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Cryptostroma corticale and fungal endophytes associated with *Fraxinus excelsior* affected by ash dieback

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Abstract
While assessing the filamentous fungi associated with woody tissues of stem collar rots, necroses, and lesions of European ash trees (*Fraxinus excelsior*) presenting symptoms of ash dieback in Germany, *Cryptostroma corticale* was recovered from three different ash trees. These isolated strains were the first report of *C. corticale* on ash and the first proof of an association of this plant pathogen with woody tissues of other tree species than *Acer* spp. in Germany. To test the pathogenicity of *C. corticale* against *F. excelsior* and to fulfil Koch's postulates, inoculation tests *in planta* with strains isolated from *Acer pseudoplatanus* and *F. excelsior* were conducted according to Henle–Koch's postulates in a greenhouse located in Göttingen. The pathogenicity tests were performed with apparently healthy ash saplings from June 2021 until January 2022. After three and seven months, neither necroses or lesions due to *C. corticale* nor disease symptoms were observed. Mostly, the inoculation wounds healed over, and *C. corticale* could not be re-isolated from the ash woody tissue. In an attempt to re-isolate the inoculated strains, the filamentous fungal endophytes of the ash woody tissues were isolated and identified. A total of 32 taxa of the Ascomycota were found, where the most common species was *Boeremia exigua*. Most frequently observed orders were *Pleosporales* (58.4%), followed by *Sordariales* (13.5%), *Hypocreales* (9.4%), and *Diaporthales* (8.7%). On average, 3.7 endophytic species were recorded on each sapling.

Keywords *Fraxinus excelsior* · *Cryptostroma corticale* · Endophytic fungi · Pathogenicity tests · Stem collar necrosis

Introduction

The anamorphic fungus *Cryptostroma corticale* (Ellis & Everh.) P.H. Greg. & S. Waller (*Ascomycota*) is the causal agent of Sooty bark disease (SBD). This species was first isolated from *Acer campestre* L. in Ontario in 1889 and called *Coniosporium corticale* Ellis & Everh. (Ellis & Everhart 1889). Phylogenetic studies based on analyses of four genes (ITS nrDNA, actin, RPB2 and β-tubulin) revealed that *C. corticale* is a member of the *Xylariaceae* Tul. & C. Tul., *Graphostromataceae* M.E. Barr, J.D. Rogers & Y.M. Ju (Ju et al. 1998; Koukol et al. 2015). Koukol et al. (2015) illustrated the affinity of *C. corticale* to the genus *Biscogniauxia* with the closest relatives being *B. bartholomaei* (Peck) Lar.N. Vassiljeva and *Graphostroma platystomum* (Schwein.) Piroz. (Koukol et al. 2015).

Further cases of SBD were subsequently observed in Canada and the USA, and it is likely that *C. corticale* is originally native to North America (Enderle et al. 2020; Gregory and Waller 1951). Major hosts of the SBD pathogen are *Acer* species (*Sapindaceae and Sapindales*), such as *A. pseudoplatanus* L., *A. saccharinum* L. (Gregory and Waller 1951), as well as less commonly *A. campestre* L. (Moreau and Moreau 1954), *A. platanoides* L. (Bencheva 2014), and *A. negundo* L. (Young 1978). There is evidence to suggest the existence of additional potential host trees, such as *Tilia* spp. and *Betula* spp. (Cochard et al. 2015), as well as *Aesculus hippocastanum* L. (Young 1978). In Europe, first observations of the Sooty bark disease were made on *A. pseudoplatanus* in Wanstead Park Essex, UK, in the year 1945 (Gregory and Waller 1951). In Germany, the first report on sycamore (*A. pseudoplatanus*) firewood diseased by *C. corticale* dates to the year 1964 (Enderle et al. 2020; Plate and Schneider 1965).

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Most likely *C. corticale* primarily infects its host tree through fresh wounds (Townrow 1953; Dickenson 1980). The warmth-loving, invasive anamorphic fungus is presumed to be an opportunistic latent pathogen switching from its endophytic lifestyle to pathogenic and saprophytic life stages when its host is stressed (Dickenson 1980). SBD of sycamore leads to mortality of the infected trees, triggered and accelerated by a tree stressor, such as drought (Gregory and Waller 1951; Dickenson and Wheeler 1981; Enderle et al. 2020). Typical disease symptoms, described in detail by Enderle et al. (2020), include the peeling of the outer stem or branch bark layer and the appearance of a brownish-black, sooty layer of conidia.. However, in years with normal temperatures and sufficient precipitation, especially in summer, the course of disease can proceed without any externally visible symptoms (Cech 2019). After infection with *C. corticale* via wounds or less likely natural openings of the tree (Dickenson 1980), the latent pathogen may survive as an endophyte. First, the fungal mycelium spreads via the xylem and later via the phloem through the wood into the heartwood (Young 1978; Dickenson and Wheeler 1981).

Due to severe drought conditions during several summers in the last two decades, outbreaks of SBD have been increasingly observed in mainland Europe (Koukol et al. 2015; Cochard et al. 2015; Bork 2018; Enderle et al. 2020). Until now, *C. corticale* is considered established in eleven European countries: Belgium (Cech 2004, 2019), Bulgaria (Bencheva 2014), Czech Republic (Koukol et al. 2015; Kelnarová et al. 2017), France (Moreau and Moreau 1951; 1954), Germany (Bork 2018; Delb et al. 2019; Rohde et al. 2019; Wenzel et al. 2019; Enderle et al. 2020), Italy (Oliveira Longa et al. 2016), the Netherlands (EPPO 2014), Norway (Spaulding 1961), the UK, and Switzerland (Cochard et al. 2015).

The conidia release of *C. corticale* poses a potential risk to human health. Persons who have intensive or occupational contact with masses of conidia can develop Pneumonitis due to *C. corticale*, also referred to as maple bark disease (MBD) or maple bark strippers' lung. The latter denotes the opportunistic mycosis of the lungs caused by inhalation of *C. corticale* conidia while stripping the bark from maple logs (Emanuel et al. 1966). MBD in humans is a hypersensitivity pneumonitis (HP) causing symptoms similar to allergic asthma, COPD, flu-like infections, influenza, and interstitial pneumonia (Braun et al. 2021; Kespohl et al. 2022).

As part of the "FraxForFuture" demonstration project, the sub-network "FraxPath" (Peters et al. 2021; Langer et al. 2022) investigates the formation of stem collar necrosis associated with trees affected by ash dieback. The fungal community associated with stem collar rots, necroses, and lesions of European ash trees (*Fraxinus excelsior* L., *Oleaceae, Lamiales*) was assessed in several German forest plots (Peters et al. 2023). To this end, classical culture-based isolation methods, *in planta* inoculations and fungal identification by ITS-barcoding and morphological characteristics, were used according to Langer (2017), Bußkamp et al. (2020), and Langer and Bußkamp (2021). In the course of this in-depth investigation of stem collar necrosis and rots of common ash, fungi associated with woody tissues of 58 ash trees from nine localities in Germany were isolated including *Cryptostroma corticale* (Peters et al. 2023). According to previous authors (Petrini 1991; Saikkonen et al. 1998; Arnold and Lutzoni 2007), we consider those fungi as endophytes that spend at least a significant amount of their life cycle within the host woody tissue without causing symptoms.

European ash dieback caused by the invasive alien fungal pathogen *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (syn. *H. pseudoalbidus* Queloz, Grünig, Berndt, T. Kowalski, T.N. Sieber & Holdenr., anamorph: *Chalara fraxinea* T. Kowalski, *Ascomycota*) was first observed in Poland at the beginning of the 1990s (Przybył 2002; Kowalski 2006). It is assumed that this fungus was introduced from Far East Asia to Europe (Gross et al. 2014; Drenkhan et al. 2014) and spreads very quickly (Timmermann et al. 2011; Enderle et al. 2019). Ash dieback and its impacts, for example stem collar necroses or root and butt rot, often have fatal consequences for the survival, growth and wood quality of *F. excelsior* (Langer et al. 2022). This disease, which is now widespread in Europe, is the most serious threat to European ash trees to date (Skovsgaard et al. 2017; Peters et al. 2021). In addition to the typical and eponymous crown symptoms of ash, such as shoot dieback and leaf necrosis, stem collar rot and necrosis were often observed on diseased trees (Husson et al. 2012; Langer 2017; Meyn et al. 2019; Enderle et al. 2017). In Germany, ash dieback has been present since 2002 at least (Schumacher et al. 2007) and is present in all regions where common ash grows (Langer 2017). To assess the impact of *C. corticale* on the health of European ash, pathogenicity tests were carried out according to the Henle–Koch postulates (Evans 1976), using the occurrence of necrosis as an indicator for pathogenicity.

Materials and methods

Fungal strains

All fungal strains used in our pathogenicity tests were identified based on morphology and ITS sequencing. The ITS sequences (containing ITS1, 5.8S and ITS2 regions) have been deposited in GenBank (Table 1), and all the strains are permanently stored in the NW-FVA strain collection. They were cultivated on malt yeast peptone agar (MYP), modified according to Langer (1994), containing 0.7% malt

Table 1 List of strains used for pathogenicity tests and isolated endophytes from European ash saplings, Freq. = Frequency (%, defined as the portion of isolated strains in relation to the total number of isolated filamentous strains), Cont. = Continuity (%, defined as number of trees from which the fungus was isolated in relation to the total number of trees), Id. = Identity, I.s. = Incertae sedis; Basis of identification: "-" indicates that no NCBI blastn result was a close match for identification or that DNA extraction was unsuccessful. Blastn conducted: 25.07.2022

Species	Order	Freq. (%)	Cont. (%)	Reference strain (NW-FVA)	Accession No	Seq. length	ITS NCBI blastn results, 25.07.2022			
							Basis of identification	Accession No	Id. (%)	References
Strains used for pathogenicity tests (*isolation source*)										
Cryptostroma corticale (*A. pseudoplatanus*)	Xylariales	0	0	5572	OP010049	623	*Cryptostroma corticale* culture CBS:216.52	MH857008	99.84	Vu et al. (2019)
Cryptostroma corticale (*A. pseudoplatanus*)		0	0	5889	OP010050	630				
Cryptostroma corticale (*F. excelsior*)		0	0	5932	OP023158	630				
Cryptostroma corticale (*F. excelsior*)		0	0	6116	OP010051	621				
Cryptostroma corticale (*F. excelsior*)		0	0	6181	OP010052	620				
Isolated endophytes										
Alternaria infectoria	Pleosporales	10	38.6	8134	OP036593	560	*Alternaria infectoria* isolate 113	MN534845	100	M Al-Khawaldeh and SE Araj unpublished
Alternaria rosae	Pleosporales	1.1	5.7	7341	OP036581	569	*Alternaria rosae* isolate AS-S-6	MK632004	100	X Nie and G Zhao unpublished
Alternaria sect. *Alternata*	Pleosporales	6.8	27.1	8099	OP036582	531	*Alternaria alternata*	OW988245	100	P Becker unpublished
Boeremia exigua	Pleosporales	26.5	71.4	7280	OP036570	509	*Boeremia exigua* var. *exigua*	MN540289	100	T Radic et al. unpublished
Chaetomium cochliodes	Sordariales	1.8	7.1	8128	OP036590	535	*Chaetomium cochliodes* strain NW-FVA5065	MT561402	100	GJ Langer and J Bußkamp unpublished
Cladosporium spp.	Dothideales	0.7	4.3	8165	OP036596	512	*Cladosporium xylophilum* strain 18BPLE003	MT645916	100	M Cambon et al. unpublished
Diaporthe cf. *rudis*	Diaporthales	5.7	30	8166	OP036597	552	*Diaporthe* sp. strain NW-FVA5281	MT561408	99.82	GJ Langer and J Bußkamp unpublished
Diaporthe sp. (*eres*-Gr.)	Diaporthales	3.0	14.3	8173	OP036599	515	*Diaporthe eres* culture CBS:145,040 (*Phomopsis oblonga*)	MK442579	100	Crous et al. (2019)
Dichotomopilus sp.	Sordariales	11.4	32.9	7293	OP036573	547	*Dichotomopilus funicola* isolate KJ01	MN893925	100	Z Xu et al. unpublished
Didymella cf. *macrostoma*	Pleosporales	2.3	10	7285	OP036572	507	*Didymella macrostoma* isolate Dm24L-19An	MN588154	100	K Patejuk, et al. unpublished
Didymella sp.	Pleosporales	4.3	15.7	8102	OP036584	500	*Didymella* sp. strain NWFVA5756	MW365344	100	Blumenstein et al. (2021)
Epicoccum nigrum	Pleosporales	2.5	14.3	7283	OP036571	510	*Epicoccum nigrum* isolate 18	MT573480	100	W Wysoczanski et al. unpublished
Fusarium oxysporum	Hypocreales	2.1	5.7	8161	OP036594	505	*Fusarium oxysporum* clone SF_967	MT530243	100	C Li unpublished

Table 1 (continued)

Species	Order	Freq. (%)	Cont. (%)	Reference strain (NW-FVA)	Accession No	Seq. length	ITS NCBI blastn results, 25.07.2022			
							Basis of identification	Accession No	Id. (%)	References
Fusarium sp.1	Hypocreales	0.7	2.9	8118	OP036585	522	*Fusarium lateritium* isolate Fl23L-19An	MN588156	100	Patejuk et al. (2021)
Fusarium sp.2	Hypocreales	0.2	1.4	8123	OP036588	507	*Fusarium graminearum* isolate NFG104	ON024864	99.80	N Kaul, et al. unpublished
Fusarium sp.3	Hypocreales	1.1	5.7	8126	OP036589	522	*Fusarium petersiae* culture CBS:143,231 strain JW14004	MG386078	100	Lombard (2017)
Fusarium sp.4	Hypocreales	0.7	2.9	7297	OP036574	516	*Fusarium* sporotrichioides strain D933	MH266059	100	Adesemoye et al. (2018)
Fusarium sp.5	Hypocreales	0.5	2.9	7302	OP036575	529	*Fusarium acuminatum*	MT635295	99.81	A Cudowski unpublished
Juxtiphoma eupyrena	Pleosporales	0.5	1.4	8132	OP036592	501	*Juxtiphoma eupyrena* culture CBS:549.70	MH859842	100	Vu et al. (2019)
Leptospora rubella	Pleosporales	0.2	1.4	7340	OP036580	564	*Leptospora rubella*	HE774478	100	Hauptman et al. (2013)
Nemania serpens	Xylariales	0.2	1.4	7335	OP036578	552	*Nemania serpens* isolate BHI-F650a	MF161316	99.64	Haelewaters et al. (2018)
Neodidymelliopsis cannabis	Pleosporales	0.2	1.4	8267	OP036602	474	*Neodidymelliopsis cannabis* culture CBS:629.76	MH861012	100	Vu et al. (2019)
Neopestalotiopsis rosae	Xylariales	1.4	4.3	8164	OP036595	512	*Neopestalotiopsis rosae* isolate KoRLI046308	MN341547	100	SY Oh et al. unpublished
Neosetophoma cerealis	Pleosporales	0.2	1.4	8174	OP036600	553	*Neosetophoma cerealis* culture CBS:443.82	MT223822	99.82	Crous et al. (2020)
Paraphoma chrysanthemicola	Pleosporales	0.7	1.4	8129	OP036591	520	*Paraphoma chrysanthemicola* isolate 8924	MK647980	99.23	van der Merwe et al. (2021)
				8175	OP036601	516	*Paraphoma chrysanthemicola* strain BAN-100	JN123358	100	Ban et al. (2012)
Pestalotiopsis scoparia	Xylariales	0.9	5.7	8100	OP036583	566	*Pestalotiopsis scoparia* culture CBS:176.25	MH854838	99.65	Vu et al. (2019)
Ploettnerulaceae	Helotiales	0.2	1.4	8268	OP036603	593	*Cadophora* sp. MUT 4401	KC339236	99.66	Panno et al. (2013)
Podospora sp.	Sordariales	0.2	1.4	7338	OP036579	496	*Podospora appendiculata* culture CBS:314.62	MH858163	98.19	Vu et al. (2019)
Stagonosporopsis sp.	Pleosporales	0.7	2.9	7314	OP036577	510	*Stagonosporopsis* sp. strain 36–3	MK100222	99.80	S Khodaei et al. unpublished
Trichoderma spp.	Hypocreales	4.1	17.1	7303	OP036587	462	*Trichoderma harzianum* clone SF_847	MT530123	98.92	C Li unpublished
				8120	OP036586	580	*Trichoderma harzianum* culture CBS:354.33	MH855457	100	Vu et al. (2019)
				7303	OP036576	573	*Trichoderma viride* culture CBS:127,113	MH864422	99.65	Vu et al. (2019)

extract (Merck, Darmstadt, Germany), 0.05% yeast extract (Fluka, Seelze, Germany), 0.1% peptone (Merck), and 1.5% agar (Fluka).

Cryptostroma corticale isolates from *Acer pseudoplatanus.*

NW-FVA 5572 (2020-54-B2u-7), Germany, Lower Saxony, Universal Transverse Mercator (UTM) 32 U 602426 5808725, leg. P. Gawehn and R. Schlößer, 25.05.2020; isolated from woody stem tissue sampled by increment boring; Accession No. OP010049

NW-FVA 5889 (B41-1 2020-50-8), Germany, Saxony-Anhalt, UTM 32 U 667800 5697482, leg. P. Gawehn and R. Schlößer, 11.05.2020; isolated from conidia; Accession No. OP010050.

Cryptostroma corticale isolates from *Fraxinus excelsior* sampled from woody stem tissue of three different trees with stem collar rots (Peters et al. 2023)

NW-FVA 5932 (ES-2020-9-14), Germany, Lower Saxony, UTM 32 U 564973 5757894, leg. S. Peters and P. Gawehn, 20.10.2020; Accession No. OP023158

NW-FVA 6116 (ES-2021-53-33) Germany, Lower Saxony, UTM 32 U 579914 5757111, leg. S. Peters and P. Gawehn, 22.02.2021; Accession No. OP010051

NW-FVA 6181 (ES-2021-49-28) Germany, Lower Saxony, UTM 32 U 579913 5757147, leg. S. Peters and P. Gawehn, 22.02.2021; Accession No. OP010052.

Plant material

Seventy two-year-old *Fraxinus excelsior* saplings (assortment 50–80 cm) were purchased from the tree nursery Schlegel & Co., Riedlingen, Germany, originating from the provenance 8 11 02-Nordostdeutsches Tiefland and replanted in 5 l pots (18.6 × 18.6 × 20 cm) containing potting compost (PROFI-LINIE Kleeschulte Topfsubstrat mineralisch: pH 6, salinity 1.5 g/l, N total: 320 mg/l, P2O5: 120 mg/l, K2O: 350 mg/l, Mg: 120 mg/l, Kleeschulte Erden GmbH & Co. KG, Briloner Straße 14, D-59602 Rüthen, Germany). At the beginning of the experiments, the plants were healthy without visible stem necroses or symptoms of ash dieback. Plant height above substrate was measured at harvest (Online Resource 1). A one-way-ANOVA (aov) was used to determine if there were any statistically significant differences for the saplings height between the treatments "inoculated with *C. corticale*", "inoculated with MYP", and "untreated". Differences were considered as statistically significant if p-value was below the threshold of 0.01.

Pathogenicity tests

To estimate the impact of five different *C. corticale* test strains (NW-FVA 5572 and 5889 sampled from sycamore, NW-FVA 5932, 6116, and 6181 isolated from common ash) on European ash, pathogenicity tests were conducted in vivo according to Henle–Koch postulates (Evans 1976). The presence of necroses was used as an indicator of pathogenicity. The inoculation experiments were performed in a foil greenhouse at the NW-FVA in Göttingen, southern Lower Saxony, Germany (UTM 32 U 563091 5710663), from the 15 June 2021 until the 20 January 2022 (7 months).

For each tested strain, ten ash saplings were inoculated with a MYP-agar plug of a one-week-old culture of the fungus. The plugs were placed in wounds made with a sterile scalpel (1–5-mm diameter) on the stem at a height of 44 cm above ground. The removed bark was replaced on top of the plug, and then the stem was wrapped with Parafilm. A set of ten untreated controls, saplings which were not inoculated at all, and mock controls, prepared by inoculating ten saplings with a sterile pure culture medium plug of MYP, were established. The trees were arranged in equal distance from each other and watered to maintain the peat adequately moist.

After three months (13 September 2021), five saplings per treatment group were sampled to check infection success and necrosis formation. The bark around the area of inoculation was peeled away for visualisation and measurement of the extent of any necroses. Lesion lengths were measured with a ruler in the vertical direction to an accuracy of 1 mm. Fungi were re-isolated from discoloured and non-discoloured stem tissue at six loci adjacent to the point of inoculation (above: isolate 1 = right at the necrosis edge, isolate 2 = 1 cm above, and isolate 3 = 2 cm above; below: isolate 4 = right at the necrosis edge, isolate 5 = 1 cm below, and isolate 6 = 2 cm below). The resulting filamentous isolates were identified. The pathogenicity tests ended seven months (20 January 2022) after inoculation when the remaining test plants were sampled and evaluated as described above.

Determination of Fungi

Isolated endophytic strains were assigned to morphotypes (MT) and identified on the basis of micro-morphological characteristics according to Bußkamp et al. (2020) and Langer and Bußkamp (2021) and/or sequencing of the ITS region (White et al. 1990). At least one representative strain of each morphotype was submitted to molecular identification, involving DNA extraction from the mycelium. Mycelium was placed in 1.5-ml Eppendorf tubes with five 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 s. 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 and sequenced using the primer pair ITS-1F

(Gardes and Bruns 1993) and ITS-4 (White et al. 1990). The PCR mixture consisted of 1 µl of DNA and 19 µl mastermix, which contained 2.5 µl 10 × buffer (Carl Roth, Karlsruhe, Germany), 1 µl of each primer (10 mmol, 2.5 µl $MgCl_2$ (25 mmol), 0.1 µl 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, USA) was used to carry out the DNA amplifications. The conditions for the amplification of the ITS region were set according to Bien et al. (2020). A 1% agarose gel was used to visualise the PCR products. The products were sent to Eurofins Scientific Laboratory (Ebersberg, Germany) for sequencing. Resulting sequences were checked and edited where necessary using BioEdit Sequence Alignment Editor (v. 7.2.5; Hall 1999) and submitted to GenBank subsequently.

For identification, the ITS sequences obtained were used in blastn searches in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/, Altschul et al. 1997). A similarity threshold of at least 98% was set for species-level identification. Blastn results were critically interpreted with emphasis on well-curated culture collections such as the Westerdijk Fungal Biodiversity Collection (CBS). Generally, morphological characteristics were used to confirm the results of molecular identification. In the case that no definite identification was possible to a specific taxonomic level, the taxon name was marked by cf. (confer) to indicate uncertainties.

At least one representative culture for each MT was stored in MYP slant tubes at 4 °C at the fungal culture collection of the NW-FVA. Frequency of isolated taxa, defined as the portion of the amount of isolated strains in relation to the total number of isolated filamentous strains, was calculated. Additionally, continuity of isolated taxa, defined as the number of trees from which the fungus was isolated in relation to the total number of trees, was calculated.

Results

Isolation of Cryptostroma corticale from ash stem collars

From three out of 58 European ash trees (*Fraxinus excelsior*), *Cryptostroma corticale* could be isolated from woody stem tissue (Fig. 1) at two locations in Lower Saxony.

Pathogenicity tests

At the end of the experiment, the height of the tested plants varied considerably independently from the harvest date. The mean plant height was 90.6 cm (min. 42, max. 148 cm), and stem diameter was 0.65 cm (min. 0.3 cm, max. 1.01 cm). One-way ANOVA showed that there were no significant differences for the saplings height between the different treatments for the incubation period of 13 and 31 weeks (Online Resource 1). No necroses were observed regardless of the treatment group studied. All plants were healthy, and calluses had formed over the loci of inoculation.

Re-isolation and isolated endophytic fungi

In total, 420 chips of stem tissue originating from 70 saplings were incubated. From these, 438 mycelial outgrowths were observed (Online Resource 2). Most filamentous fungi grew out between one and three weeks after incubation of the tissue sample. Some of the observed fungal mycelia were omitted due to obvious repetitions or contaminations. From 2.1% of all incubated segments, no outgrowth was detected, while 12.3% yielded yeasts. *Cryptostroma corticale* could not be re-isolated as an endophyte nor isolated from the mock- or untreated controls.

The resulting pure culture isolates were all *Ascomycota* assigned to 32 taxa, and all but one species (*Ploettnerulaceae* sp.) could be identified to genus or species level (Table 1). Most frequently observed orders were *Pleosporales* (58.4%), followed by *Sordariales* (13.5%), *Hypocreales* (9.4%), *Diaporthales* (8.7%), *Xylariales* (1.6%), *Dothideales* (0.7%), and *Helotiales* (0.2%). Between one and eight different species were found in the studied woody tissue per sapling (Table 2). On average, 3.7 species were recorded on each sapling. The most frequent taxa were *Boeremia exigua* (Desm.) Aveskamp, Gruyter & Verkley (26.5%), *Dichotomopilus* sp. (11.4%), *Alternaria infectoria* E.G. Simmons (10.0%), *Alternaria* sp.—*alternata*-Gr. (8.9%), and *Diaporthe* cf. *rudis* (5.7%). The most abundant species in respect to continuity was *B. exigua,* which was isolated from 71.4% of all studied ash saplings. The second most continuous species was *A. infectoria* (38.6%) followed by *Dichotomopilus* sp. (32.9%) and *Diaporthe* cf. *rudis* (30%).

Discussion

The main result of this study was that Koch's postulates could not be fulfilled for *Cryptostroma corticale* on *Fraxinus excelsior*. Therefore, the species could not be proven as a causal agent of disease in *F. excelsior*, although it was found in association with stem collar necrosis. In the *in planta* experiments under the environmental conditions of our pathogenicity tests, *C. corticale* could not be

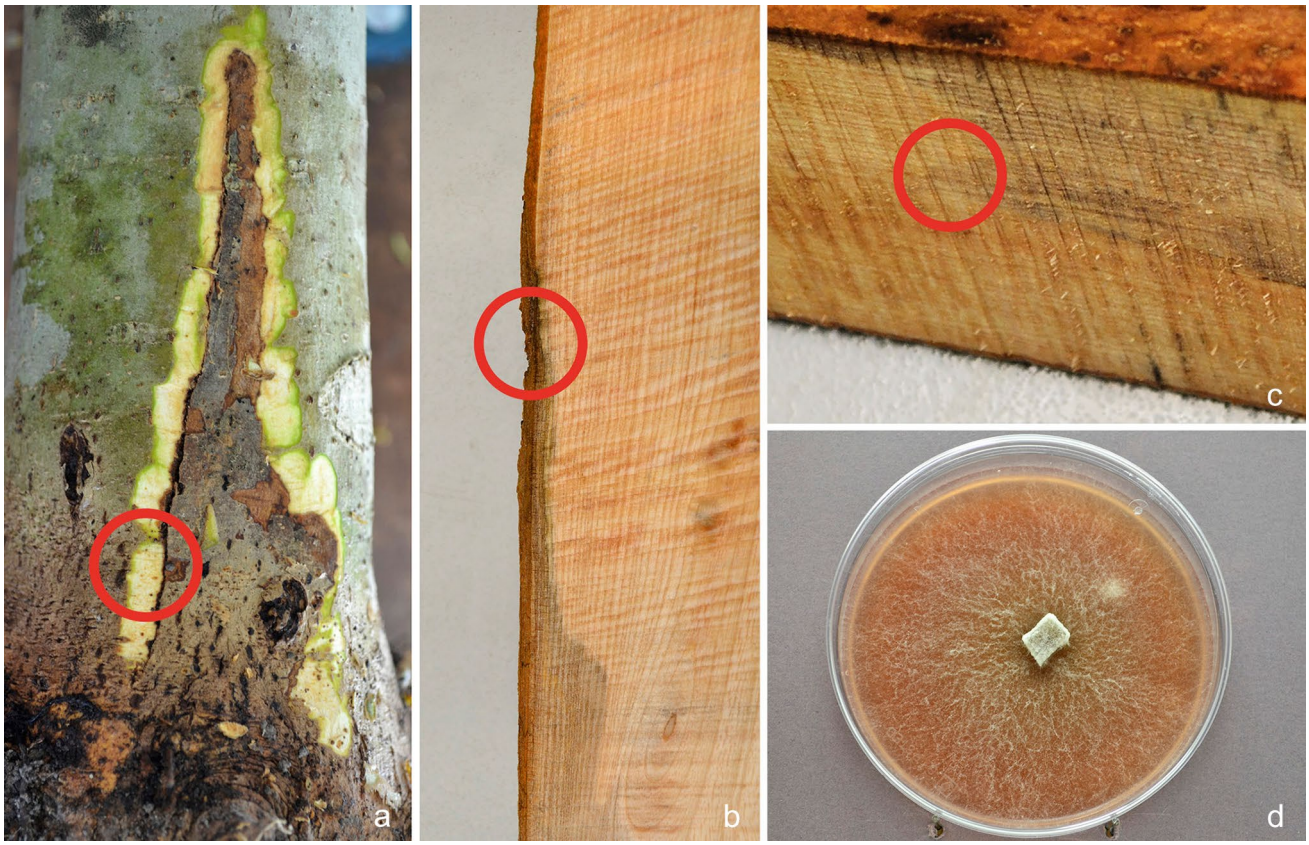

Fig. 1 *Cryptostroma corticale*, **a–c** Isolation loci on woody tissues associated with a stem collar necrosis caused by *Hymenoscyphus fraxineus* on European Ash (*Fraxinus excelsior*); **a** NW-FVA 5932; **b** NW-FVA 6116, and **c** NW-FVA 6181; **d** 7-day-old pure culture of strain NW-FVA 5932 on MYP-Agar

re-isolated. This suggests that the fungus cannot infect sterile-wounded, healthy tissue of young ash trees, in contrast to its ability to enter and infect sycamore through fresh wounds (Townrow 1953; Dickenson 1980). May be the time of year plays a role in the success of infection (Dickenson 1980). Unpublished concurrent inoculation tests with sycamore saplings, using an identical methodology, were partially successful with inoculation with the strain NW-FVA 5932 (original host tree *F. excelsior*) and unsuccessful with inoculation with the strain NW-FVA 5889 (original host tree *A. pseudoplatanus*). Only one out of five sycamore saplings were successfully infected by the *C. corticale* strain isolated from ash. This suggests that the infection on 6 June 2022, i.e. in that early summer, was rather unfavourable for a successful infection under the environmental conditions prevailing at that time. Another explanation for the failure to infect the young stems could be to lack of sufficient nutrients to allow the fungal inoculum to break down cell walls, as in the case of leaf scars (Dickenson 1980).

All isolated endophytic species could be assigned to the *Ascomycota* which fits the results on endophytes of seedling woody tissues of other tree species, for example 2-year-old *Pinus sylvestris* (Blumenstein et al. 2021). Blumenstein et al. (2021) isolated 18 different endophytic *Ascomycota* species including *Alternaria* spp., *Diaporthe* spp., *Epicoccum nigrum*, *Microsphaeropsis olivacea* (Bonord.) Höhn., *Sydowia polyspora* (Bref. & Tavel) E. Müll., and *Truncatella conorum-piceae* (Tubeuf) Steyaert. The most frequently isolated fungus in the Scots pine seedlings was a species determined as *Didymellaceae* sp. The latter species occurred in all tested Scots pine trees and is identical with *Didymella* sp. found in this study. In another study, 18 filamentous endophytic fungal species were isolated from woody stem tissue of three *Fagus sylvatica* saplings (6–17-year-old, mean: 9.6 years old; Langer and Bußkamp 2021). With the exception of the white-rot fungus *Coprinellus micaceus* (*Agaricaceae and Basidiomycota*), all isolated beech endophytes of the aforementioned study were members of the *Ascomycota*. A reason for the absence or low frequency of endophytic *Basidiomycota* on ash might be the young age or the vigour of the plants. So far, the chronological genesis of the tree endophyte community of seedlings has not yet been

Table 2 Comparison of the seven treatment groups (ten saplings per group) in respect to number of fungal isolates, isolated taxa in total and per tree, as well as most continuously isolated fungal species (ash = *Fraxinus excelsior*; sycamore = *Acer pseudoplatanus*)

Inoculated *C. corticale* strain (NW-FVA) / Treatment group	Original host of inoculated strain	No of isolates	Taxa isolated in total	Isolated taxa per tree (mean)	Most continuous isolated species							
					First	Saplings (%)	Second	Saplings (%)	Third	Saplings (%)
5572	Sycamore	70	19	1–6 (4.2)	*Dichotomopilus* sp.	60	*Boeremia exigua*	50	*Chaetomium* sp.	40
5889	Sycamore	62	16	2–4 (3.6)	*Boeremia exigua*	90	*Dichotomopilus* sp.	60	*Alternaria* sect. *Alternata*	30
5932	Ash	51	12	1–4 (3.2)	*Alternaria infectoria*	90	*Alternaria* sect. *Alternata*	50	*Boeremia exigua*	30
6116	Ash	59	18	2–6 (3.9)	*Boeremia exigua*	70	*Alternaria infectoria*	50	*Alternaria* sect. *Alternata Diaporthe* cf. *rudis*	40
6181	Ash	59	8	1–5 (3.3)	*Boeremia exigua*	70	*Dichotomopilus* sp.	60	*Diaporthe* cf. *rudis*	30
Mock- control		72	20	1–8 (4.5)	*Boeremia exigua*	90	*Diaporthe* cf. *rudis*	50	*Diaporthe* sp. (*eres*-Gr.)	40
Untreated control		65	16	1–7 (3.4)	*Boeremia exigua*	100	*Alternaria infectoria*	40	*Diaporthe* cf. *rudis*	40
	In total	438	Mean	3.7						

fully elucidated. However, it is known that leaves of forest trees do not harbour endophytes at the time of budding (Toti et al. 1993; Scholtysik et al. 2013). Moreover, it is assumed that fungal tree endophytes are vertically (Rodriguez et al. 2009) or horizontally transmitted by spores (Wilson and Carroll 1994; Helander et al. 2007; Scholtysik et al. 2013). Horizontally transmitted endophytes are species-rich and tend to colonise tissues in a broad range of host species (Helander et al. 2007; Rodriguez et al. 2009; Suryanarayanan 2011). The composition of endophytic assemblages in forest trees differ between geographically distinct locations (Peršoh et al. 2010, 2013; Guerreiro et al. 2017, 2022), which indicates that these assemblages are influenced by environmental factors such as temperature and humidity (Zimmerman and Vitousek 2012). Furthermore, common ash leaves, shoots and stems have different assemblies of fungal endophytes (Unterseher et al. 2007). This explains why the species composition of ash endophytes differed in the study of Bilański and Kowalski (2022) compared to the results on woody tissues of ash stems. The latter authors isolated 97 different fungal taxa from asymptomatic leaf petioles of *F. excelsior* collected in southern Poland. *Ascomycota* accounted for 94.6% of these species, whereas 5.4% were *Basidiomycota*. The most abundant species was *Nemania serpens* (Pers.) Gray, which was isolated in 38.0% of the studied petioles followed by *Diaporthe eres* Nitschke (33.6%), *Fraxinicola fraxini* (Aderh.) Crous, M. Shen & Y. Zhang ter (26.4%), *Diaporthe* sp. 1 (20.4%), *Alternaria* sp. 1 (14.8%), *Colletotrichum acutatum* J.H. Simmonds (14.8%), *Nemania diffusa* (Sowerby) Gray (14.0%), *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (12.4%), and *Colletotrichum* sp. (12.4%). *Nemania* serpens was also isolated in this study but significantly less frequently (0.2%), whereas *Boeremia exigua* (26.5%) and *Dichotomopilus* sp. (11.4%) were the most frequently observed species in the stems of the ash saplings.

The total amount of endophytic species isolated from woody ash tissue in this study (32) is comparable to the results of previous investigations (Butin and Kowalski 1986; Kowalski and Kehr 1992; Unterseher et al. 2005). In contrast to findings that *Diaporthales* dominate endophytic fungal communities in angiosperms (Sieber 2007), our results showed that the endophytic community of ash saplings from forest tree nurseries was dominated by *Pleosporales* (58.4%), followed by *Sordariales* (13.5%), *Hypocreales* (9.4%), and *Diaporthales* (8.7%). The predominance of *Pleosporales* in the endophytic community of the examined ash saplings is in agreement with the results of Blumenstein et al. (2021) and Bußkamp et al. (2021) on Scots pine seedlings. In addition to the differences in geography, tree age and tissue type between the

above-mentioned studies, the origin of the trees could have an influence on the detected endophytic communities, since nursery trees were used for the isolation of endophytes in this study. Lade et al. (2022) found that the nursery origin had significant effects on the fungal community structure in graft and root tissues of grapevine. However, whether these findings can be transferred to forestry plants needs to be tested, considering that grapevines are intensively processed in the nursery.

Although the pathogenic behaviour of *C. corticale* on European ash could not be demonstrated in this study, the actual role of this fungus in the necrotic tissue of the sampled trees remains unknown and needs to be further investigated. Accidental colonisation in this newly discovered fungus-host association can be ruled out as it is highly unlikely that such a case would be observed three times in two different locations. In contrast, infection with *Hymenoscyphus fraxineus* and subsequent fungi in ash stem collar necrosis and the host weakness caused by these fungi may have provoked and facilitated the entry of the wound parasite *C. corticale*. However, as an increase in SBD severity on sycamore has been recognised in the recent years, due to extended drought events, the discovered fungus-host relationship with ash needs to be taken into account when predicting the spread of *C. corticale,* and SBD outbreaks in the near future in the face of climate change.

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Author contributions SP sampled the *Cryptostroma corticale* strains from European ash. GL and JB planned and conducted the pathogenicity tests. GL performed the isolation of the endophytes, the necroses measurements, and the re-isolation of the fungi. SB performed the DNA-Isolation, PCR, and species identification. GL analysed the data and wrote the first draft. SB, JB, and SP contributed to the manuscript.

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Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The authors declare that they have no other conflict of interest.

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