Fungi associated with stem collar necroses of Fraxinus excelsior affected by ash dieback

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Abstract

In recent decades the vitality and physical stability of European ash trees in Germany have been reduced by European ash dieback, especially when associated with stem collar necroses and rots. This study was carried out to investigate the composition of the fungal communities associated with stem collar necroses. Filamentous fungi were isolated from 58 ash trees out of nine forest stands in northern, eastern, and central Germany. Obtained isolates were identified to a genus or species level by means of morphological and molecular analyses. In total 162 morphotypes including endophytic, saprotrophic and pathogenic fungi were isolated. For 33 species found no prior reports from *Fraxinus excelsior* were recognised, including *Cryptostroma corticale* and *Diplodia sapinea*. None of the identified species were found at all studied sites, though *Diplodia fraxini* was the most common fungi with regard to frequency within all isolates, occurring at seven sample sites. This species is followed by *Hymenoscyphus fraxineus*, *Armillaria* spp., *Neonectria punicea*, *Diaporthe* cf. *eres*, *Fusarium* cf. *lateritium*, and *Paracucurbitaria* sp. in order of frequency within all isolates. The aforementioned species are characterised and analysed in respect to their occurrence in stem collar necroses and at sample sites. The influence of site conditions on the fungal composition was described for five intensively sampled sites with a minimum of five studied trees (Schwansee, Rhüden, Berggießhübel, Satrup, and Schlangen). The sampling site of Schlangen was further subdivided into four subplots with different positions in the terrain. In the remaining four extensive sample sites either one or two trees, respectively, were sampled and analysed (Oranienbaumer Heide, Woltershausen, Wolfenbüttel, and Neuhege). Over all sample sites fungal communities of symptomatic stem tissue are similar concerning the most frequent fungi, but vary greatly according to singularly isolated fungi.

1. Introduction

Since the early 1990s, the European ash (*Fraxinus excelsior* L.) is threatened by European ash dieback, caused by *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (*Helotiaceae*, Ascomycota). First disease reports came from Poland and Lithuania (Przybyl 2002; Lygis et al. 2005). In Germany, the severe disease was observed since 2002 and the causal agent was first proven in the year 2006 (Heydeck et al. 2005; Schumacher et al. 2007). Meanwhile, this invasive fungal pathogen has become widespread in Europe. Affected European ash (hereafter referred to as ash) trees of all ages show a broad range of symptoms, such as leaf necrosis, wilting, shoot blight, inner bark discolorations, sunken cankers, epicormic shoots, as well as stem collar, and root necrosis, or rather rot. Reduction of tree stability and increase of mortality of ash is often connected to stem collar necrosis, which progresses towards xylem and heartwood. Stem collar necrosis can occur on trees with or without crown symptoms of ash dieback, but are often observed on diseased trees (Schumacher et al. 2009; Husson et al. 2012; Enderle et al. 2017; Langer 2017; Meyn et al. 2019). Stem collar necroses are defined as basal lesions with necrotic tissue on the outside and the inside of the stem mainly caused by fungi (Langer 2017). The actual shape of stem collar necroses depends on different factors, such as individual progress or associated fungi. Advanced necroses are often associated with wood rot caused by fungi colonising the stem following the initial infection by *H. fraxineus*.

Even though many fungi are reported from *F. excelsior* (981 different species according to the USDA website, Farr and Rossman 2022, retrieved on 17.06.2022) fungi associated with necrotic stem tissue of ash, specifically in Germany, have rarely been described (Enderle et al. 2017; Langer 2017; Meyn et al. 2019). Most frequently isolated from the necroses at the collar base of diseased ash trees were *Armillaria* spp., *Diaporthe eres* Nitschke, *Diplodia* spp., *Fusarium avenaceum* (Fr.) Sacc., *F. lateritium* Nees, *Fusarium solani* (Mart.) Sacc. (syn. *Neocosmospora solani* (Mart.) L. Lombard & Crous), *H. fraxineus*, and *Neonectria punicea* (J. C. Schmidt) Castl. & Rossman (Lygis et al. 2005; Langer 2017; Meyn et al. 2019; Linaldeddu et al. 2020). A strong evidence for *H. fraxineus* being the causal agent of ash dieback was given by Chandelier et al. (2016), who proved occurrence of *H. fraxineus* in the majority of symptomatic tissue of ash stem collar. Langer (2017) confirmed frequent isolation from stem collar necroses and the assignment as primary agent. But not every type of stem collar necrosis must be primarily caused by *H. fraxineus*. Langer (2017) showed that basal necroses can be caused by *Phytophthora* under special site conditions, as found in floodplain forests or by *Armillaria* spp. on weakened ash trees. The path of infection by *H. fraxineus* still remains unknown and little is understood about the influence of environmental factors on stem collar necroses. Site characteristics, such as moisture content, are assumed to affect disease severity. Kenigsvalde et al. (2010) and Marçais et al. (2016) determined that disease severity correlates positively with soil humidity conditions. It has been suggested that stem collar necroses development and extent is also related to moist conditions or humid topographical positions (Marçais et al. 2016). Therefore the design of this study covers a wide range of water supply types at sampling sites. The composition of the forest stands combined with their nutrient and water availability could be a factor in assessing differences in fungal diversity per stand. Most trees moderately and severely damaged due to ash dieback were observed at forest sites with a high soil organic matter content and a neutral to slightly alkaline soil pH (Turczański et al. 2019). Hence, it can be suspected that fungal composition depends on soil and water availability just as well (Linaldeddu et al. 2011; Salamon et al. 2020).

This study is conducted as part of the demonstration project FraxForFuture and the sub-network FraxPath (Langer et al. 2022). The aims of this research are to fill knowledge gaps concerning the α-diversity of cultivable *Dikarya* Hibbett, T. Y. James & Vilgalys associated with stem collar necroses of trees affected by ash dieback and the composition of their fungal communities. Therefore, fungi associated with necrotic stem bases of ash were isolated and identified from 58 ash trees in order to determine the continuity and the frequency of *H. fraxineus* and secondary fungi. The role of the most frequent fungi in the process of stem collar necroses formation is discussed.

2. Materials And Methods

2.1 Sampling sites

In total, six federal states of Germany (Lower Saxony, Schleswig-Holstein, Saxony, Saxony-Anhalt, Thuringia and North Rhine-Westphalia) were investigated. Nine mixed broad-leaved forest stands with a substantial share of *F. excelsior* affected by ash dieback were selected in order to cover different sites with a wide range of soil water supply types (Table 1, Fig. 1). The sample sites are located in northern, eastern, and central Germany with sub-oceanic to sub-continental temperate zones. All sites are eutrophic and cover the most common substrates of ash stands in Germany. Basic soil and geological data were acquired by using geological maps with a high resolution (scale 1:25000) of the respective federal geology departments and the forest inventory and forest

site mapping data sets of the federal forestry authorities. Additional data from soil core sampling, soil profiles and soil analyses were available for the sampling sites of Rhüden, Berggießhübel, Schwansee, and Schlangen because these sites are part of other studies associated with soil inventories: The site of Rhüden is part of the national forest soil inventory. Schwansee and Berggießhübel correspond to the intensive monitoring plots “TH_1 Schwansee” and “SN_2 Bienhof” of the research cluster FraxForFuture (Langer et al. 2022). The largest forest stand continuously including ash trees investigated in this study (Schlangen) has a pronounced relief and was divided into four subplots to investigate different positions in the terrain. In this case, intensive soil exploration were conducted to differentiate between the four subplots, including pedological assessments and soil sampling from soil profiles.

Table 1
Sampling site information sorted by sampling date; all forest sites are eutrophic.

Forest site (Coordinates UTM)	Sampling classification (sample number; sample date)	Meters above sea level	Exposition and Inclination in °	Climate	Soil water supply	Soil and bedrock	Mixture of tree species in stand
Oranienbaumer Heide (33 U 318931 5730457)	extensive sampling (1; 19.10.2020)	90	flat	sub-continental	slightly moist, fluctuating ground water regime	diluvial sands, boulder clay	*Quercus robur,* *Fagus sylvatica, Carpinus betulus, Betula pendula, Fraxinus excelsior*
Woltershausen (32 U 564973 5757894)	extensive sampling (2; 20.10.2020 and 04.03.2021)	289	SSE, moderate inclination (9°-18°)	weakly sub-atlantic	slightly moist	clayey loam over cretaceous limestone	*Fagus sylvatica,* *Acer pseudoplatanus,* *Fraxinus excelsior*
Wolfenbüttel (32 U 632092 5778288)	extensive sampling (1; 27.10.2020)	238	SE, slight inclination (0°-9°)	weakly sub-atlantic	moist, high water holding capacity	colluvial deposit over upper Muschelkalk (triassic limestone)	*Fagus sylvatica,* *Acer pseudoplatanus,* *Fraxinus excelsior*
Schwansee (32 U 646432 5660792)	intensive sampling (8; 10.11.2020)	163	flat	weakly sub-continental	gleysol, ground water influenced	carbonatic lake gravel deposit, lake sediment	*Fraxinus excelsior, Quercus robur, Populus nigra,* *European alder*
Rhüden (32 U 579914 5757111)	intensive sampling (10; 22.02.2021)	235	WSW, strong inclination (18°-27°)	weakly sub-atlantic	slightly dry, well drained, low water holding capacity	lower Muschelkalk, (triassic limestone) with shallow loess cover	*Fraxinus excelsior, Fagus sylvatica,* *Acer pseudoplatanus, Prunus avium*
Neuhege (32 U 593054 6010661)	extensive sampling (1; 08.06.2021)	53	ESE, slight inclination (0°-9°)	moderately sub-atlantic	stagnosol/gleysol, ground water influenced	marly till, boulder clay	*Quercus robur,* *Alnus glutinosa, Fraxinus excelsior, Fagus sylvatica*
Berggießhübel (33 U 426320 5629803)	intensive sampling (11; 01.07.2021)	475	NNW, slight inclination (0°-9°)	sub-continental, sub-montane	slightly moist, slope water influenced, slightly stagnic	basalt and gneiss solifluction soil with loamy loess cover	*Betula pendula,* *Acer pseudoplatanus,* *Fraxinus excelsior, Tilia cordata,* *Quercus robur,* *Prunus avium,* *Sorbus aucuparia*
Satrup (32 U 546373 6072699)	intensive sampling (5; 06.07.2021)	49	ESE, slight inclination (0°-9°)	moderately sub-atlantic	slightly moist, high water holding capacity, slightly stagnic	marly till, boulder clay	*Fagus sylvatica, Fraxinus excelsior*
Schlangen 1 (32 U 492453 5740809)	intensive sampling (3; 01.11.2021)	274	valley bottom, NNW, slight inclination (0°-9°)	moderately sub-atlantic	moist, high water holding capacity, slightly stagnic	colluvial deposit, silty loam	*Fagus sylvatica, Fraxinus excelsior, Acer pseudoplatanus, Prunus avium*
Schlangen 2 (32 U 492621 5741084)	intensive sampling (6; 08.11.2021)	299	upper slope, S, strong inclination (18°-27°)	moderately sub-atlantic	slightly dry, low water holding capacity	cretaceous limestone, very shallow loess cover (clayey loam)	*Fagus sylvatica, Fraxinus excelsior, Acer pseudoplatanus, Prunus avium*
Schlangen 3 (32 U 492515 5740880)	intensive sampling (4; 01.11.2021)	305	upper slope, NNW, slight inclination (0°-9°)	moderately sub-atlantic	slightly moist, moderate water holding capacity	cretaceous limestone, moderate loess cover (clayey loam)	*Fraxinus excelsior, Fagus sylvatica,* *Acer pseudoplatanus, Prunus avium*

Forest site (Coordinates UTM)	Sampling classification (sample number; sample date)	Meters above sea level	Exposition and Inclination in °	Climate	Soil water supply	Soil and bedrock	Mixture of tree species in stand
Schlangen 4 (32 U 492738 5741014)	intensive sampling (6; 01., 02., 08.11.2021)	316	hill top, W, slight inclination (0°-9°)	moderately sub-atlantic	slightly moist, moderate water holding capacity	cretaceous limestone, moderate loess cover (clayey loam)	*Fagus sylvatica,* *Acer pseudoplatanus, Fraxinus excelsior, Prunus avium*

The shallow sites (Rhüden, Schlangen 2) in exposed terrain positions on limestone with high coarse soil fractions and low water storage capacities represent the driest end of the ecological niche of ash. Several other sites have moderate (Schlangen 3, Schlangen 4, Wolterhausen) or high water storage capacities (Wolfenbüttel, Schlangen 1) because of medium to deep loamy loess covers or colluvial deposits. The remaining sites are primarily characterised by either stagnic soil conditions (Satrup, Berggießhübel), slope water influence (Berggießhübel) or groundwater influence (Oranienbaumer Heide, Schwansee, Neuhege). Soil substrate, water retention capacity, terrain relief, climate and presence/absence of groundwater or stagnic soil properties (Table 1) were combined to create a ranking of site water supplies of the sampling sites (Online Resource 1 and Online Resource 2).

Between one and nineteen trees were excavated and sampled per site. Hence, the sampling sites were divided into intensive and extensive sampling sites. The intensive sampling sites had a minimum of five and a maximum of 19 sample trees. Intensive sampling was conducted in Rhüden (10 sampled trees), Satrup (5), Berggießhübel (11), Schwansee (8), and Schlangen (19). Each intensive sampling site or subplot was of 0.2–0.5 ha in size. The extensive sampling sites with only one or two examined ash trees, were taken into account only for fungal occurrence: Woltershausen (2 sampled trees), Wolfenbüttel (1), Neuhege (1), and Oranienbaumer Heide (1). These individual trees were included to increase the sample set and the distribution of investigated stem collar necroses and their associated fungi.

2.2 Sampled trees

In total 58 ash trees were sampled, including six trees initially selected as control trees (two in Berggießhübel, one in Satrup, and three in Schlangen; Online Resource 3). The diameter at breast height of the sampled ash trees ranged from approximately 7–25 cm. The age of the sample trees ranged from 15 up to 80 years. The majority was approximately 40 years old. Classification of stem base and crown condition of the studied trees was carried out according to the guidelines of Peters et al. (2021b, a). Additionally, the neighbouring tree species occurring in the studied stands were noted (Table 1).

Ash trees were felled in the years 2020–2021 and cut at least 15 cm above the visible necrotic area. Subsequently, trunk bases and the uppermost parts of the main roots were dug out with picks and shovels. Depending on soil structure (rock content) final roots were cut by chainsaw with a .325" Rapid Duro 3 (RD3), 1.6 mm chainsaw chain (STIHL AG & Co. KG, Dieburg, Germany). The stem collars were transported to laboratory in clean and marked plastic bags.

2.2 Isolation of fungi

In preparation for fungal isolation, tree stems were cleaned with a coarse brush under tap water. After air-drying overnight, the samples were processed in the laboratory. Each sample was photographed for documentation. For surface sterilisation, the samples were sprayed with 70% ethanol all over the necrosis and wiped with a paper towel. Bark was removed from symptomatic stem areas at the transition zones of living and dead woody tissue. Depending on the thickness of the bark either a sterilised knife or a scalpel was used for removal of the bark. The exposed tissue was disinfected with ethanol again. For chipping of wood tissue samples a chisel and hammer were used. All tools were sterilised by flame shortly before each use. The number of wood chips taken from each tree varied depending on the size of the necrosis. Three of the 5–10 mm long wood chips were placed in a 90 mm petri dish containing malt yeast peptone (MYP) agar, modified according to Langer (1994) containing 0.7% malt extract (Merck, Darmstadt, Germany), 0.05% yeast extract (Fluka, Seelze, Germany), 0.1% peptone (Merck) and 1.5% agar (Fluka). Once the surface of necroses was processed, stem collars were cut longitudinally with a band saw and carefully sanded for better visualisation of the discolorations. The longitudinal sections were treated according to the isolation method for the surface of necrosis described above. Wood chips were taken at the edge of the necroses and at the transition areas of different discolorations. The process was repeated with the cross section of the stem (Fig. 2).

The petri dishes containing wood chips were incubated at room temperature under ambient daylight for four weeks. The cultures were checked for outgrowing mycelia once a week. Emerging mycelia of filamentous fungi were sub-cultured into pure cultures. The pure cultures were grouped into morphotypes (MT) based on similarity of cultural characteristics. At least one representative culture for each MT was stored in MYP slants at 4°C at the fungal culture collection of the Northwest German Forest Research Institute (NW-FVA). Beside the MT assignment, contaminated or overgrown fungi were summarised under “Fungus sp.”.

The frequency of each isolated fungal MT within all isolates (f_{MT}) was specified as the percentage of this particular MT in all outgrowing isolates. To measure the ratio of morphotype isolates to isolation attempts, the frequency of isolated fungal MTs in relation to the total amount of wood chips (f_{WC}) is used. Continuity of isolated MTs is defined as the number of sampled trees where the MT was detected in relation to the total number of sampled trees.

Analyses of the fungal diversity found in this study were conducted using RStudio (v. 4.1.2, R Core Team 2021). The packages used were tidyverse (Wickham et al. 2019), ggplot 2 (Wickham 2016) and ggVennDiagram (Gao 2021).

2.3 Molecular analysis

For molecular analysis, at least one representative strain from each MT was chosen. Mycelium was placed in 1.5 ml Eppendorf tubes with three glass beads (3 mm) and 150 µl of TE buffer (10 ml 1 mmol Tris HCl (pH 0.8), 2 ml 0.5 mmol EDTA; Carl Roth, Karlsruhe, Germany), and crushed in a Mixer Mill MM 200 (Retsch, Haan, Germany) with 25 vibrations per second for 90 seconds. Subsequently, genomic DNA was extracted following the protocol of Izumitsu et al. (2012).

The 5.8S nuclear ribosomal gene with the two flanking internal transcribed spacers ITS-1 and ITS-2 (ITS region) was amplified for all strains using the primer pair ITS-1F (Gardes and Bruns 1993) and ITS-4 (White et al. 1990). Additionally, for a selection of strains belonging to *Armillaria*, a partial sequence of the translation elongation factor 1α (*EF-1α*) was amplified using the primer pair EF595F + EF1160R (Kauserud and Schumacher 2001). The PCR mixture consisted of 1 µl of DNA and 19 µl mastermix which contained 2.5 µl 10x PCR reaction buffer (with 20 mM $MgCl_2$, Carl Roth, Karlsruhe, Germany), 1 µl of each primer (10 mmol), 2.5 µl $MgCl_2$ (25 mmol), 0.1 µl Roti®-Pol Taq HY Taq polymerase (Carl Roth, Karlsruhe, Germany) and 2.5 µl of 2 mmol dNTPs (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Each reaction was topped up to a volume of 20 µl by adding sterile water. A StepOnePlus™ PCR System (Applied Biosystems, Waltham, Massachusetts, US) was used to carry out the DNA amplifications. The PCR conditions for the amplification of the ITS and *EF-1α* regions were set according to Bien et al. (2020) and Guo et al. (2016), respectively. A 1% agarose gel was used to visualise the PCR products. The products were sent to Eurofins Scientific Laboratory (Ebersberg, Germany) for sequencing. Initially, PCR samples of the ITS region were sequenced using the forward reaction (primer ITS-1F). In case of imprecise results, additionally reverse reactions (primer ITS-4) were sequenced. PCR products of the *EF-1α* sequence region were sequenced by the respective forward and reverse reactions. All resulting sequences were visually checked and edited as follows using BioEdit Sequence Alignment Editor (v. 7.2.5; Hall 1999). Consensus sequences were generated, for all strains with forward and reverse sequences available. Defective sequence beginnings and ends were trimmed and erroneous nucleotide allocations corrected. Sequences were submitted to GenBank (Table 2).

Table 2

List of isolated fungi sorted alphabetically within orders; fungi causing wood rot are marked with *; probable first reports of species isolated from *F. excelsior* a
Basidiomycota. Datum Blast: 22./23.06.2022

Species	Division	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results
									Basis of identification
Acremonium sp.	A	*Hypocreales*	8031	OP023272	1	0.07%	2%	1	*Acremonium variecolor*
Akanthomyces sp.[FR]	A	*Hypocreales*	6958	OP023220	1	0.07%	2%	1	*Akanthomyces muscarius*
Alternaria infectoria	A	*Pleosporales*	5940	OP023162	3	0.20%	5%	2	*Alternaria infectoria*
Alternaria sp.	A	*Pleosporales*	6119	OP023191	5	0.33%	5%	2	*Alternaria angustiovoidea*
Angustimassarina sp. 1	A	*Pleosporales*	5951	OP023165	2	0.13%	3%	1	*Angustimassarina lonicerae*
Angustimassarina sp. 2	A	*Pleosporales*	6207	OP023203	4	0.26%	3%	2	*Angustimassarina lonicerae*
Armillaria spp.*	B	*Agaricales*	5952	OP023166	158	10.46%	50%	5	*Armillaria gallica* *Armillaria cepistipes*
Ascocoryne sp. 1*	A	*Helotiales*	7103	OP023244	3	0.20%	3%	2	*Ascocoryne* sp.
Ascocoryne sp. 2*	A	*Helotiales*	8030	OP023271	1	0.07%	2%	1	*Ascocoryne solitaria*
Aureobasidium pullulans	A	*Dothideales*	6176	OP023196	5	0.33%	7%	3	*Aureobasidium pullulans*
Beauveria bassiana	A	*Hypocreales*	6979	OP023247	2	0.13%	2%	1	*Beauveria bassiana*
Beauveria pseudobassiana	A	*Hypocreales*	6001	OP023182	1	0.07%	2%	1	*Beauveria pseudobassiana*
*Biscogniauxia nummularia**	A	*Xylariales*	5926	OP023156	3	0.20%	5%	1	*Biscogniauxia nummularia*
Bispora sp.*	A	*Incertae sedis*	8329	OP023300	1	0.07%	2%	1	*Bispora antennata*
*Bjerkandera adusta**	B	*Polyporales*	5943	OP023163	5	0.33%	9%	3	*Bjerkandera adusta*
Botrytis cf. *cinerea*	A	*Helotiales*	6874	OP023212	6	0.40%	9%	3	*Botrytis cinerea*
Cadophora dextrinospora [FR]	A	*Helotiales*	7026	OP023226	11	0.73%	10%	3	*Cadophora dextrinospora*
Cadophora melinii	A	*Helotiales*	6985	OP023248	1	0.07%	2%	1	*Cadophora melinii*
Cadophora ramosa [FR]	A	*Helotiales*	6194	OP023202	8	0.53%	7%	4	*Cadophora ramosa*
Cadophora sp. 1	A	*Helotiales*	7100	OP023258	3	0.20%	3%	2	*Cadophora malorum*
Cadophora sp. 2	A	*Helotiales*	8478	OP023311	1	0.07%	2%	1	*Cadophora malorum*
Calycina herbarum [FR]	A	*Helotiales*	8287	OP023287	1	0.07%	2%	1	*Calycina herbarum*
Camposporium sp.	A	*Incertae sedis*	8336	OP023307	1	0.07%	2%	1	*Camposporium ramosum*

Species	Division	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results
									Basis of identification
Cephalotrichiella penicillata FR	A	*Microascales*	7042	OP023232	1	0.07%	2%	1	*Cephalotrichiella penicillata*
Ceratobasidiaceae sp. 1	B	*Cantharellales*	8003	OP023265	2	0.13%	3%	1	*Ceratobasidium* sp.
Ceratobasidiaceae sp. 2	B	*Cantharellales*	8005	OP023267	1	0.07%	2%	1	*Ceratobasidiaceae* sp.
Chaetomiaceae sp.	A	*Sordariales*	8332	OP023303	1	0.07%	2%	1	*Chaetomium* sp.
Chaetomium globosum	A	*Sordariales*	8326	OP023297	1	0.07%	2%	1	*Chaetomium globosum*
Chaetomium sp.	A	*Sordariales*	8323	OP023295	1	0.07%	2%	1	*Chaetomium subaffine*
Chloridium virescens var. *caudigerum*	A	*Chaetosphaeriales*	8290	OP023290	6	0.40%	7%	1	*Chloridium virescens* var. c*audigerum*
Cladosporium sp.	A	*Capnodiales*	7029	OP023229	2	0.13%	3%	1	*Cladosporium iridis*
Clonostachys sp.	A	*Hypocreales*	5985	OP023174	1	0.07%	2%	1	*Clonostachys rosea f. catenulata*
Coniochaeta velutina FR	A	*Coniochaetales*	8334	OP023305	1	0.07%	2%	1	*Coniochaeta velutina*
Coprinellus cf. *domesticus**	B	*Agaricales*	6011	OP023184	1	0.07%	2%	1	*Coprinellus domesticus*
Coprinellus cf. r*adians* 1*	B	*Agaricales*	6962	OP023221	1	0.07%	2%	1	*Coprinellus radians*
Coprinellus cf. r*adians* 2*	B	*Agaricales*	8590	OP023312	1	0.07%	2%	1	*Coprinellus radians*
*Coprinellus disseminates**	B	*Agaricales*	5991	OP023177	12	0.79%	10%	2	*Coprinellus disseminatus*
*Coprinellus micaceus**	B	*Agaricales*	6048	OP023189	22	1.46%	19%	3	*Coprinellus micaceus*
Cordycipitaceae sp.	A	*Hypocreales*	6965	OP023223	1	0.07%	2%	1	*Samsoniella hepiali*
Cosmospora sp. 1	A	*Hypocreales*	7044	OP023234	1	0.07%	2%	1	*Cosmospora* sp.
Cosmospora sp. 2	A	*Hypocreales*	7095	OP023262	3	0.20%	5%	2	*Cosmospora lavitskiae*
*Cryptostroma corticale** FR	A	*Xylariales*	5932	OP023158	3	0.20%	5%	2	*Cryptostroma corticale*
Cyclothyriella rubronotata FR	A	*Pleosporales*	8333	OP023304	1	0.07%	2%	1	*Cyclothyriella rubronotata*
Diaporthe cf. *eres*	A	*Diaporthales*	5924	OP023155	58	3.84%	43%	7	*Diaporthe eres*
Diaporthe cf. *rudis*	A	*Diaporthales*	6131	OP023194	4	0.26%	7%	1	*Diaporthe rudis*
Didymella sp. 1	A	*Pleosporales*	5939	OP023161	1	0.07%	2%	1	*Didymella macrostoma*
Didymella sp. 2	A	*Pleosporales*	5982	OP023173	1	0.07%	2%	1	*Didymella* sp.
Didymella sp. 3	A	*Pleosporales*	6147	OP023193	1	0.07%	2%	1	*Didymella pinodella*
Didymella sp. 4	A	*Pleosporales*	6875	OP023213	1	0.07%	2%	1	*Didymella prosopidis*

Species	Division	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results
									Basis of identification
Didymellaceae sp.	A	*Pleosporales*	8289	OP023289	1	0.07%	2%	1	*Didymella* sp.
Diplodia fraxini	A	*Botryosphaeriales*	5921	OP023153	280	18.49%	71%	7	*Diplodia fraxini*
Diplodia sapinea FR	A	*Botryosphaeriales*	5979	OP023170	1	0.07%	2%	1	*Diplodia sapinea*
Geotrichum candidum FR	A	*Saccharomycetales*	6963	OP023222	4	0.26%	3%	1	*Geotrichum candidum*
Dothiorella sp.	A	*Botryosphaeriales*	5944	OP023164	1	0.07%	2%	1	*Dothiorella* sp.
Entoleuca sp.	A	*Xylariales*	6018	OP023187	2	0.13%	3%	1	*Entoleuca* sp.
Eutypa cf. *petrakii* var. *hederae*	A	*Xylariales*	5994	OP023179	5	0.33%	3%	1	*Eutypa petrakii* var. *hederae*
*Eutypa lata**	A	*Xylariales*	5937	OP023160	20	1.32%	3%	2	*Eutypa lata*
Exophiala sp.	A	*Chaetothyriales*	8390	OP023310	4	0.26%	7%	1	*Exophiala* sp.
*Flammulina velutipes**	B	*Agaricales*	8037	OP023274	4	0.26%	2%	1	*Flammulina velutipes*
*Fomitopsis betulina** FR	B	*Polyporales*	8324	OP023296	1	0.07%	2%	1	*Fomitopsis betulina*
*Fomitopsis pinicola**	B	*Polyporales*	7048	OP023253	1	0.07%	2%	1	*Fomitopsis pinicola*
Fusarium cf. *lateritium*	A	*Hypocreales*	5919	OP023151	53	3.50%	34%	6	*Fusarium lateritium*
Fusarium sambucinum	A	*Hypocreales*	6043	OP023188	1	0.07%	2%	1	*Fusarium sambucinum*
Fusarium solani	A	*Hypocreales*	6003	OP023183	15	0.99%	12%	2	*Fusarium solani*
Fusarium sp. 1	A	*Hypocreales*	5920	OP023152	6	0.40%	9%	2	*Fusarium iranicum*
Fusarium sp. 2	A	*Hypocreales*	5933	OP023159	1	0.07%	2%	1	*Fusarium sporotrichioides*
Fusarium stercicola	A	*Hypocreales*	7072	OP023275	1	0.07%	2%	1	*Fusarium stercicola*
Geotrichum sp.	A	*Saccharomycetales*	6955	OP023237	2	0.13%	3%	2	*Geotrichum* sp.
Gliomastix sp.	A	*Hypocreales*	6957	OP023219	2	0.13%	3%	1	*Gliomastix murorum* var. *felina*
Graphium sp.	A	*Microascales*	8007	OP023269	1	0.07%	2%	1	*Graphium* sp.
*Heterobasidion annosum**	B	*Russulales*	6111	OP023195	2	0.13%	3%	1	*Heterobasidion annosum*
Humicolopsis cephalosporioides FR	A	*Helotiales*	7045	OP023235	1	0.07%	2%	1	*Humicolopsis cephalosporioides*
Hymenoscyphus fraxineus	A	*Helotiales*	5922	OP023154	179	11.85%	53%	8	*Hymenoscyphus fraxineus*
Hypholoma cf. *acutum**	B	*Agaricales*	6345	OP023209	2	0.12%	3%	2	*Hypholoma fasciculare*
Hypocreales sp.	A	*Hypocreales*	6930	OP023217	1	0.07%	2%	1	*Albifimbria verrucaria*

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									Basis of identification
*Hypoxylon fragiforme**	A	*Xylariales*	6113	OP023192	4	0.26%	7%	2	*Hypoxylon fragiforme*
*Hypoxylon howeanum**	A	*Xylariales*	7046	OP023251	1	0.07%	2%	1	*Hypoxylon howeanum*
*Hypoxylon rubiginosum**	A	*Xylariales*	6051	OP023190	6	0.40%	7%	3	*Hypoxylon rubiginosum*
Ilyonectria sp. 1	A	*Hypocreales*	7051	OP023252	4	0.26%	7%	2	*Ilyonectria lusitanica*
Ilyonectria sp. 2	A	*Hypocreales*	7068	OP023255	1	0.07%	2%	1	*Ilyonectria liliigena*
Ilyonectria sp. 3	A	*Hypocreales*	8271	OP023281	1	0.07%	2%	1	*Ilyonectria protearum*
*Jackrogersella cohaerens**	A	*Xylariales*	8292	OP023292	3	0.20%	5%	1	*Jackrogersella cohaerens*
Jackrogersella sp.	A	*Xylariales*	6192	OP023200	4	0.26%	7%	3	*Jackrogersella* sp.
Juxtiphoma eupyrena	A	*Pleosporales*	8182	OP023280	2	0.13%	2%	1	*Juxtiphoma eupyrena*
Kalmusia sp.	A	*Pleosporales*	7032	OP023231	1	0.07%	2%	1	*Kalmusia longispora*
*Kuehneromyces mutabilis**	B	*Agaricales*	5988	OP023176	1	0.07%	2%	1	*Kuehneromyces mutabilis*
Lasionectria sp.	A	*Hypocreales*	6191	OP023199	1	0.07%	2%	1	*Lasionectria vulpina*
Laxitextum bicolor	B	*Russulales*	8006	OP023268	1	0.07%	2%	1	*Laxitextum bicolor*
Lepteutypa fuckelii	A	*Amphisphaeriales*	8284	OP023284	1	0.07%	2%	1	*Lepteutypa fuckelii*
Leptodontidium sp.	A	*Helotiales*	8338	OP023309	1	0.07%	2%	1	*Leptodontidium* sp.
Leptosillia muelleri FR	A	*Xylariales*	6208	OP023204	2	0.13%	3%	2	*Leptosillia muelleri*
Lophiotrema rubi FR	A	*Pleosporales*	8327	OP023298	1	0.07%	2%	1	*Lophiotrema rubi*
Lophiotrema sp.	A	*Pleosporales*	8337	OP023308	1	0.07%	2%	1	*Lophiotrema mucilaginosis*
Metapochonia bulbillosa FR	A	*Hypocreales*	6876	OP023214	2	0.13%	3%	2	*Metapochonia bulbillosa*
Metapochonia suchlasporia FR	A	*Hypocreales*	8272	OP023282	1	0.07%	2%	1	*Metapochonia suchlasporia*
Microcera cf. *larvarum*	A	*Hypocreales*	6195	OP023210	3	0.20%	5%	3	*Microcera larvarum*
Microcera rubra FR	A	*Hypocreales*	7028	OP023228	1	0.07%	2%	1	*Microcera rubra*
Microsphaeropsis olivacea	A	*Pleosporales*	7039	OP023250	1	0.07%	2%	1	*Microsphaeropsis olivacea*
*Mycoacia nothofagi** FR	B	*Polyporales*	8043	OP023278	1	0.07%	2%	1	*Mycoacia nothofagi*
Nectriaceae sp. 1	A	*Hypocreales*	5987	OP023175	3	0.20%	5%	2	*Cylindrocarpon* sp.
Nectriaceae sp. 2	A	*Hypocreales*	8286	OP023286	2	0.13%	3%	1	*Dialonectria ullevolea*
*Nemania serpens**	A	*Xylariales*	6193	OP023201	6	0.40%	9%	3	*Nemania serpens*

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Neoascochyta sp.	A	*Pleosporales*	6968	OP023225	3	0.20%	5%	2	*Neoascochyta exitialis*
Neobulgaria sp.*	A	*Helotiales*	8331	OP023302	1	0.07%	2%	1	*Neobulgaria* sp.
Neocucurbitaria acerina FR	A	*Pleosporales*	5981	OP023172	1	0.07%	2%	1	*Neocucurbitaria acerina*
Neocucurbitaria sp.	A	*Pleosporales*	7031	OP023230	1	0.07%	2%	1	*Neocucurbitaria cava*
Neofabraea sp.	A	*Helotiales*	6125	OP023198	8	0.53%	9%	4	*Neofabraea kienholzii*
Neonectria punicea	A	*Hypocreales*	5980	OP023171	123	8.14%	33%	6	*Neonectria punicea*
Neopyrenochaeta acicola FR	A	*Pleosporales*	8328	OP023299	1	0.07%	2%	1	*Neopyrenochaeta acicola*
Neopyrenochaeta sp.	A	*Pleosporales*	8293	OP023293	2	0.13%	2%	1	*Neopyrenochaeta fragariae*
Neosetophoma sp.	A	*Pleosporales*	5928	OP023157	1	0.07%	2%	1	*Neosetophoma rosigena*
Nigrograna mycophila	A	*Pleosporales*	8330	OP023301	1	0.07%	2%	1	*Nigrograna mycophila*
*Obolarina dryophila** FR	A	*Xylariales*	6324	OP023207	1	0.07%	2%	1	*Obolarina dryophila*
Oliveonia sp.	B	*Cantharellales*	8004	OP023266	1	0.07%	2%	1	*Oliveonia* sp.
Paracucurbitaria sp.	A	*Pleosporales*	5999	OP023181	31	2.05%	34%	6	*Paracucurbitaria corni*
Paraphaeosphaeria neglecta	A	*Pleosporales*	6014	OP023186	5	0.33%	7%	3	*Paraphaeosphaeria neglecta*
Penicillium daleae FR	A	*Eurotiales*	6931	OP023218	2	0.13%	3%	1	*Penicillium daleae*
Peniophora cf. *cinerea* 1*	B	*Russulales*	6180	OP023208	5	0.33%	7%	3	*Peniophora cinerea*
Peniophora cf. *cinerea* 2*	B	*Russulales*	8039	OP023277	1	0.07%	2%	1	*Peniophora cinerea*
Peniophora cf. *incarnata**	B	*Russulales*	7034	OP023249	1	0.07%	2%	1	*Peniophora incarnata*
*Peniophora laeta** FR	B	*Russulales*	8036	OP023273	1	0.07%	2%	1	*Peniophora laeta*
*Peniophora lycii**	B	*Russulales*	5995	OP023180	19	1.26%	10%	3	*Peniophora lycii*
*Peniophora quercina** FR	B	*Russulales*	8044	OP023279	1	0.07%	2%	1	*Peniophora quercina*
*Peniophora rufomarginata** FR	B	*Russulales*	7027	OP023227	1	0.07%	2%	1	*Peniophora rufomarginata*
Peniophora sp. 1*	B	*Russulales*	6927	OP023215	1	0.07%	2%	1	*Peniophora lycii*
Peniophora sp. 2*	B	*Russulales*	7226	OP023263	1	0.07%	2%	1	*Peniophora lycii*
Peniophora sp. 3*	B	*Russulales*	8038	OP023276	1	0.07%	2%	1	*Peniophora lycii*
Pezicula cf. *sporulosa*	A	*Helotiales*	7104	OP023168	5	0.33%	3%	1	*Pezicula sporulosa*
Pezicula cf. *rostrupii*	A	*Helotiales*	5976	OP023245	1	0.07%	2%	1	*Pezicula rostrupii*

Species	Division	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results
									Basis of identification
Pezicula sp. 1	A	*Helotiales*	6112	OP023205	1	0.07%	2%	1	*Pezicula radicicola*
Pezicula sp. 2	A	*Helotiales*	7154	OP023260	4	0.26%	3%	2	*Pezicula* sp.
Pezicula sp. 3	A	*Helotiales*	7060	OP023240	1	0.07%	2%	1	*Pezicula ericae*
Phaeosphaeria sp.	A	*Pleosporales*	7321	OP023264	1	0.07%	2%	1	*Phaeosphaeria glyceriae-plicatae*
Phanerochaete sordida s. lat. Gruppe*	B	*Polyporales*	7052	OP023254	1	0.07%	2%	1	*Phanerochaete sordida*
Phialocephala fortinii FR	A	*Helotiales*	7056	OP023238	1	0.07%	2%	1	*Phialocephala fortinii*
Phialocephala piceae FR	A	*Helotiales*	7038	OP023216	8	0.53%	9%	2	*Phialocephala piceae*
Phialocephala sp.	A	*Helotiales*	6952	OP023236	1	0.07%	2%	1	*Phialocephala oblonga*
*Phlebia radiata**	B	*Polyporales*	6013	OP023185	3	0.20%	5%	2	*Phlebia radiata*
Pleosporales sp.	A	*Pleosporales*	8335	OP023306	1	0.07%	2%	1	*Phoma herbarum*
Pseudeurotiaceae sp. 1	A	*Thelebolales*	6966	OP023224	1	0.07%	2%	1	*Geomyces asperulatus*
Pseudeurotiaceae sp. 2	A	*Thelebolales*	7152	OP023246	1	0.07%	2%	1	*Pseudogymnoascus pannorum*
Pseudogymnoascus sp.	A	*Thelebolales*	6432	OP023211	1	0.07%	2%	1	*Pseudogymnoascus appendiculatus*
Pseudopithomyces chartarum FR	A	*Pleosporales*	8294	OP023294	1	0.07%	2%	1	*Pseudopithomyces chartarum*
Ramularia sp.	A	*Mycosphaerellales*	7043	OP023233	1	0.07%	2%	1	*Ramularia collo-cygni*
Rhexocercosporidium sp.	A	*Helotiales*	7097	OP023257	1	0.07%	2%	1	*Rhexocercosporidium* sp.
Rhizoctonia fusispora	B	*Cantharellales*	7057	OP023239	1	0.07%	2%	1	*Rhizoctonia fusispora*
Sarocladium cf. *dejongiae*	A	*Hypocreales*	7061	OP023241	1	0.07%	2%	1	*Sarocladium dejongiae*
*Schizopora paradoxa**	B	*Hymenochaetales*	7155	OP023261	1	0.07%	2%	1	*Schizopora paradoxa*
Sclerostagonospora cycadis	A	*Pleosporales*	8285	OP023285	1	0.07%	2%	1	*Sclerostagonospora cycadis*
*Scytalidium album** FR	A	*Helotiales*	6322	OP023206	1	0.07%	2%	1	*Scytalidium album*
*Scytalidium lignicola**	A	*Helotiales*	7062	OP023242	1	0.07%	2%	1	*Scytalidium lignicola*
*Sistotrema oblongisporum** FR	B	*Cantharellales*	8008	OP023270	3	0.20%	3%	1	*Sistotrema oblongisporum*
Sordariales sp.	A	*Sordariales*	7071	OP023256	1	0.07%	2%	1	*Podospora tetraspora*
Sporothrix sp.	A	*Ophiostomatales*	8291	OP023291	1	0,07%	2%	1	*Sporothrix* sp.
Steccherinum sp.*	B	*Polyporales*	8283	OP023283	1	0,07%	2%	1	*Steccherinum bourdotii*

Species	Division	Order	NW-FVA ID	ITS Accession no.	n isolates	Frequency (%)	Continuity (%)	Sites isolated from	ITS NCBI Blast results
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Stereum sp.*	B	*Russulales*	5970	OP023167	6	0,40%	5%	2	*Stereum armeniacum*
Thelonectria sp.	A	*Hypocreales*	5993	OP023178	1	0,07%	2%	1	*Thelonectria* sp.
Thysanophora penicillioides FR	A	*Eurotiales*	7063	OP023243	1	0,07%	2%	1	*Thysanophora penicillioides*
*Trametes versicolor**	B	*Polyporales*	6137	OP023197	5	0,33%	9%	2	*Trametes versicolor*
Vexillomyces sp. FR	A	*Leotiales*	7102	ON809459	1	0.07%	2%	1	*Vexillomyces verruculosus*
*Xylaria longipes**	A	*Xylariales*	8288	OP023288	1	0,07%	2%	1	*Xylaria longipes*
*Xylaria polymorpha**	A	*Xylariales*	5978	OP023169	7	0,46%	12%	3	*Xylaria polymorpha*

2.4 Identification of fungi

Only cultivatable Dikarya fungi were investigated, however, yeasts were not taken into account. Only isolates which are clearly growing from the wood chips were determined. Obvious contamination with filamentous fungi was not considered. The genus *Trichoderma* was not included in the analyses, because it is difficult to assess whether these very fast growing fungi were contaminations or real outgrowth from the wood.

MTs were assigned to fungal taxa based on morphological observation and molecular analysis of representative strains following the method of Guo et al. (2000). For fungal taxon determination blastn searches based on ITS sequences were conducted on the GenBank database (http://www.ncbi.nlm.nih.gov/genbank, Altschul et al. 1997) excluding uncultured/environmental sample sequences from the search. Results were critically interpreted with emphasis on well-curated culture collections, such as the Westerdijk Fungal Biodiversity Collection (CBS). In general, blastn results on a species level below a threshold of 98% identity were not trusted to be accurate enough for final determination. In case no definite affiliation was possible to a specific taxonomic level the identification was marked by cf. (confer) to indicate uncertainties. The results were re-checked against literature and previously identified cultures from the institute's collection for confirmation. Additionally to blastn searches, extended analyses for taxon determination on a species level were conducted for isolates belonging to *Diplodia* and *Armillaria* due to the considerable number of isolates from these genera. Phylogenetic analyses were conducted based on an ITS sequence-dataset and an ITS-*EF-1α* concatenated sequence-dataset for *Diplodia* and *Armillaria* isolates, respectively, including appropriate reference sequences retrieved from GenBank. Both analyses were performed using RAxML v. 8.2.11 (Stamatakis 2006, 2014) as implemented in Geneious R11 (Kearse et al. 2012) using the GTRGAMMA model with the rapid bootstrapping and search for best scoring ML tree algorithm including 1,000 bootstrap replicates (Online Resource 4 and 5).

As a rule, current names were applied according to the nomenclatorial database MycoBank (Robert et al. 2005). Two exceptions from this generally applied rule have been made in the case of the MTs designated here as *Fusarium solani* s. l. and *Armillaria gallica*. In the case of *F. solani* the authors are aware that there is a currently unsettled discussion about correct delimitation of this species, or rather species complex. Here we follow the "classic" nomenclature substantiated by Geiser et al. (2021) obverse that promoted by Lombard et al. (2015) and Sandoval-Denis et al. (2019) who place said species complex in the genus *Neocosmospora* (*N. solani* (Mart.) L. Lombard & Crous). The currently applied name for *A. gallica*, according to MycoBank is *A. lutea* Gillet. However, Marxmüller (1992) stated that *A. lutea* is a nomen ambiguum and the later introduced name *A. gallica* Marxm. & Romagn. is to be used (Burdsall and Volk 1993).

3. Results

3.1 Sampled trees

The crown health status regarding ash dieback of the sampled trees ranged from vital and nearly without dieback symptoms to almost dead. All sampled trees (Online Resource 3), except for the six planed control trees, had obvious stem collar necroses through discoloured, sunken, and in some cases ruptured bark. In cases of ruptured bark, subjacent stem collars were neither completely rotting nor dead. During processing of the sample trees in the laboratory two out of the six control trees (tree 32 and 42, Online Resource 3) showed necrotic woody tissue inside the stem base. Consequently, these two trees were transferred to and analysed as sample trees (n = 54).

3.2 Isolated Fungi

In total, 4,401 wood chips of stem collar tissue originating from 58 trees were incubated. 1,511 mycelial outgrows (from which 226 were not identifiable due to contaminations; marked as Fungus sp.) from 1,413 wood chips (32%) were observed. 958 chips (22%) showed no outgrowth at all after four weeks of incubation, while 960 (22%) chips had been overgrown by fast growing fungi from adjacent wood chips before outgrowth could be recognised. The remaining 1,070 (24%) wood chips were colonised or contaminated by yeasts, mould or fungi which do not belong to Dikarya. The resulting pure culture isolates were assigned to 162 MTs (excluding *Trichoderma* spp.) and all but ten could at least be identified to a genus level. 89 isolates could be identified to a species level (Table 2).

The majority of the isolated filamentous fungi from all samples were *Ascomycota* (132 MTs, 77.8%), 36 MTs (22.2%) belonged to the division of *Basidiomycota.* Within the *Ascomycota*, the most frequently observed orders (Fig. 3) were *Hypocreales* (23.0%) followed by *Pleosporales* (22.2%), and *Helotiales* (19.8%). The *Basidiomycota* fungi were mainly represented by *Russulales* (36.1%), *Agaricales* (25.0%), and *Polyporales* (22.2%).

Despite the diversity of 162 detected fungal MTs, only a few fungi occurred with high f_{MT}. 67 MTs (41%) were isolated more than once and from these only 13 fungi (8%) were obtained ten or more times. The remaining 95 fungi (59%) were only isolated once. Between one and 27 different fungi were found per stem collar. On average, nine MTs were recorded on each tree. None of the identified fungi were found at all sites (including extensive sampling sites) and merely 46 MTs (28%) were found at more than one site. In total, 116 fungi (72%) were found solely at one site (the four subplots of Schlangen are considered as one site).

Although morphologically similar to *Diplodia mutila* (Fr.) Mont., in the phylogenetic analysis based on ITS sequences, the majority of *Diplodia* isolates from this study are placed clearly within a clade of strains of *Diplodia fraxini* (Fr.) Fr, including its ex-neotype (Online Resource 4). One strain from this study (NW-FVA 5979) is placed within a clade formed by strains of *Diplodia sapinea* (Fr.) Fuckel including its ex-epitype strain and the ex-type strain of the synonymised *Diplodia intermedia*. The ITS sequences of this clade differ by one nucleotide, however, the ITS sequence of NW-FVA 5979 is identical to that of the ex-epitype strain of *D. sapinea*. Preliminary morphological observation of strains of *Armillaria* indicated affiliation to *A. gallica*. However, *A. gallica* cannot be clearly distinguished morphologically from *Armillaria cepistipes* Velen. (Tsopelas 1999). Of the 35 isolates included in the phylogenetic analysis of *Armillaria*, 33 isolates are placed within a clade containing reference strains of *A. gallica*. The two remaining isolates are placed within a clade of *A. cepistipes* (Online Resource 5). Since only a selection of *Armillaria* isolates was included in the phylogenetic analysis due to limited lab resources, a clear distinction could not be made for all isolates of this genus. Hence, isolates of *Armillaria* are combined and referred to as *Armillaria* spp. in the final assessment of this study.

The most frequent MTs isolated were *D. fraxini* (21.8%), *H. fraxineus* (13.9%), *Armillaria* spp. (12.3%), and *N. punicea* (9.6%), which account for nearly half of the isolated fungi. Perithecia of *N. punicea* were frequently observed on the ash bark above the necrotic lesions. All other isolated fungi were less frequent with < 4% proportion. The most abundant MTs in respect to continuity beside the aforementioned *D. fraxini* (71% continuity), *H. fraxineus* (53%), *Armillaria* spp. (50%), and *N. punicea* (33%), were *Diaporthe* cf. *eres* (43% continuity / 3.8% f_{MT}), *Fusarium* cf. *lateritium* (34% / 3.5%), and *Paracucurbitaria* sp. (34% / 2.1%). These MTs were also most abundant in regard to occurrence at the nine sample sites (*H. fraxineus* occurs at eight sites, *Diaporthe* cf. *eres* and *D. fraxini* at seven sites, *Fusarium* cf. *lateritium*, *N. punicea*, and *Paracucurbitaria* sp. at six sites). When taking into account only the intensive sampling sites with at least five stem collars studied, there is an overlap of five occurring fungi: *Diaporthe* cf. *eres*, *D. fraxini*, *H. fraxineus*, *N. punicea*, and *Paracucurbitaria* sp.

The ash dieback pathogen *H. fraxineus* was isolated from 31 of the 54 stem collar necroses (57%). It was isolated at all studied sites except at Wolfenbüttel, where only a single tree with stem collar necrosis was sampled. The fungus was detected in both, young and advanced necroses, but not in the four control samples without symptomatic tissue. *Hymenoscyphus fraxineus* was isolated less frequently at the investigated sites with better water supply (soil water supply and climate combined; Online Resource 2).

147 MTs (91%) were only isolated from necrotic stem tissue. Almost one-third of all MTs according to their identification are able to decay wood (Table 2). A significant proportion of the isolated species were found here for the first time associated with *F. excelsior*, for example: three isolates from necrotic stem collar tissue were assigned to the MT identified as *Cryptostroma corticale* (Ellis & Everh.) P.H. Greg. & S. Waller and one isolate from stem collar tissue was identified as *D. sapinea*. The isolation of *Paracucurbitaria* sp. from the examined samples is the first proof of this genus from stem collar necroses of *F. excelsior*. Furthermore, one isolate from necrotic stem collar tissue was preliminarily assigned to the genus *Vexillomyces* S. Bien, C. Kraus & Damm based on ITS sequence comparison. Further morphologic as well as multi-locus phylogenetic investigations based on additional DNA regions (ribosomal large subunit, *EF-1α* and a 200-bp intron of the glyceraldehyde-3-phosphate dehydrogenase) revealed this isolate to represent a novel yet undescribed species of *Vexillomyces* (Tan et al. 2022). The MTs, which only occurred once in asymptomatic control samples were assigned to *Akanthomyces* sp., *Cephalotrichiella penicillata* Crous, and *Sclerostagonospora cycadis* Crous & G. Okada. The following fungi were isolated from stem collar necroses as well as from symptomless controls: *Alternaria infectoria* E.G. Simmons, *Alternaria* sp., *Diaporthe* cf. *eres*, *Exophiala* sp., *Hypoxylon fragiforme* E.G. Simmons, *Jackrogersella* sp., *Paracucurbitaria* sp., *Peniophora* sp. 1 and sp. 2, *Phaeosphaeria* sp., *Pseudeurotiaceae* sp., and *Sistotrema oblongisporum* M.P. Christ. & Hauerslev.

3.4 Fungal communities in stem collar necroses

The number of MTs isolated from stem collar necroses at the different sites ranged from one (Wolfenbüttel, one sample tree) to ten (Woltershausen, two sample trees) at the extensive sites and from 29 (Schwansee, eight sample trees) to 87 (Schlangen, 19 sample trees) at the intensive sites. Hence, the fungal communities at the sites studied differed in their species composition and diversity (Fig. 4). In Schlangen 52 MTs (32% of all 162 isolated MTs of this study) out of 87 MTs were found exclusively at this site. Nevertheless, the most common MTs in Schlangen are identical with the most frequent MTs over all sampling sites (*Armillaria* spp., *D. fraxini*, *H. fraxineus*, and *N. punicea*). *Diplodia fraxini* was isolated from almost 80% and *H. fraxineus* from almost 70% of the trees studied at this site. 54 MTs were found in the samples from the site of Berggießhübel and 30 (19%) of those occurred exclusively at this site. Likewise, the most common MTs at Berggießhübel were *D. fraxini*, *H. fraxineus*, and *Armillaria* spp. However, *N. punicea* was isolated only once there. At the Rhüden study site, 32 MTs were found, 13 (8%) of which were found exclusively at this site. The most frequent MTs were the same as the most abundant MTs in respect to continuity from all samples - except *N. punicea*, which was isolated only once in Rhüden. 31 MTs were found in the Satrup samples, 12 of which (8%) exclusively at this site. The most common MTs in Satrup were identical with the four most common MTs in Schlangen. At Schwansee, 29 fungal MTs were isolated in total, 12 of which (8%) were found exclusively at this site. At this location the most common MTs besides *D. fraxini* and *N. punicea* were *Coprinellus* species and *F. solani* s. l.

4. Discussion

4.1 Fungi associated with ash stem collars

In total, 162 fungi were isolated from ash stem collars and differentiated within this study. About half of these fungi were isolated from stem collar necroses in comparable studies as well (Lygis et al. 2005; Langer 2017; Meyn et al. 2019; Kranjec Orlović et al. 2020). Though 87 taxa (54%) isolated here were not reported by the aforementioned studies. It has to be taken into account that different samples sizes and sample site numbers lead to differing numbers of species. Considering the mentioned studies, Meyn et al. (2019) had the smallest sample size with four trees at one sample site and reported the smallest diversity with 16 fungal species. Langer (2017) isolated more fungal species (35) from 32 sample trees at seven sample sites. The correlation between sampling size and reported fungal diversity is also confirmed by Kranjec Orlović et al. (2020) with 68 fungal species isolated from 90 sample trees examined at three sample sites. The non-negligible impact of the number of sample sites on the detectable fungal species diversity is shown in this study with 162 MTs isolated from a smaller sample size (58 trees), but a higher number of sample sites (9) compared to the study of Kranjec Orlović et al. (2020). Other factors, such as number of incubated wood chips or the isolation method can also have influence on the number of isolated species. But overall, there seems to be a positive correlation between sample size or number of sampled sites and species richness. Langer (2017) observed that, advanced of stem collar necroses result in a higher number of isolated species. This could be confirmed in the present study when taking into account only the stem collar necroses with isolation of *H. fraxineus*.

Similar to studies focusing on endophytes of tree woody tissues (Bußkamp et al. 2020; Langer et al. 2021) in this study the majority of fungi isolated belong to Ascomycota (77.8%). A reason for the lower frequencies of *Basidiomycota* compared to *Ascomycota* might be that fungi belonging to the former often need longer incubation periods in order to grow out from incubated woody tissues (Oses et al. 2008) but since the incubated increment segments were kept for four weeks on nutrient media, it has to be assumed that enough time was given for fungi to grow out. However, the proportion of Basidiomycota (22.2%) in this study is higher than in the aforementioned studies. The reason for this discrepancy might be the focus on different woody tissue types in the mentioned studies, and hence detection of differing fungal communities with divergent ecological functions. Basidiomycota isolated from woody tissues are often related to wood rot, because lignin is primarily decomposed by this fungal group and therefore they are more likely to be found in diseased or necrotic rather than asymptomatic woody tissue (Eriksson et al. 1990; Bugg et al. 2011). Hence, the occurrence of white and brown rot fungi in stem collar necroses is not unusual. Typical white rot fungi like *Armillaria* spp., *Coprinellus* spp., *Bjerkandera adusta* (Willd.) P. Karst., *Peniophora* spp., *Trametes versicolor* (L.) Lloyd, and few brown rot fungi like *Fomitopsis* spp. have been isolated from stem collar necroses in this study. The majority of soft rot fungi isolated here pertain to Ascomycota and the following representatives of this group were found: *Biscogniauxia nummularia* (Bull.) Kuntze, *Hypoxylon* spp., *Jackrogersella* sp., *Nemania serpens* (Pers.) Gray, and *Xylaria* spp. (all *Xylariales*). Beside the occurrence of white and brown rot fungi the frequent association of xylarialean wood decay fungi with stem collar necroses make it plausible that affected ash trees have a massive loss of stability and tend to topple over even without wind as a supporting factor.

Approximately one-third of the isolated MTs detected in this study were listed for *F. excelsior* in the USDA fungal database (Farr and Rossman 2022). Only one of the most frequent MTs of this study, *N. punicea*, is not listed there, but other species from the genus *Neonectria* are mentioned. However, *N. punicea* was described as one of the most frequent MTs associated with stem collar necroses in the context of ash dieback (Langer 2017; Meyn et al. 2019). The other most abundant MTs isolated in our study *Armillaria* spp., *D. fraxini*, *H. fraxineus*, *Diaporthe* cf. *eres*, *Fusarium* cf. *lateritium*, and *Paracucurbitaria* sp. were already described to be associated with ash (Chandelier et al. 2016; Haňáčková et al. 2017; Langer 2017; Meyn et al. 2019; Linaldeddu et al. 2020; Kowalski and Bilański 2021; Barta et al. 2022). Langer (2017) investigated stem collar necroses of 32 ash trees and determined the aforementioned species as well – except for *Paracucurbitaria* sp. Meyn et al. (2019) isolated *D. fraxini* (labelled as *Botryosphaeria stevensii)*, *H. fraxineus*, *N. punicea*, and *Diaporthe* cf. *eres* as well. The most commonly isolated species from stem collar necroses in the present study, except *Armillaria* sp. and *Paracucurbitaria* sp., were also isolated in high frequency by Linaldeddu et al. (2020), although they focussed on symptomatic branches of damaged ash trees in Italy. The absence of *Armillaria* sp. in branches was anticipated, because it is a soil-borne root and stem rot fungus, colonising its host through rhizomorph growth (Morrison 2004).

In this study, isolates belonging to the genus *Armillaria* were among the most consistently detected MTs, where *A. gallica* was detected more frequently. Additionally to our isolations from stem collar necroses, mycelial fans and rhizomorphs of *Armillaria* spp. were observed at all sample sites of this study and the majority of further studied ash stands diseased by ash dieback. *Armillaria* species are common soil colonisers in Europe and therefore are probably existing in most forest sites even before ash dieback occurs (Morrison 2004; Lygis et al. 2005; Bakys et al. 2009b). They are considered as secondary pathogens and wood decaying fungi infecting stressed trees, which explains their occurrence in advanced stem necroses and root rot (Chandelier et al. 2016). On the one hand, *Armillaria* spp. can colonise stem collars after the necrosis has already formed by *H. fraxineus*. On the other hand, they are also able to independently attack a weakened ash tree without a stem collar necroses due to *H. fraxineus* (Langer 2017). As in our study, the occurrence of *A. gallica* and *A. cepistipes* associated with stem collar necroses of trees affected by ash dieback has been shown by Chandelier et al. (2016) in Belgium. Enderle et al. (2017) also detected *A. gallica* in stem collar rots in south-western Germany. These results are in contrast to investigations by Lygis et al. (2005), who determined *A. cepistipes* as most frequent in Lithuania. The different distribution of the latter two *Armillaria* species can be explained with the sampling in different regions of Europe and varying site characteristics and altitudes. The distribution of *A. cepistipes* appears to correspond with an inverse relationship between latitude and altitude (Guillaumin et al. 1993). For example, *A. cepistipes* occurred in low altitudes at northern latitudes and in higher altitudes at southern latitudes. However both species are widely distributed and common in Europe (Legrand et al. 1996; Marxmüller and Holdenrieder 2000). In the south of Germany (Bavaria) *A. cepistipes* tends to occur at higher altitudes (montane) and also colonises conifers, whereas *A. gallica* was not collected at sites above 600 m.a.s.l. (meters above sea level) (Marxmüller and Holdenrieder 2000). According to (Tsopelas 1999), *A. cepistipes* occurred more often in higher altitudes, whereas *A. gallica* is predominant in beech forests. In this study, *A. cepistipes* was present for forest sites at approx. 300 m.a.s.l. consisting of mixed stand compositions with European beech (Schlangen 1 and Schlangen 4). The occurrence of *A. cepistipes* at both low and high altitudes (up to 1750 m.a.s.l.) in Germany, is probably related to the fact that there are also regions with continental climate with relatively cold winters over a wide range of altitudes (Guillaumin et al. 1993). According to field observations by Guillaumin et al. (1993) the pathogenicity of *A. cepistipes* is lower than that of *A. gallica*, which is a

common weak parasite of hardwoods. Nevertheless, regardless of which of the two species caused infection, *Armillaria* spp. most likely accelerate the decline of ash dieback affected ash trees (Chandelier et al. 2016) and reduce stem stability.

The most frequently isolated species in our study *D. fraxini* has been recognised as the dominant species in comparable studies as well. Linaldeddu et al. (2020) determined that many reports of *D. fraxini* on ash have earlier been assigned to *D. mutila* s. l. Phylogenetic analyses showed, that most of the *Diplodia* strains isolated in this study, although morphologically similar to *D. mutila*, certainly match with *D. fraxini*. It is an aggressive pathogen known to cause bark lesions and wood discoloration or to enlarge necroses, which are primarily caused by *H. fraxineus* (Alves et al. 2014; Linaldeddu et al. 2020, 2022). Kowalski et al. (2017) classified it as the second most pathogenic fungus after *H. fraxineus*, though it was not mentioned as a frequent coloniser of *F. excelsior* before ash dieback disease occurred (Kowalski et al. 2016). These facts might indicate that infections with *H. fraxineus* facilitate the colonisation of affected ash trees by *D. fraxini*. Another possible explanation for the more frequent occurrence of *D. fraxini* could be global warming because this species benefits from warm temperatures of around 25° C (Alves et al. 2014). In our opinion, *D. fraxini* plays an important role in ash dieback disease and contributes undoubtedly to a greater damage extent, in particular at stem collar necroses. Beside the latter very frequent *Diplodia* species, to the knowledge of the authors, this is the first report of *D. sapinea* on ash. In contrast to the study by Linaldeddu et al. (2020), the species *D. subglobosa* could not be isolated in our analysis, maybe because they investigated branches and not stem collar necroses.

Neonectria punicea has a large host spectrum, including *F. excelsior* (Hirooka et al. 2013). However, this fungus has rarely been documented from this particular host species before (Langer 2017; Meyn et al. 2019; Karadžić et al. 2020). *N. punicea* was found to be associated with stem collar necroses and cankers of European ash in Germany (Langer 2017; Meyn et al. 2019) and it is able to cause necroses in juvenile ash trees (Karadžić et al. 2020). Its perithecia were observed frequently on the bark above the necrotic ash tissue (ibid. and Karadžić et al. 2020). *Neonectria punicea* is mainly known to be a secondary pathogen, but can also express an endophytic lifestyle (Langer 2017). Species of the genus *Neonectria* invade through natural entrances, like lenticels or artificial wounds, for infection (Flack and Swinburne 1977; Salgado-Salazar and Crouch 2019).

The isolation of strains assigned to *Diaporthe* cf. *eres* were made from diseased and also from healthy woody ash tissue in this study. This is in agreement with insights that *Diaporthe eres* can live as a plant pathogen, endophyte or saprotroph and has a wide host range as well as a widespread distribution (Udayanga et al. 2014; Linaldeddu et al. 2020). This species often produces its tiny fruit bodies on dead woody tissues (Kowalski et al. 2016). In a study by Kowalski et al. (2017), D. *eres* showed the least virulence and caused significantly milder disease symptoms on *F. excelsior* plants than the other tested fungal species. *Diaporthe eres* could be considered as a weak pathogen in comparison to ash dieback on *F. excelsior*. In case of tree weakening by *H. fraxineus* the early endophytic presence of *D. eres* favours a fast pathogenic attack (Kowalski et al. 2016).

In this study *Fusarium lateritium* Nees has been isolated frequently from symptomatic tissue and once from healthy wood tissue. The species is already known from *F. excelsior* in association with bacterial ash canker (Riggenbach 1956) but its virulence seems to be low in comparison with other fungal species (Bakys et al. 2009b). Kowalski et al. (2017) showed, that *F. lateritium* causes none or only small necroses on *F. excelsior*. In general, *Fusarium* spp. have a wide host range and are reported as the most common endophytes in ash bark and wood (Kowalski and Kehr 1992; Sieber 2007; Bakys et al. 2009b; Kowalski et al. 2016). The facts described above, indicate that *F. lateritium* is able to colonise the bark and woody tissue of ash independently of *H. fraxineus*. In association with ash dieback though it is more likely that the species contributes to the stem collar necroses as secondary pathogen. Thereby, it is non-essential, whether acceleration of ash dieback is established by shifting from endophytic to pathogenic lifestyle or colonising the tree as a secondary pathogen after tree weakening.

As far as it is known, the isolation of *Paracucurbitaria* sp. from the examined samples is the first proof of this genus in stem collar necroses. It was not isolated by Langer (2017) and Meyn et al. (2019) from rootstock. However, Kowalski and Bilański (2021) detected *Paracucurbitaria* sp. in previous year's ash leaf petioles in Poland, Barta et al. (2022) isolated it from ash twigs in Slovakia, and Haňáčková et al. (2017) reported *Paracucurbitaria corni* (Bat. & A. F. Vital) Valenz.-Lopez, Stchigel, Guarro & Cano as an endophyte of ash leaves and seeds. Therefore the occurrence of species from the genus *Paracucurbitaria* in plant material of *F. excelsior* is not striking, but its high frequency in stem collar necroses was unanticipated. It can be assumed that the high frequency of *Paracucurbitaria sp.* is no coincidence, because its detection in stem collar necroses of ash is increasing in ongoing research at the NW-FVA since sampling for this study.

Besides *D. sapinea*, there are a few species, which, to the knowledge of the authors, have not been previously reported from *F. excelsior* (Table 2). One of them is *C. corticale*, known as the causal agent of the sooty bark disease on maples. Its main host is *Acer pseudoplatanus* L., but it has been proven that *C. corticale* can colonise other maple species as well as *Aesculus hippocastanum* L. (Enderle et al. 2020). This species was found at sampling sites with sycamore. In addition to the first reports of ash as a host, one strain belonging to the genus *Vexillomyces* was isolated and recognised as undescribed species. The genus *Vexillomyces* was described in 2020 for two species (*V. palatinus*, *V. verruculosus*) isolated from spore traps attached to vine shoots. No host organism is known for these species. Later several species of *Claussenomyces* and *Tympanis* were transferred to the genus (Baral and Quijada 2020). The respective species are known from dead or living angiosperm and gymnosperm wood, however, only for *V. atrovirens* (syn. *Claussenomyces atrovirens*) an affiliation to the host genus *Fraxinus* could be recognised (Dennis 1986).

4.2 Role of *Hymenoscyphus fraxineus* in stem collar necroses

The ash dieback pathogen *H. fraxineus* could not be isolated from all of the 54 symptomatic stem collars. Only in about half of the trees, the fungus could be determined. It has been already reported by several authors, that the ash dieback pathogen could not be frequently isolated from symptomatic tissue of ash (Przybyl 2002; Bakys et al. 2009a; Enderle et al. 2017). A possible explanation for this could be its slow growth, unfavourable sampling conditions for the pathogen or too advanced necroses with antagonistic activity of other colonisers (Kowalski and Holdenrieder 2009; Hauptman et al. 2013; Gross et al. 2014; Langer 2017). Often, *H. fraxineus* could be solely isolated from recently discoloured woody tissues of the stem collar necroses (Fig. 2) and is probably supressed in the older parts of the necroses already colonised by secondary fungi. The aforementioned reasons might have contributed to the moderate

isolation success of the ash dieback pathogen in this study. Perhaps, fungal community analysis by means of culture-independent methods, such as high throughput sequencing or qPCR could detect *H. fraxineus* more frequently than by culture based isolation, since these methods have the potential to detect inactively present fungi (spores) or even DNA residues if the initial fungus has been suppressed by secondary invaders (Lindahl et al. 2013). Our results on the f_{MT} and continuity of the association and localisation of *H. fraxineus* in basal stem necroses confirm the assumption, that this pathogen is very often the main or primary causal agent triggering stem collar necroses. Either way, *H. fraxineus* is confirmed as the main pathogenic agent of the ash dieback epidemic (Kowalski 2006; Bakys et al. 2009a; Kowalski and Holdenrieder 2009; Gross et al. 2014). The only lack of evidence of *H. fraxineus* at the study site Wolfenbüttel could be explained by the meagre sample size (single tree, very low number of extracted wood chips). It can be assumed that *H. fraxineus* may have been isolated if a larger wood chip number or sample tree size was examined. According to information from a co-researcher in FraxPath, *H. fraxineus* was present in branches of the sample trees at the study site Wolfenbüttel (Maia Ridley, personal communication).

4.3 Fungal communities in stem collar necroses

Kowalski and Kehr (1992) and Sieber (2007) concluded that the fungal communities in *F. excelsior* are mainly dominated by fungi belonging to *Diaporthales* (*Diaporthe* spp.) followed by *Pezicula* species. In contrast, fungal taxa identified in this study mainly belong to *Hypocreales*, followed by *Pleosporales* and *Helotiales*. Fungi belonging to the *Diaporthales* (summarised under the category “others” in Fig. 3) account only for a tiny proportion of 1.6% of all isolated ascomycetes from our sampling, including the fifth frequent isolated taxon. Furthermore, *Pezicula* species are less frequent in this study with 12 isolates representing five MTs. *Pezicula cinnamomea* (DC.) Sacc. determined by Kowalski and Kehr (1992) as the second most isolated fungus, could not be isolated in this study. This discrepancy in the diversity could be explained by the fact that Kowalski and Kehr (1992) and Sieber (2007) focused on endophytes isolated from asymptomatic plant material. Another possible explanation might be the different geographical regions of the discussed studies, or the use of different plant tissue material, or sampling and isolation methods. The composition of fungal orders inhabiting asymptomatic plant material might not represent the composition in symptomatic stem tissue in specific.

The fact, that the composition of fungi isolated in this study differs with only a little overlap between the sampling sites, leads to the assumption that adding further sampling sites would reveal new sets of fungi not recorded in this study. The observation of significantly different occurrences of fungal taxa between forest sites is confirmed by Bilański and Kowalski (2022). In the study of Meyn et al. (2019) only two species were found in all sample trees and many of the identified fungi were single isolates. Similarly, Kranjec Orlović et al. (2020) revealed just few predominating taxa representing half of all fungal isolates from stem bases of *Fraxinus angustifolia* Vahl. In addition, species represented only by a single isolate make up one-third of all isolates in the study by the latter authors.

4.3 Relation of the most common fungi to the site characteristics

It is generally accepted that European ash trees independently of age class and site conditions are infected by *H. fraxineus* (Pautasso et al. 2013). However, the extent of ash dieback in the crown and stem collar necroses and tree mortality, most likely depend on many different factors. Susceptibility of ash trees to the pathogen, the range of subsequent colonising fungal species (Langer et al. 2022), tree vitality, or the environmental context of the forest site and stand (Havrdová et al. 2017) are some examples. Ash tree vitality is encouraged at fertile and (moderately) wet soils, conditions which are preferred by ash (Walentowski et al. 2017). It has to be taken into account that for this study only a selection of forest sites from a rather narrow area out of the wide range of European ash was investigated. An optimal soil and water supply with a sufficient percentage of ash trees was fundamental. Furthermore, the selection of sample trees was subjected to different restrictions. For example the condition of a diameter at breast height less than 25 cm because of logistics and processing abilities in the lab. Besides that, trees with very advanced necroses like completely necrotic or rotten stem base or dead trees were not suitable for investigation.

Our preliminary results indicate that *H. fraxineus* was isolated less frequent at sites with higher water availability (Online Resource 2). This is in accordance with the guess that the fungal composition of stem collar necroses depends on soil and water availability of the forest stand (Linaldeddu et al. 2011; Salamon et al. 2020). As mentioned before, this assumption refers only to the selection of the investigated forest sites. One explanation could be, that secondary fungi have more favourable conditions at sites with higher water availability and thus are able to overgrow *H. fraxineus* faster than at drier sites.

For the other most common fungi *Armillaria* spp., *Diaporthe* cf. *eres*, *D. fraxini*, *Fusarium* cf. *lateritium*, *N. punicea*, and *Paracucurbitaria* sp. no significant correlation between f_{WC} and water balance could be determined (Online Resource 2). Assuming that *H. fraxineus* as the sole pathogen influences the extent of damage caused by stem collar necrosis, this this would be in contrast to the suggestion of several authors that stands with wet soil conditions show a higher probability that the individual trees affected by *H. fraxineus* exhibit greater damage (Gross et al. 2014; Erfmeier et al. 2019). At Schwansee, the wettest sampling site, *H. fraxineus* was only isolated twice. However, the stem collar necroses were most advanced at these sampling trees, where a lower isolation rate of *H. fraxineus* was generally expected, as mentioned previously.

It was noticeable, that *D. fraxini* and *N. punicea* had a significantly different f_{WC} at the various sampling sites (Online Resource 2), but there was no indication for a correlation with the site characteristics water supply, soil and bedrock, climate, or mixture of trees. However, it was observed that ash trees with a low f_{WC} of *D. fraxini* had a thinner bark. Compared with *D. fraxini* and *N. punicea*, the MTs *Diaporthe* cf. *eres*, *Fusarium* cf. *lateritium*, and *Paracucurbitaria* sp. had a consistent f_{WC} over all sites. But *Fusarium* cf. *lateritium* was not isolated at Satrup and at the valley bottom in Schlangen. Due to the lower amount of isolations in this study, the authors assume there is also a lower probability of occurrence in stem collar necroses.

Armillaria species were not present at all studied sites and could not be isolated from the trees in Schwansee. This result is contradictory to those of Enderle et al. (2017), who found older necroses to be more often colonised by *Armillaria* spp. The progress of the necroses formation was clearly visible by their partially ruptured wood surface and presence of fruiting bodies on the necrotic stem areas of wood decay fungi, such as *Coprinellus* sp. and *Xylaria polymorpha* (Pers.) Grev. (Liers et al. 2011). Furthermore, the absence of *Armillaria* spp. isolates in Schwansee, the moistest of all sampling sites which is influenced by its

ground water, do not correspond to preference of *Armillaria* species for continuously moist soil conditions (Whiting and Rizzo 1999). A possible explanation for the lack of this species in Schwansee, could be the specific forest site background as a former lake. The area was earlier used as fishpond until the 18th century. Thus, the soil was subjected to special formation conditions (Welk 2017) and perhaps it was not possible for *Armillaria* spp. to colonise the soil like in other forest sites.

Many of the other MTs detected in this study were isolated just once, which may indicate no direct correlation with the investigated forest sites, thus site characteristics like soil and water supply relatedness cannot be assumed. However, it cannot be ruled out that the one-time isolated fungi occur in other forest sites, than the investigated ones, too. As well as a higher abundancy is theoretical possible. Additionally, it is to be expected that the composition of fungi might differ according to tree age, tree species composition, forest management type, season and the like (Scholtysik et al. 2013; Tomao et al. 2020). For example, a more diverse tree species composition at a forest site could contribute to the occurrence of a wider spectrum of fungi colonising a tree (Cavard et al. 2011; Kowalski et al. 2016; Krah et al. 2018; Tomao et al. 2020). This is confirmed by the isolation of sycamore typical fungi like *C. corticale* und *C. rubronotata* from *F. excelsior* in stands with maple trees. It is furthermore supported by the fact that the most mixed intensive sampling site of Berggießhübel has one of the highest fungal diversity in relation to its sample tree amount. Besides its diverse tree species composition, in addition Berggießhübel is the most eastern sampling site. Satrup is the most northern sampling site and shows also high fungal diversity despite its smallest sample tree amount. This observation suggests that widely varying sites in Germany lead to differing fungal communities. Furthermore, a possible underestimation of fungal diversity in the studied trees may occur since not all fungi are detectable through standardised culture based methods or in general (Guo et al. 2001; Allen et al. 2003; Unterseher 2007; Muggia et al. 2017).

4.4 Conclusion and Outlook

This study provided new insights on the fungal diversity and communities of endophytes, primary and secondary pathogens, wood decaying fungi, and saprophytes associated with stem collar necroses of European ash trees. A rich fungal composition inhabiting symptomatic stem tissue has been revealed with four frequent species occurring at most of the studied forest sites, but with little overlap between the sites. The fungal species richness detected in this study (162) is considerably higher compared to previous investigations in which 16–75 different species were detected (Lygis et al. 2005; Enderle et al. 2017; Langer 2017; Meyn et al. 2019). This difference in diversity can be explained by the larger sampling size (not only tree number, but also amount of wood chips taken) and the partially greater number of sites studied. Single trees with only about 20 studied chips of stem collar tissue each (Oranienbaumer Heide, Wolfenbüttel) had the fewest amount of isolated MTs. Further studies on stem collar necroses can increase the knowledge of fungal biodiversity on *F. excelsior*, as the first proved fungi in this study show.

The ash dieback pathogen was isolated from only about half of the trees sampled. Different reasons like its slow growth can cause a low isolation rate of the primary pathogen. Nevertheless it can be assumed, that stem collar necroses are commonly initiated by this fungus. The occurrence of several pathogenic fungi from necrotic stem tissue of ash beside *H. fraxineus* is striking, because of their high f_{MT}. It was shown that the different fungal communities of the sample trees are largely dominated by three MTs (*D. fraxini*, *Armillaria* spp. and *N. punicea*) next to *H. fraxineus* representing almost 50% of all isolates. They are considered to play a major role in the progression of stem collar necroses and rot and therefore also contribute to a loss of tree stability. The remaining fungi which were isolated from the stem collars necroses turned out to be very diverse with much lower f_{MT}, in the majority of cases were represented with only one isolate. Overall, the synergistic interaction of different pathogens in the context of ash dieback, for example *H. fraxineus* and *D. fraxini* or *N. punicea*, can lead to a larger damage in contrast to infection by only one pathogen (Marçais et al. 2010). In this context, *N. punicea* poses a serious threat to planted ash forests and natural regenerations of *F. excelsior*, especially if another host tree species, such as European beech (*Fagus sylvatica* L.) is in mixture. European beech is potentially an inoculum reservoir of *N. punicea* for future infections of ash stem collars (Karadžić et al. 2020). Therefore, in the future the susceptibility of ash to form stem collar necroses and to be diseased by *D. fraxini* and *N. punicea* should be considered in breeding programs to develop more resistant ash trees in relation to ash dieback.

However stem collar necrosis types caused by other fungi than *H. fraxineus* or *Phytophthora* spp. (Langer 2017), should not be disregarded. The results of this study show, that at least one fungal pathogen can be found in the necrosis without evidence of *H. fraxineus.* For example, one of the control samples, which turned out to have necrotic tissue inside the wooden body, was colonised by *Armillaria* sp. In this case, it is likely that the fungus attacked the weakened tree independently of a pre-colonisation of the stem collar by *H. fraxineus*.

Since in this study no correlation between the site factors and fungal occurrence could be calculated because most of the isolated fungi were only detected once, further studies should be carried out at additional comparable forest sites. Inventories of stem collar necroses at a higher number of locations may reveal dependence of MTs to forest side conditions and their individual role in the fungal communities in detail. Future studies need to be conducted in order to estimate potentially high risk characteristics of forest sites for pronounced and fast advancing stem collar necroses and rot. Additionally, the investigation of genotypes of *H. fraxineus* associated with single stem collar necroses could help to better understand the path of infection with *H. fraxineus* and the secondary colonisation by other fungi.

Declarations

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Conflict of Interest

The authors declare that they have no conflict of interest.

Statement of contributions

The study including sampling, lab work, and analysis was primarily conducted by S. Peters with support from G. Langer, S. Bien and S. Fuchs. The first draft of the manuscript was written by S. Peters and revised by G. Langer, S. Bien, J. Bußkamp and E. Langer.

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Figures

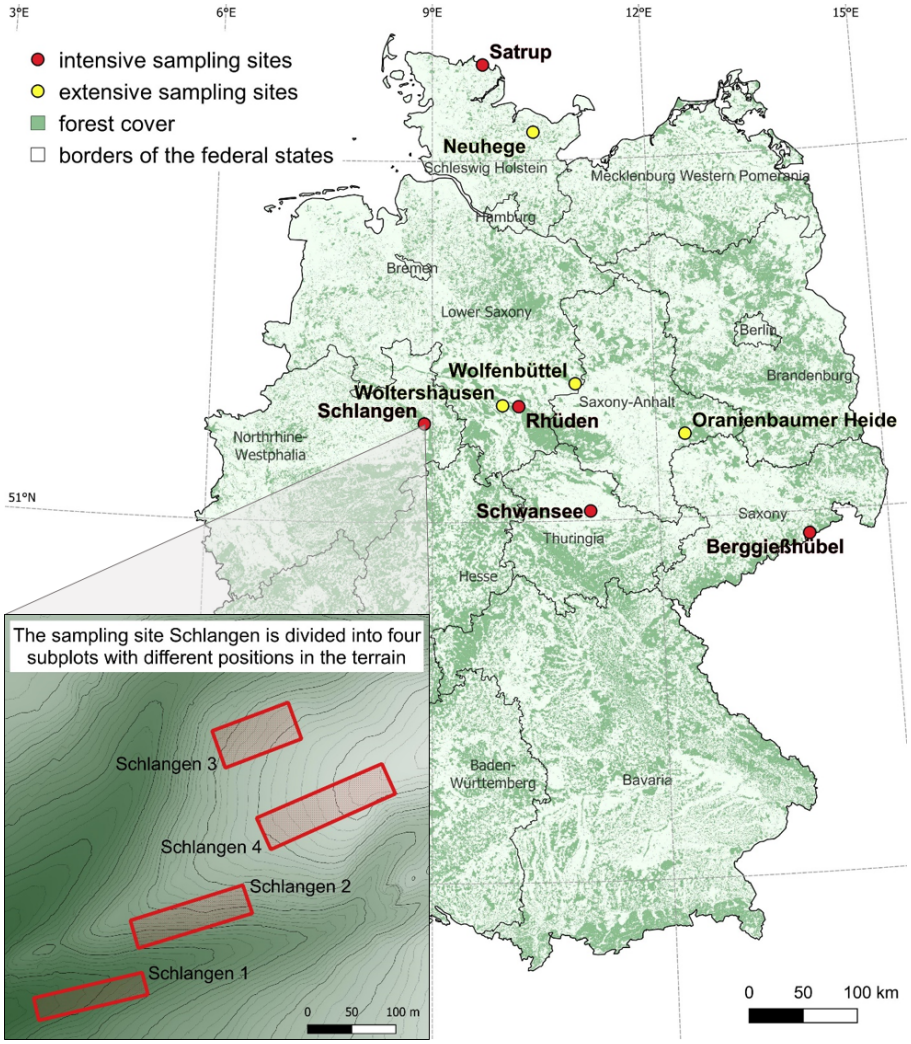

Figure 1

Sampling sites in Germany divided in intensive (red) and extensive (yellow) sampling sites with a detailed view of the special study site Schlangen and its feature of splitting in four subplots with different terrain positions (QGIS 3.24)

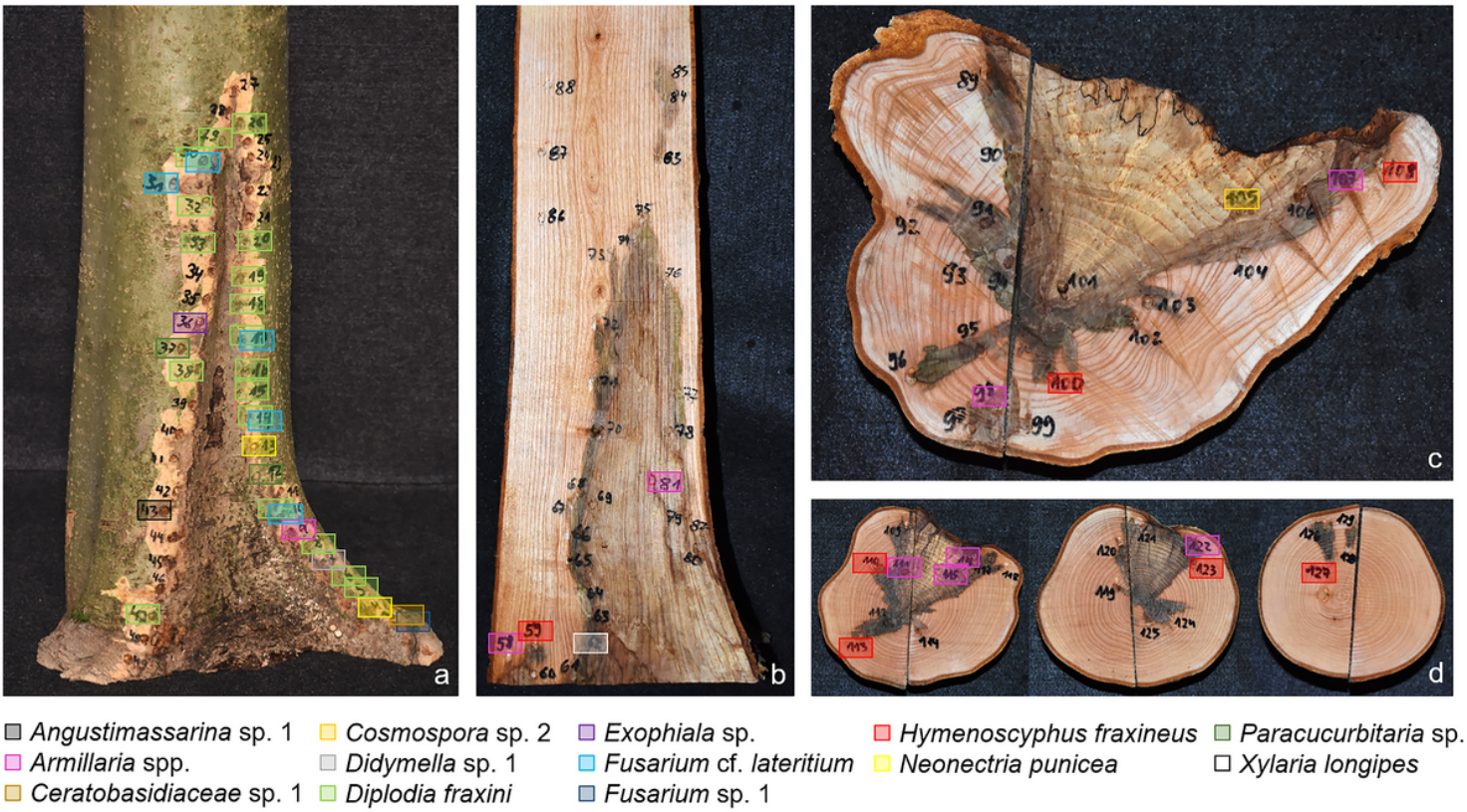

Figure 2

Fungi associated with the stem collar necrosis of ash tree 53 from the sampling site Schlangen 2. Isolation loci are numbered. a) Sampled stem base from the outside, b) longitudinal-section of stem base with visible wood discoloration, c) basal cross-section of stem base d) cross-section of the stem base above ground level every 10 – 15 centimetres

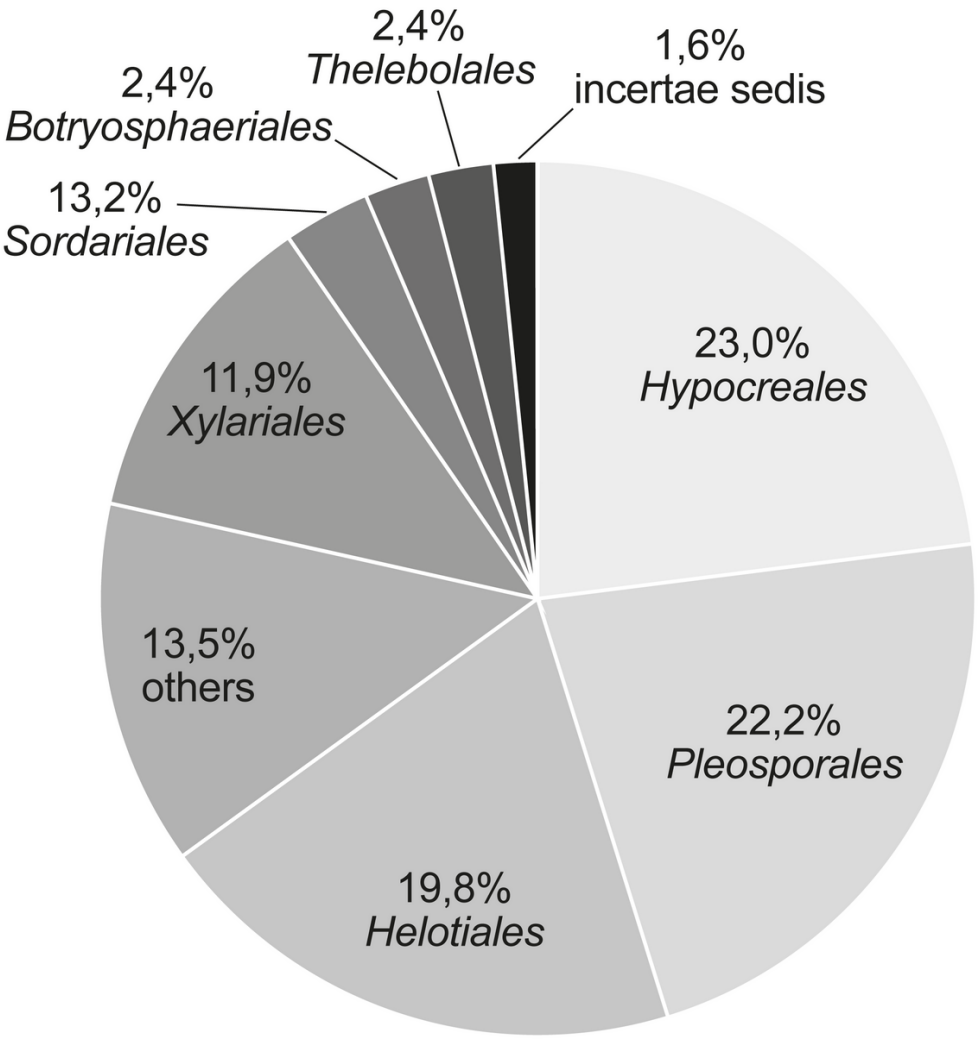

Figure 3

Isolated orders of the *Ascomycota,* n = 132 of the isolated morphotypes belonging to the *Ascomycota* (Microsoft PowerPoint 2013)

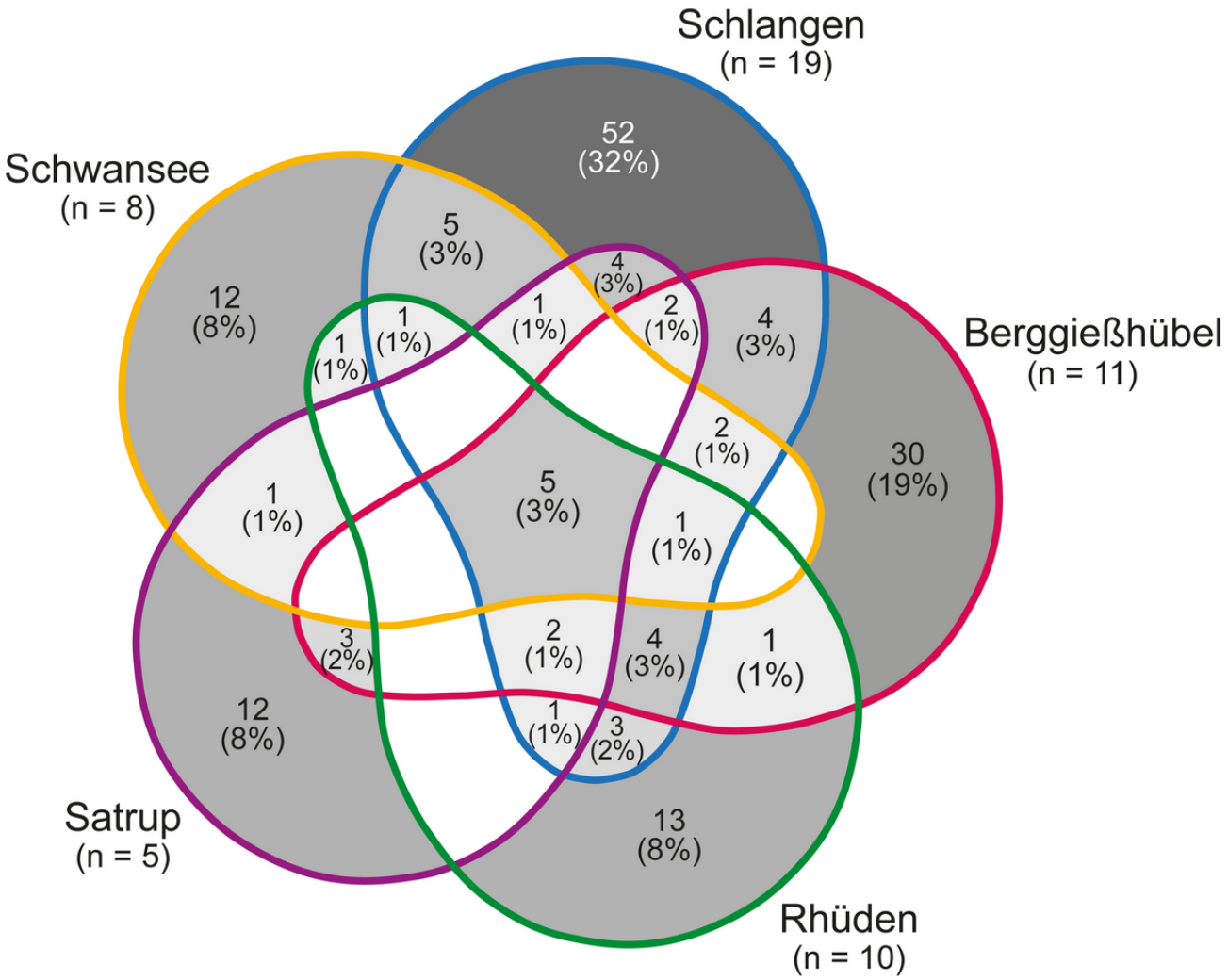

Figure 4

Overlap between the fungi isolated from the main sites (with at least five samples) with the indication of how many of the isolated fungi were found at each site and between the different sites (absolute number as well as percantage), empty white areas indicating no overlap between the respective sites. Five fungi at the overlap of all sites are *Diaporthe* cf. *eres*, *Diplodia fraxini*, *Hymenoscyphus fraxineus*, *Neonectria punicea*, and *Paracucurbitaria* sp. (RStudio 4.1.2)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ESM1Frequency.xlsx
- ESM2Frequency.tif
- ESM3Overviewsampledtreesandmorphotypes.xlsx
- ESM4PhylogeneticanalysisDiplodia.pdf
- ESM5PhylogeneticanalysisArmillaria.pdf