A total of 130 soil samples within this study were quantified for *H. fraxineus* DNA markers with a total of 23 positive samples ranging from 864 to 10 detected copies (Fig. 3). The rehearsal series comprised samples as part of the *H. fraxineus* distribution study carried out at four sites in 2022 followed by a soil layer study carried out at one

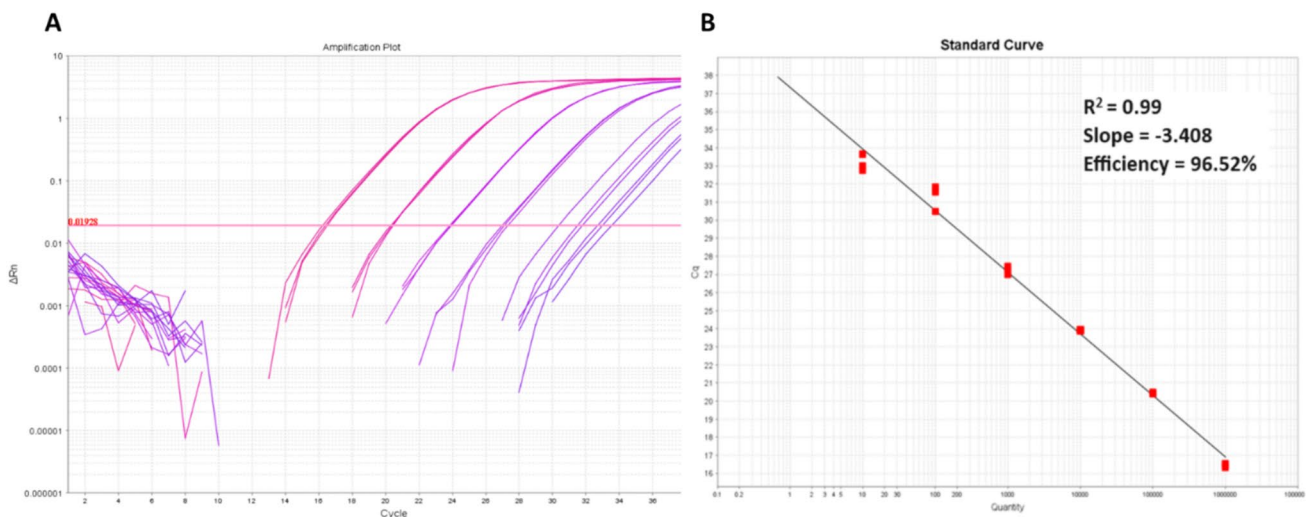


Fig. 2 Plot of amplification **A** and standard curve **B** for the *H. fraxineus* (ITS) target (Chandelier et al. 2010). A dilution series 1×10^0 to 1×10^6 of ITS gBlocks™ was measured in three technical replicates per concentration. Amplification plots **A** for ITS gBlocks™ contain-

ing the *H. fraxineus* template. Standard curve **B** obtained by plotting (red dots) the threshold numbers of the technical replicate's qPCR cycles against the absolute copy number of each standard. Coefficient of determination, slope and efficiency are stated **B**

Table 4 Mean C_q values as a mean of three technical replicates and standard deviation for synthetic gBlock standards (*H. fraxineus* target) in ascending concentration from 1×10^0 to 1×10^6 copies per reaction. The duplex assay (Cf-F/R/S; CTO189fC/RT1r/TMP1) has a detection limit of 10 copies per reaction for the *H. fraxineus* template

Copy number	Mean C_q values	Standard deviation
1×10^0	Not detected	X
1×10^1	33	± 0.46
1×10^2	31	± 0.72
1×10^3	27	± 0.21
1×10^4	23	± 0.04
1×10^5	20	± 0.03
1×10^6	16	± 0.11

site in 2023. We provided evidence that DNA markers of *H. fraxineus* are detectable in soil samples collected from ash stands in varying quantities, which are subsequently referred to as pathogen-load.

Distribution of *H. fraxineus* in soil

As part of the pathogen distribution study, 70 soil samples from four study sites were tested for the presence of *H. fraxineus*. All soil samples were positive for the quality control template AOB (Table S1), demonstrating sufficient DNA quality and quantity in the DNA extracts. The highest pathogen distribution in the tested plots was observed at the site in Grabenstätt with 50% positive plots (5 of 10), followed by Kaisheim with 35% (7 of 20), Emmendingen with 15% (3 of 20) and Schorndorf with 5% (1 of 20) (Fig. 3 & 4). The distribution of positive plots showed the variation in pathogen-load between individual plots, highlighting plots and neighbouring plots with high pathogen-loads. This was particularly evident in plots 4 to 6 at the Kaisheim study site and 17 to 19 at the Emmendingen site (Fig. 3). The quantification of *H. fraxineus* confirmed a heterogeneous pathogen-load across all sites, with particularly high levels at the Grabenstätt (plot 4: 864 copies/40 ng) and Kaisheim

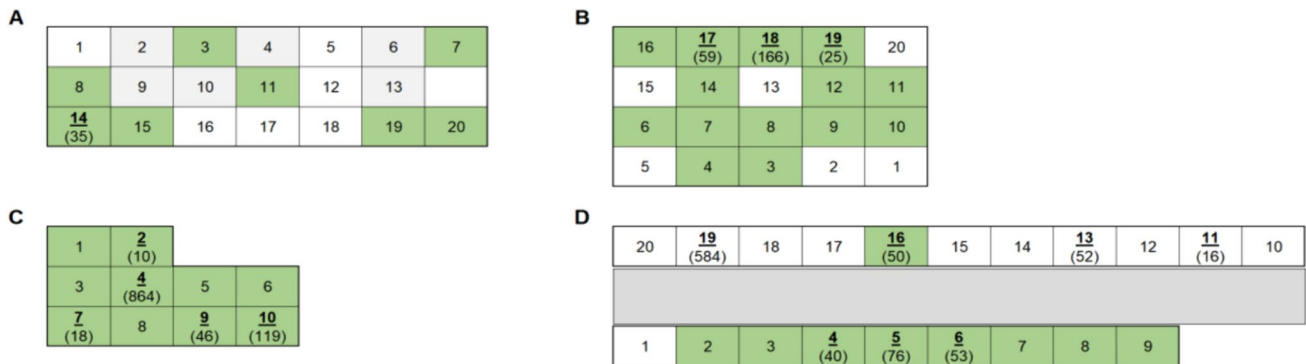
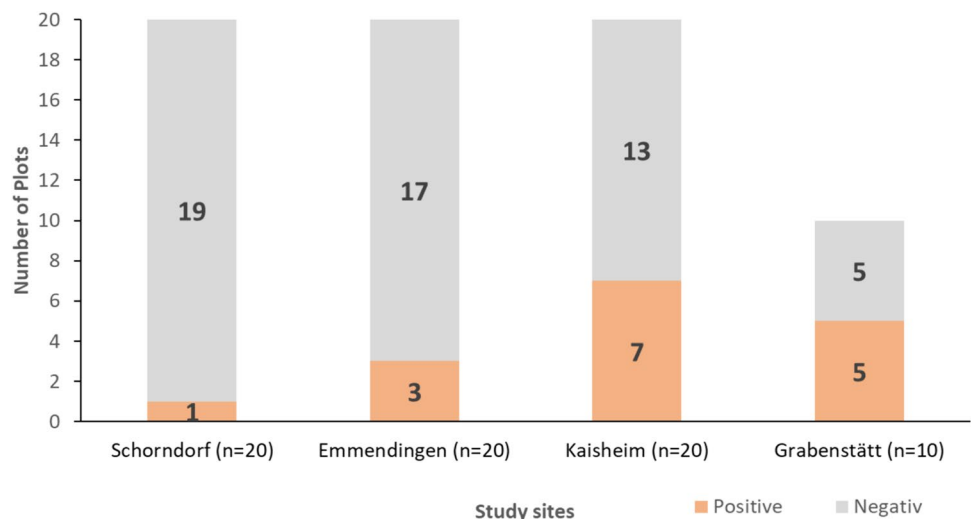


Fig. 3 Plot design of the study sites Schorndorf **A**, Emmendingen **B**, Grabenstätt **C** and Kaisheim **D**. Plots are numbered, with bold and underlined numbers representing plots with positive *H. fraxineus* detections and green plots representing plots with at least one ash tree

with leaves. Absolute quantification data are given in parentheses as copies per 40 ng of template DNA. Each plot is 100 m² in size. The grey area in **D** marks the area between the plots which is not drawn to scale

Fig. 4 Plots per study site grouped as positive and negative tested for *H. fraxineus* in duplex assay. Number of samples is displayed in brackets; absolute numbers of plots per group are displayed within the column chart as numbers



(plot 19: 584 copies/40 ng) study sites. Nonetheless, plot 10 (10 copies/40 ng) and plot 11 (16 copies/40 ng) at the Grabenstätt and Kaisheim sites, respectively, displayed low pathogen-loads (Fig. 3). Moreover, the majority of the plots tested positive had at least one ash tree, whereas only three plots tested positive despite having no ash tree on the plot (Fig. 3). Besides the detection via qPCR, the presence of *H. fraxineus* was confirmed through PCR and subsequent sequencing of the samples. Five sequences were acquired and aligned with the positive control (*H. fraxineus* 1431 liquid culture), as well as five different *H. albidus* strains (Fig. 5). All sequences acquired from the soil DNA template correspond 100% with the positive control and a *H. fraxineus* sequence from the database. However, they display nine nucleotide disparities compared to *H. albidus* sequences.

Soil layer study

H. fraxineus DNA was identified in all three sampling depths at the Schorndorf site, with spatial variation in pathogen-load observed. The quantity of positive tested samples declined

gradually with increasing depth: 30% at 0–5 cm, 15% at 5–10 cm and 10% at 10–15 cm (Table 5 & Fig. 6). Absolute quantification varied between 282 copies/40 ng DNA for the highest and 13 copies/40 ng for the lowest quantified sample (Table 5). The mean quantity per sampling depth was 27 copies/40 ng at 0–5 cm, 9 copies/40 ng at 5–10 cm and 20 copies/40 ng at 10–15 cm (Table 5), indicating that the pathogen-load decreases with increasing soil depth. However, mean quantity was calculated with negative samples equivalent to zero copies. Given the detection limit of ten copies per reaction, the assumption of negative samples corresponding to zero copies may introduce a bias in the mean quantity.

Detection of *H. fraxineus* on passive spore traps

The two spore trap filters yielded positive results for the AOB marker. Both spore traps were exposed to ambient air in a sequence of seven days in late June and tested positive for *H. fraxineus* at a quantity of 25 and 11 copies/40 ng, respectively. These results also support the use of the qPCR assay for measuring the pathogen-load on spore traps with filter matrix. Therefore, the entire process, encompassing

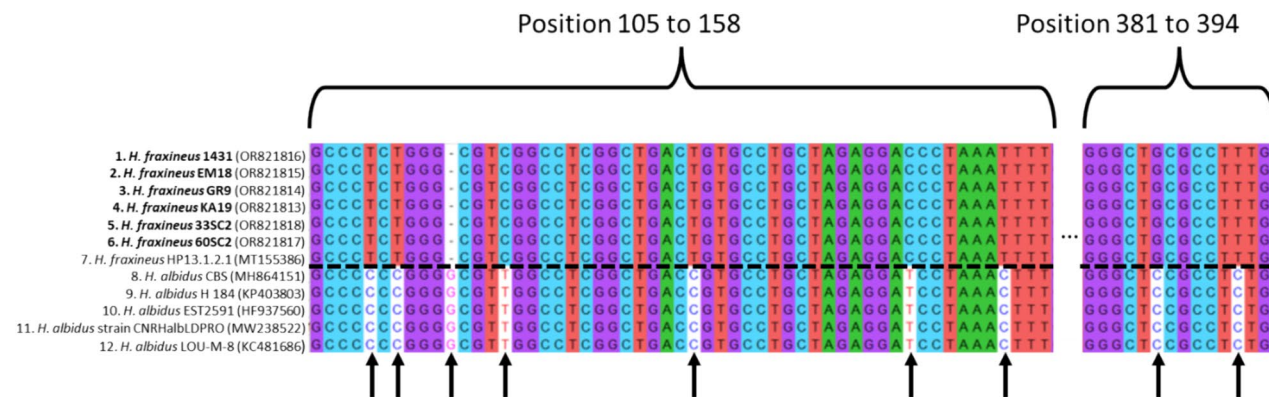


Fig. 5 Nucleotide alignment for the partial rDNA and ITS sequences of *H. fraxineus* and *H. albidus* fragmented to display the mismatches (arrows). Strains are indicated on the left, with their respective accession numbers in parentheses. Strains in bold are obtained

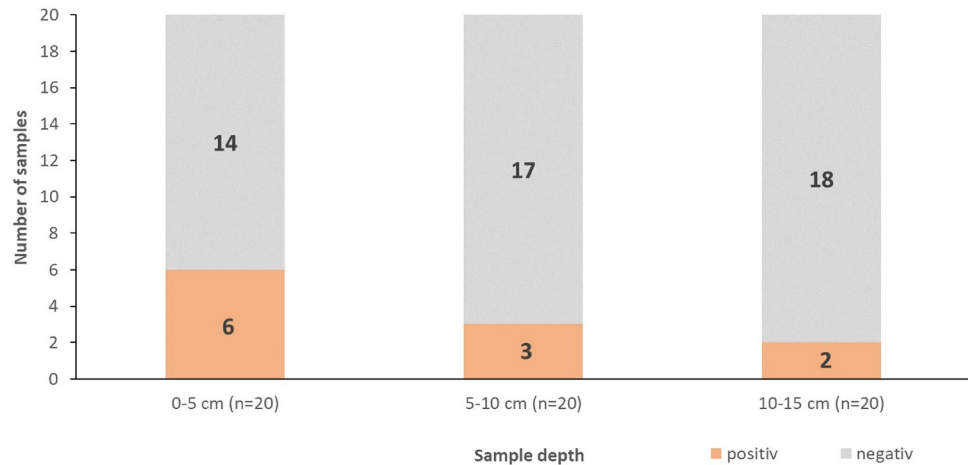
from templates generated of this study. The dotted line is employed to highlight between the two species. Curly parentheses are utilized to denote nucleotide positions as counted in *H. fraxineus* 1431 (Acc. No. OR821816)

Table 5 Soil layer samples from the Schorndorf site with positive detection of *H. fraxineus* displayed with values of absolute quantification for sample 1 to 6, whereas samples 7 to 20 are negative in all sampling depths (not shown). The quantities displayed within soil

layers are mean values of three technical replications displayed as copies/40 ng input DNA and layers with no detection of *H. fraxineus* are marked with X. The mean quantity for each soil layer was calculated based on a sample size of 20

Depth	Soil layer samples						Mean quantity	Location
	1	2	3	4	5	6		
0 to 5 cm	26	16	13	252	13	236	28	Schorndorf
5 to 10 cm	35	X	X	41	X	71	7	
10 to 15 cm	16	X	X	X	X	282	15	

Fig. 6 Samples per sampling depth were grouped as positive and negative for *H. fraxineus* in duplex assay. The number of samples is displayed in brackets, and absolute numbers of plots per group are displayed within the column bar as numbers



sample preparation, DNA extraction and the quantification of *H. fraxineus* markers, was successfully employed on two spore trap samples.

Differences in pathogen-load between study sights

The hypothesis that there are no differences in number of positive detected plots between study sites was tested with a binomial approach. Using the generalised linear mixed models (GLIMMIX, SAS v9.4, www.sas.com), a p-value of 0.0634 was obtained. Given that the p-value is greater than the alpha level of 0.05, the hypothesis cannot be rejected. In light of these findings, it is not possible to draw any conclusions regarding the differences between the study sites.

Discussion

Since the first detection of *H. fraxineus* several studies were conducted to study the diseases and develop management strategies to conserve common ash in a forest environment (Langer et al. 2022; Schumacher 2011). Ascospores of *H. fraxineus* were monitored in various studies and several environmental factors such as humidity, precipitation and temperature are associated with ascospore abundance (Aguayo et al. 2018; Chandelier et al. 2014; Dvořák et al. 2016, 2023). Further, ascospore abundance was found to decrease rapidly within 50 m from the inoculum source and with increasing height (Chandelier et al. 2014; Dvořák et al. 2023; Grosdidier et al. 2020). Landscape architecture, especially the resulting microclimates, is another crucial factor influencing the ascospore flight (Grosdidier et al. 2020). The aforementioned factors could lead to a variable pathogen-load even at a small spatial scale as observed in this study, which may explain the spatial distribution of ADB, especially concerning stem collar necrosis (Enderle

et al. 2013). Integration of two already established protocols (Chandelier et al. 2010; Hermansson and Lindgren 2001) was successfully implemented with a detection limit of 10 copies for the *H. fraxineus* template, as per current literature in singleplex studies where qPCR was found to be even more sensitive towards fungal detection than high-throughput sequencing approaches (Chandelier et al. 2021). The detection of the AOB template as a quality control measure was unavoidable, given that soil is a complex matrix where the absolute quantity of extracted DNA and, in particular, the quality of the extracted DNA may be influenced by a variety of inhibitors, which could result in a false negative interpretation of the results (Manfredini et al. 2021; Wang et al. 2017). Our results suggest an ubiquitous abundance of AOB within the soil of different ash stands, which is consistent with previous findings in German forest soils (Krüger et al. 2021). We conclude that there is no interference of soil type on the molecular detection of *H. fraxineus*, as AOB were detected in all samples, despite the presence of three different soil types. Furthermore, specificity of the oligonucleotides designed by Chandelier et al. 2011 was previously discussed (EPPO 2013) and interference with *H. albidus* DNA might occur. Therefore the qPCR was occasionally verified by a species-specific PCR assay (EPPO 2013; Johansson et al. 2010).

Distribution of *H. fraxineus* in soil

Soil monitoring is a frequently used method to assess the abundance of microorganisms such as *Beauveria bassiana* after biocontrol introduction in agricultural settings (Manfredini et al. 2021; Tartanus et al. 2021). Our study was conducted to investigate the spatial distribution of *H. fraxineus*, expressed as DNA marker copy numbers per 40 ng input DNA as a proxy for pathogen-load. Heterogeneous distribution of pathogen-load was seen on all four study sites, indicating an uneven distribution of pathogen propagules. The

majority of plots exhibiting pathogen-loads were situated in proximity to ash trees, which suggests a potential influence on pathogen-load formation and ash density. The clone plantation in Grabenstätt is characterised by the highest abundance of ash trees, the highest pathogen-load within a single plot and the highest percentage of plots that tested positive. Thus, there appears to be an influence between the abundance of ash trees and pathogen-load, which is consistent with previous research (Laubray et al. 2023) that found a link between quantity of infected rachis and ash density. In addition to ash density, grass cover is another factor that affects the quantity of apothecia and, therefore, indirectly influences the potential amount of ascospores that can be produced (Mansfield et al. 2018). Another method to describe the pathogen-load within an ash stand was applied in Poland, where several plots in ash stands were counted for ash rachis and apothecia to estimate infectious material (Kowalski et al. 2013). The results are consistent with this work, describing variability in the pathogen-load detected within an ash stand and between ash stands.

Restricting the detected pathogen-load to either spores or mycelium is not possible due to the PCR method used, which cannot differentiate between these two. Since ascospore abundance in ash stands is particularly high in June (Cracknell et al. 2023; Dvořák et al. 2016, 2023; Laubray et al. 2023) and at ground level (Chandelier et al. 2014) one may speculate that a high proportion of detected pathogen-load is constituted by ascospores deposited on soil. Nevertheless, mating, overwintering and the formation of pseudosclerotia and ascospores occur on leaf residues of *Fraxinus* spp. (Gross and Holdenrieder 2013; Marçais et al. 2023). Furthermore, *H. fraxineus* has the ability to grow in soil inoculated with conidia, even in the absence of ash tissue, and can infect ash roots through the soil (Fones et al. 2016). Therefore, in addition to ascospores, the mycelium and conidia of *H. fraxineus* can be detected in leaf residues and soil, which may contribute to the pathogen-load. As a result, the term "pathogen-load" is employed to describe the quantified DNA markers of *H. fraxineus*. In contrast to previous studies where *H. fraxineus* infectious material was monitored (Kowalski et al. 2013), the employed term pathogen-load in our study refers to infectious and non-infectious material simultaneously. Soil samples are more advantageous than spore traps in that their sample sizes can be easily adjusted, enabling the expected level of resolution to match the study's design.

Moist conditions, mainly caused by flooding and topography, are suspected to influence *H. fraxineus*-associated collar necrosis (Enderle et al. 2013; Marçais et al. 2016). Moreover, heavy clay soils in combination with high precipitation and water logging are associated with root infections (Baxter et al. 2023). High pathogen-load and moist conditions may increase stem necrosis incidence, leading to a higher likelihood of tree death. One mitigation measure to overcome

ash dieback disease is the selection of plus-trees with tolerance to *H. fraxineus* (Stener 2018). Our research reveals that pathogen-load varies within soils and may contribute to stem canker formation. As a result, it is recommended that the monitoring of pathogen-load in plus tree selections is given greater importance, as this variable may have been previously undervalued.

Distribution of *H. fraxineus* within different soil depths

The pathogen-load of *H. fraxineus* is assumed to deposit through input from wind-dispersed spores and leaf residues containing mycelium. As ascospore abundance and infected leaf residues are described as ubiquitous in ash stands (Chandelier et al. 2014; Dvořák et al. 2023; Laubray et al. 2023; Marçais et al. 2023), it can be assumed that deposition on the soil differs due to topography, microclimates and fauna, rather than ascospore abundance. Another peculiarity within the soil layers is that the depths between 5 and 15 cm were only found positive if the first layer was also positive. As leaching of soluble nutrients from *F. excelsior* leaf litter was described (Hagen-Thorn et al. 2006; Nykvist 1959), it is assumed that vertical displacement or growth of *H. fraxineus* occurs after deposition on the foliage and soil surface. However, the longevity of the pathogen-load in the soil, its potential for accumulation and the translocation processes are uncertain.

Detection of *H. fraxineus* on passive spore traps

The successful detection of *H. fraxineus* on passive spore traps was undertaken in various countries using paper filters and different matrices as adhesives (Chandelier et al. 2014; Grosdidier et al. 2018; Laubray et al. 2023). Extraction of DNA from these paper filters primarily involves macerating the entire trap and then utilizing this matrix for additional extraction techniques. The method outlined in this study utilised a one-hole puncher to prepare random sections of the entire paper filter. This allows the use of easy-to-handle standard laboratory containers (2 ml) for all extraction procedures, including maceration. Commercially available bead beaters can be utilized to efficiently perform maceration, as demonstrated for both pure ascospores and soil medium (Wang et al. 2023).

Conclusion

Soil samples examined in this study show heterogeneous pathogen-loads of *H. fraxineus* within the topsoil of all tested ash stands. Despite the high ascospore pressure described in stands, the deposition of *H. fraxineus* propagules seems to be heterogeneous and accumulates

in hotspots. Those hotspots are discussed to be influenced by microclimates and topography. Pathogen accumulation in soil and relocation in deeper layers is suggested. The pathogen-load composition cannot be further specified in *H. fraxineus* compartments like ascospores, or mycelia given the applied methods limitations. Hence the measurements include all compartments of *H. fraxineus* containing DNA. Nevertheless, a high proportion of ascospores can be assumed due to the high pressure of those described in several studies. The aforementioned technique for detecting pathogen-load through soil samples has the potential for monitoring pathogen pressure in areas such as new ash plantations but also selected tolerant or sensible ash trees, allowing for an assessment of current pathogen pressure.

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Author contributions JWB was responsible for the initial drafting of the manuscript and the conduct of the field trials. The study was designed by JWB and MK. Spore trap trials were designed and performed by GK and SJO. The design and evaluation of synthetic gene fragments was conducted by CZ. All authors were involved in the writing, review and editing of the manuscript.

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Declarations

Conflicts of interest The authors confirm that there are no conflicts of interest.

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References

- Aguayo J, Fourrier-Jeandel C, Husson C, Ioos R (2018) Assessment of passive traps combined with high-throughput sequencing to study airborne fungal communities. *Appl Environ Microbiol* 84:e02637-e2717
- Baxter E, Cooke LR, Spaans F, Grant IR, McCracken AR (2023) The introduction of *Hymenoscyphus fraxineus* to Northern Ireland and the subsequent development of ash dieback. *Forest Pathol* 53:e12789
- Bengtsson SBK, Barklund P, von Brömssen C, Stenlid J (2014) Seasonal pattern of lesion development in diseased *Fraxinus excelsior* infected by *Hymenoscyphus pseudoalbidus*. *PLoS ONE* 9:e76429
- Chamberlain AC, Chadwick RC (1972) Deposition of spores and other particles on vegetation and soil. *Annals of Applied Biology* 71:141–158
- Chandelier A, André F, Laurent F (2010) Detection of *Chalara fraxinea* in common ash (*Fraxinus excelsior*) using real time PCR. *Forest Pathol* 40:87–95
- Chandelier A, Delhaye N, Helson M (2011) First report of the ash dieback pathogen *Hymenoscyphus pseudoalbidus* (Anamorph *Chalara fraxinea*) on *Fraxinus excelsior* in Belgium. *Plant Dis* 95:220
- Chandelier A, Helson M, Dvorak M, Gischer F (2014) Detection and quantification of airborne inoculum of *Hymenoscyphus pseudoalbidus* using real-time PCR assays. *Plant Pathol* 63:1296–1305
- Chandelier A, Gerarts FS, Martin G, Herman M, Delhaye L (2016) Temporal evolution of collar lesions associated with ash dieback and the occurrence of *Armillaria* in Belgian forests. *Forest Pathol* 46:289–297
- Chandelier A, Hulin JS, Martin G, Debode F, Massart S (2021) Comparison of qPCR and metabarcoding methods as tools for the detection of airborne inoculum of forest fungal pathogens. *Phytopathology* 111:570–581
- Cracknell DJ, Peterken GF, Pommerening A, Lawrence PJ, Healey JR (2023) Neighbours matter and the weak succumb: Ash dieback infection is more severe in ash trees with fewer conspecific neighbours and lower prior growth rate. *J Ecol* 111:2118–2133
- Drenkhan R, Sander H, Hanso M (2014) Introduction of mandshuriana ash (*Fraxinus mandshurica* Rupr.) to Estonia: Is it related to the current epidemic on European ash (*F. excelsior* L.)? *Eur J Forest Res* 133:769–781
- Dvořák M, Rotkova G, Botella L (2016) Detection of Airborne Inoculum of *Hymenoscyphus fraxineus* and *H. albidus* during Seasonal Fluctuations Associated with Absence of Apothecia. *Forests* 7(1):1
- Dvořák M, Štoidl P, Rost M (2023) Vertical spread of *Hymenoscyphus fraxineus* propagules. *NB* 84:231–246
- Eisen A-K, Fussi B, Šikoparija B, Jochner-Oette S (2022) Aerobiological pollen deposition and transport of *Fraxinus excelsior* l at a small spatial scale. *Forests* 13(3):424
- Enderle R, Peters F, Nakou A, Metzler B (2013) Temporal development of ash dieback symptoms and spatial distribution of collar rots in a provenance trial of *Fraxinus excelsior*. *Eur J Forest Res* 132(5–6):865–876
- Enderle R, Metzler B, Riemer U, Kändler G (2018) Ash Dieback on Sample Points of the National Forest Inventory in South-Western Germany. *Forests* 9(1):25
- Enderle, R. Fussi, B. Lenz, H.D. Langer, G. Nagel, R. and Metzler, B.** (2017) Ash dieback in Germany: research on disease development, resistance and management options. *Dieback of European Ash (Fraxinus spp.) - consequences and guidelines for sustainable management* 89–105
- EPPO (2013) PM 7/117 (1) *Hymenoscyphus pseudoalbidus*. *EPPO Bulletin* 43:449–461

- Fones HN, Mardon C, Gurr SJ (2016) A role for the asexual spores in infection of *Fraxinus excelsior* by the ash-dieback fungus *Hymenoscyphus fraxineus*. *Sci Rep* 6:34638
- Gadoury D, MacHardy WE (1986) Forecasting Ascospore Dose of *Venturia inaequalis* in Commercial Apple Orchards. *Phytopathology* 76:112
- Grosdidier M, Scordia T, Ioos R, Marçais B (2020) Landscape epidemiology of ash dieback. *J Ecol* 108:1789–1799
- Grosdidier, M. Ioos, R. Husson, C. Cael, O. Scordia, T. and Marçais, B.** (2018) Tracking the invasion. dispersal of *Hymenoscyphus fraxineus* airborne inoculum at different scales. *FEMS Microbiol Ecol*, **94**,5 fty049
- Gross A, Holdenrieder O (2013) On the longevity of *Hymenoscyphus pseudoalbidus* in petioles of *Fraxinus excelsior*. *For Path* 43:168–170
- Gross A, Grünig CR, Queloz V, Holdenrieder O (2012a) A molecular toolkit for population genetic investigations of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Forest Pathol* 42:252–264
- Gross A, Zaffarano PL, Duo A, Grünig CR (2012b) Reproductive mode and life cycle of the ash dieback pathogen *Hymenoscyphus pseudoalbidus*. *Fungal Genet Biol* 49:977–986
- Hagen-Thorn A, Varnagirytė I, Nihlgård B, Armolaitis K (2006) Autumn nutrient resorption and losses in four deciduous forest tree species. *For Ecol Manage* 228:33–39
- Haňáčková Z, Koukol O, Čmoková A, Zahradník D, Havrdová L (2017) Direct evidence of *Hymenoscyphus fraxineus* infection pathway through the petiole-shoot junction. *Forest Pathol* 47:e12370
- Hermansson A, Lindgren PE (2001) Quantification of ammonia-oxidizing bacteria in arable soil by real-time PCR. *Appl Environ Microbiol* 67:972–976
- Husson C, Caël O, Grandjean JP, Nageleisen LM, Marçais B (2012) Occurrence of *Hymenoscyphus pseudoalbidus* on infected ash logs. *Plant Pathol* 61:889–895
- Ioos R, Kowalski T, Husson C, Holdenrieder O (2009) Rapid in planta detection of *Chalara fraxinea* by a real-time PCR assay using a dual-labelled probe. *Eur J Plant Pathol* 125:329–335
- Johansson SBK, Vasaitis R, Ihrmark K, Barklund P, Stenlid J (2010) Detection of *Chalara fraxinea* from tissue of *Fraxinus excelsior* using species-specific ITS primers. *Forest Pathol* 40:111–115
- Köhn M (1928) Bemerkungen zur mechanischen Bodenanalyse. III. Ein neuer Pipettapparat. *Zeitschrift Für Pflanzenernährung, Düngung, Bodenkunde* 11:50–54
- Kowalchuk GA, Stephen JR, de Boer W, Prosser JI, Embley TM, Woldendorp JW (1997) Analysis of ammonia-oxidizing bacteria of the beta subdivision of the class Proteobacteria in coastal sand dunes by denaturing gradient gel electrophoresis and sequencing of PCR-amplified 16S ribosomal DNA fragments. *Appl Environ Microbiol* 63:1489–1497
- Kowalski T (2006) *Chalara fraxinea* sp. nov. associated with dieback of ash (*Fraxinus excelsior*) in Poland. *Forest Pathol* 36:264–270
- Kowalski T, Holdenrieder O (2009) Pathogenicity of *Chalara fraxinea*. *Forest Pathol* 39:1–7
- Kowalski T, Bialobrzeski M, Ostafinska A (2013) The occurrence of *Hymenoscyphus pseudoalbidus* apothecia in the leaf litter of *Fraxinus excelsior* stands with ash dieback symptoms in southern Poland. *Acta Mycol* 48:2
- Kräutler K, Treitler R, Kirisits T (2015) *Hymenoscyphus fraxineus* can directly infect intact current-year shoots of *Fraxinus excelsior* and artificially exposed leaf scars. *Forest Pathol* 45:274–280
- Krüger M, Potthast K, Michalzik B, Tischer A, Küsel K, Deckner FF, Herrmann M (2021) Drought and rewetting events enhance nitrate leaching and seepage-mediated translocation of microbes from beech forest soils. *Soil Biol Biochem* 154:108153
- Langer GJ, Fuchs S, Osewold J, Peters S, Schrewe F, Ridley M, Kätzel R, Bubner B, Grüner J (2022) FraxForFuture—research on European ash dieback in Germany. *J Plant Dis Prot* 129:1285–1295
- Laubray S, Buée M, Marçais B (2023) Evidence of a component Allee effect for an invasive pathogen: *Hymenoscyphus fraxineus*, the ash dieback agent. *Biol Invasions* 25:2567–2582
- Manfredini A, Malusà E, Costa C, Pallottino F, Mocali S, Pinzari F, Canfora L (2021) Current methods, common practices, and perspectives in tracking and monitoring bioinoculants in soil. *Front Microbiol* 12:698491
- Mansfield JW, Galambos N, Saville R (2018) The use of ascospores of the dieback fungus *Hymenoscyphus fraxineus* for infection assays reveals a significant period of biotrophic interaction in penetrated ash cells. *Plant Pathol* 67(6):1354–1361
- Mansfield J, Brown I, Papp-Rupar M (2019) Life at the edge - the cytology and physiology of the biotroph to necrotroph transition in *Hymenoscyphus fraxineus* during lesion formation in ash. *Plant Pathol* 68(5):908–920
- Marçais B, Husson C, Godart L, Caël O (2016) Influence of site and stand factors on *Hymenoscyphus fraxineus* -induced basal lesions. *Plant Pathol* 65(9):1452–1461
- Marçais B, Giraudel A, Husson C (2023) Ability of the ash dieback pathogen to reproduce and to induce damage on its host are controlled by different environmental parameters. *PLoS Pathog* 19:e1010558
- McKinney LV, Thomsen IM, Kjær ED, Bengtsson S, Nielsen LR (2012) Rapid invasion by an aggressive pathogenic fungus (*Hymenoscyphus pseudoalbidus*) replaces a native decomposer (*Hymenoscyphus albidus*): a case of local cryptic extinction? *Fungal Ecol* 5:663–669
- McMullan M, Rafiqi M, Kaithakottil G, Clavijo BJ, Bilham L, Orton E, Percival-Alwyn L, Ward BJ, Edwards A, Saunders DGO, Accinelli G, Wright J, Verweij W, Koutsovoulos G, Yoshida K, Hosoya T, Williamson L, Jennings P, Ioos R, Husson C, Hietala AM, Vivian-Smith A, Solheim H, MacClean D, Fosker C, Hall N, Brown JKM, Swarbrick D, Blaxter M, Downie JA, Clark MD (2018) The ash dieback invasion of Europe was founded by two genetically divergent individuals. *Nat Ecol Evol* 2:1000–1008
- Nykvist N (1959) Leaching and decomposition of litter in experiments on leaf litter of *fraxinus excelsior*. *Oikos* 10:190
- Peters S, Langer G, Kätzel R (2021) Bonitur geschädigter eschen im kontext des eschentriebsterbens. *AFZ-der Wald* 12:28–31
- Peters S, Fuchs S, Bien S, Bußkamp J, Langer GJ, Langer EJ (2023) Fungi associated with stem collar necroses of *Fraxinus excelsior* affected by ash dieback. *Mycol Progress* 22:1–29
- Queloz V, Grünig CR, Berndt R, Kowalski T, Sieber TN, Holdenrieder O (2011) Cryptic speciation in *Hymenoscyphus albidus*. *Forest Pathol* 41(2):133–142
- Schumacher J (2011) The general situation regarding ash dieback in Germany and investigations concerning the invasion and distribution strategies of *Chalara fraxinea* in woody tissue I. *EPPO Bulletin* 41(1):7–10
- Schumacher J, Wulf A, Leonhard S (2007) First record of *Chalara fraxinea* T. KOWALSKI sp. nov. in Germany—a new agent of ash decline. *Journal for Cultivated Plants* 59:121–123
- Schumacher J, Kehr R, Leonhard S (2010) Mycological and histological investigations of *Fraxinus excelsior* nursery saplings naturally infected by *Chalara fraxinea*. *Forest Pathol* 40(5):419–429
- Stener L-G (2018) Genetic evaluation of damage caused by ash dieback with emphasis on selection stability over time. *For Ecol Manage* 409:584–592
- Tartanus M, Furmanczyk EM, Canfora L, Pinzari F, Tkaczuk C, Majchrowska-Safaryan A, Malusà E (2021) Biocontrol of *Melolontha spp* grubs in organic strawberry plantations by entomopathogenic fungi as affected by environmental and

- metabolic factors and the interaction with soil microbial biodiversity. *Insects* 12:127
- Timmermann V, Børja I, Hietala AM, Kirisits T, Solheim H (2011) Ash dieback: pathogen spread and diurnal patterns of ascospore dispersal, with special emphasis on Norway*. *EPPO Bulletin* 41:14–20
- Wang H, Qi J, Xiao D, Wang Z, Tian K (2017) A re-evaluation of dilution for eliminating PCR inhibition in soil DNA samples. *Soil Biol Biochem* 106:109–118
- Wang, T.W. Strickland, D.A. Haredy, Y. Cox, K.D. and Hodge, K.T.** (2023) A quantitative PCR assay for detection of the mycotoxigenic plant pathogen and food spoiling mold *Paecilomyces niveus* in fruit, food, and soil. *bioRxiv*, 2023–02
- Zhao Y-J, Hosoya T, Baral H-O, Hosaka K, Kakishima M (2013) *Hymenoscyphus pseudoalbidus*, the correct name for *Lambertella albida* reported from Japan. *Mycotaxon* 122(1):25–41

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