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ORIGINAL ARTICLE

Endophytic community in juvenile *Acer pseudoplatanus* and pathogenicity of *Cryptostroma corticale* and other associated fungi under controlled conditions

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Abstract

The causative agent of sooty bark disease, *Cryptostroma corticale*, has, for some years, caused increased damage to maples (*Acer* spp.) in Germany and throughout Europe. It has been stated that this pathogen can occur latently in *Acer*. In this study, the composition of endophytic fungi of woody stem tissues of sycamore (*Acer pseudoplatanus*) saplings were analysed in order to investigate whether *C. corticale* is present in young, visually healthy maple trees from natural regeneration. The most abundant taxa of the 30 isolated endophytes were *Diaporthe* cf. *rudis* and *Petrakia irregularis*. An association of five fungal species (*Biscogniauxia mediterranea*, *Coniochaeta velutina*, *Gibellulopsis catenata*, *Neocucurbitaria quercina*, *Tangerinosporium thalitricola*) with *A. pseudoplatanus* was reported for the first time. *Cryptostroma corticale* was not present in the juvenile sycamore stems. Furthermore, its pathogenicity was studied in comparison to other pathogens associated with *A. pseudoplatanus*, namely *Diplodia mutila*, *Dothiorella* sp., *Nectria cinnabarina*, *Neonectria coccinea*, *Neonectria punicea* and *Stegonsporium pyriforme*. The longest necroses were induced by *C. corticale* followed by *N. coccinea*. In a trial with a *C. corticale* spore suspension sprayed directly on stem bark, the fungus could be re-isolated from woody tissue, however necroses were evoked only when the bark was wounded prior to infection. The results from the experiments presented here function as additional evidence for the ability of *C. corticale* to establish endophytically within the host and, in particular, for the ability of the fungus to establish a successful infection/entrance through intact bark.

Keywords Fungal endophytes · Pathogenicity tests · Sooty bark Disease · Sycamore maple · Aggressiveness · Infection pathway

Introduction

Sycamore or sycamore maple (*Acer pseudoplatanus* L., Sapindaceae), is a broadleaved tree species of high economic and ecological value that commonly grows in mixed forests of Central Europe (Hein et al. 2009; Leslie 2005; Spiecker et al. 2009). In Europe, use of sycamore trees in managed forests is expected to increase due to their high adaptability to a wide range of site conditions, high competitiveness and good natural regeneration in the context of evident climatic changes (Simon et al. 2010; Vacek et al. 2017).

Due to the known high number of associated fungal species, sycamore is also considered a "fungal-friendly" tree species (Butin and Kowalski 1986). However, sycamore maple is associated with a number of serious bark- and

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vascular diseases in Europe. Nectria-canker (causal agent *Nectria cinnabarina* (Tode) Fr.), Verticillium blight (causal agent *Verticillium* spp.) or diseases caused by *Armillaria* spp., *Kretzschmaria deusta* (Hoffm.) P.M.D. Martin, *Laetiporus sulphureus* (Bull.) Murrill, *Pholiota squarrosa* (Vahl) P. Kumm., and fungal-like organisms such as *Phytophthora* spp. constitute a considerable threat to the vitality and break resistance of sycamore trees. In addition, the following pathogens are known to be associated with sycamore: *Cryptostroma corticale* (Ellis & Everh.) P.H. Greg. & S. Waller, *Diplodia mutila* (Fr.) Mont. (Abdollahzadeh et al. 2014; Kuch et al. 2014), *Dothiorella* sp., *Neonectria coccinea* (Pers.) Rossman & Samuels (Langer et al. 2013), *Neonectria punicea* (J.C. Schmidt) Castl. & Rossman, and *Stegonsporium pyriforme* (Hoffm.) Corda (Tomiczek et al. 2005; Voglmayr and Jaklitsch 2008).

In particular, the invasive pathogen *C. corticale* (Ascomycota), inducing the bark- and vascular sooty bark disease (SBD), is widely distributed in Europe and has an increasing importance as a fatal maple pathogen. Sycamore maple was the most affected tree species in the disease outbreaks of SBD in Central Europe following the exceptional warm and dry year of 2018 (Bork 2018; Delb et al. 2016; Rohde et al. 2019; Schlößer et al. 2023; Wenzel et al. 2019).

The advanced phase of the disease is characterized by the mass production of conidial spores between different layers of the bark, which peels off and releases the spores. Prolonged exposure to *Cryptostroma* spore masses poses a potential hazard to humans, particularly to woodworkers, as it can provoke hypersensitivity pneumonitis (Farmers lung disease; Spoerke and Rumack 1994; Braun et al. 2021).

Based on phylogenetic studies, the anamorphic plant pathogenic fungus *C. corticale* could by be assigned to the *Xylariales*, in the family *Xylariaceae* Tul. & C. Tul., or rather *Graphostromataceae* M.E. Barr, J.D. Rogers & Y.M. Ju family (Ju et al. 1998; Koukol et al., 2014). As expected from a xylarialean fungus, *C. corticale* also shows a potential for wood degradation (Ogris et al. 2021). The closest relatives are species of *Biscogniauxia* Kuntze and *Graphostroma* Piroz. (Koukol et al., 2014).

Cryptostroma corticale has often been described as an example for latent pathogens (Enderle et al. 2020; Kelnarová et al. 2017), which can remain symptomless in the woody tissue over an extended period of time following infection (endophytic/latent phase; Verhoeff 1974; Schulz and Boyle 2005). However, clear evidence for endophytism of *C. corticale* in maple was not provided until recently when Schlößer et al. (2023) isolated this fungus from symptomless woody tissue of *A. pseudoplatanus* in healthy forest stands with no signs of SBD in the vicinity. Previous reports of isolations from symptomless woody tissue originated from trees already showing symptoms of SBD, such as wood discoloration or defoliation (e.g. Kelnarová et al. 2017; Tropf 2020). In addition, conidial spores of *C. corticale* produced on stored wood logs originating from regions with no known disease cases at the time have been reported (Plate and Schneider 1965; Cech 2019). In general, little is known about the endophytic fungal community within above-ground woody tissue of this host, as these fungi have rarely been addressed in the case of *A. pseudoplatanus* (e.g. Kowalski and Kehr 1992). The most extensive dataset about this particular community is provided by the study of Schlößer et al. (2023), who report a total of 91 different fungal taxa from 30 to 65 year-old trees in Germany. Several other studies about endophytic fungal communities of *A. pseudoplatanus* focus on other tissue types, such as leaves (e.g. Pehl and Butin 1994; Unterseher et al. 2007; Schlegel et al. 2018). Further studies on endophytic fungal communities were conducted on *Acer campestre* L., *A. platanoides* L., and *A. tataricum* subsp. *ginnala* (Maxim.) Wesm. (Qi et al. 2009, 2012), A. *macrophyllum* Pursh and *A. saccharum* Marshall (Vujanovic and Brisson 2002) or *A. truncatum* Bunge (Sun et al. 2011). Nevertheless, in all of the studies mentioned above, mature and established stands of trees with a significant history of possible infection events were sampled. Information on the early succession of the wood-inhabiting community of sycamore trees is inadequate due to the lack of data about endophytic fungi colonizing young maples.

In order to better understand the endophytic fungal communities in woody tissue of juvenile *A. pseudoplatanus* trees and to further reveal their particular role, i.e. pathogenic or latently pathogenic, as well as to understand the pathogenic potential of *Cryptostroma corticale*, the aims of this study were (1) to investigate the endophytic fungal community in *A. pseudoplatanus* saplings originating from natural regeneration and (2) to compare aggressiveness of *Cryptostroma corticale* within the plant stem with those of other important pathogens of *A. pseudoplatanus*. Additionally, (3) to compare the ability of *C. corticale* spores to penetrate through wounded and unwounded bark tissue.

Materials and methods

Sycamore saplings were sampled in the summer of 2020 in Hesse, Germany (UTM 32U 499,050 5,588,838), in a forest stand containing mainly *A. pseudoplatanus* with natural regeneration. Furthermore, *Fagus sylvatica* L. and to a lower extent *Fraxinus excelsior* L., *Betula pendula* Roth, *Quercus* sp. and *Picea abies* (L.) H.Karst. were present as well. *A. pseudoplatanus* saplings showing no visible external damage were selected for sampling. Sycamore saplings with an age of approx. 10 years and a

stem diameter between 1.5 and 3 cm were sampled. The trees were cut between 10 and 20 cm and again at 1.1 m above the ground, so that sticks with ca. 1 m length were obtained as a sample. If there was visible wood discoloration the tree was discarded. In total, 130 sapling stem sticks (SSS) were sampled and stored for later processing in water filled plastic containers and covered with plastic bags to avoid dehydration.

Isolation of fungal endophytes

Ten of the 130 SSS were randomly selected for isolation of endophytic fungi from wood and bark. These SSS were brush cleaned under running tap water. Forty-five pieces of wood, 15 from the top, middle and bottom of each sample SSS, were surface sterilised (60 s in 70% ethanol, 5 min in 4% NaOCl, 60 s in 70% ethanol) and dried on sterile filter paper. Wood chips, partially with bark attached, were removed from the stems using a sterile scalpel and, for each, three chips were plated on Malt Yeast Peptone agar medium (MYP) plates (Langer 1994; Bußkamp et al. 2020). The plates were incubated at room temperature and diffuse daylight and checked every two to three days for fungal growth. Hyphal tips of developing fungi were transferred to MYP medium with a sterilised needle. Isolated fungi were grouped into morphotypes based on cultural and morphological features. At least one representative culture for each morphotype was preserved on MYP slants at 4 °C in the fungal culture collection of the Northwest German Forest Research Institute (NW-FVA), Göttingen, Germany.

DNA extraction and sequencing Genomic DNA of one randomly selected isolate per morphotype was extracted using the method of Keriö et al. (2020). The 5.8 S nuclear ribosomal gene with the two flanking internal transcribed spacers ITS-1 and ITS-2 were amplified using the primer pair ITS-1 and ITS-4 (White et al. 1990). The reaction mixture for PCR contained 1 µL of DNA template (1/10 dilution), 2.5 µL 10x buffer (CoralLoad, QIAGEN, Germany), 1.25 µL of each primer (10 mM), 0.5 µL $MgCl_2$ (25 mM), 0.25 µL *Taq* polymerase (0.5U, QIAGEN, Germany) and 0.5 µL of 10 mM dNTPs. Each reaction was made up to a final volume of 25 µL with sterile water. DNA amplifications of ITS were carried out in a PCR-Thermocycler (BIOMETRA T-GRADIENT) programmed for an initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94, 52 and 72 °C for 30, 60 and 60 s, respectively, with a 10 min extension at 72 °C on the final cycle. The PCR products were visualised on a 1% agarose gel, washed using the NucleoSpin™ Gel and PCR Clean-up-Kit (Macherey-Nagel, Düren, Germany) and sequenced by GATC Biotech AG (Ebersberg, Germany). The DNA sequences generated in this study were deposited in GenBank (Tables 1 and 2).

Table 1 List of strains used for pathogenicity tests with accession number and origin. All strains were isolated from *Acer pseudoplatanus*

Taxon name	Strain No.	GenBank accession	Origin and date of isolation
Cryptostroma corticale	NW-FVA 4962	OP819900	Germany, Hesse, 2019
Diplodia mutila	NW-FVA 4947	OP819899	Germany, Hesse, 2019
Dothiorella sp.	NW-FVA 5344	OP819901	Germany, Hesse, 2019
Nectria cinnabarina	NW-FVA 0007	OP819896	Germany, Lower Saxony, 2009
Neonectria coccinea	NW-FVA 2478	OP819897	Germany, Lower Saxony, 2014
Neonectria punicea	NW-FVA 3096	OP819898	Germany, Schleswig-Holstein, 2016
Stegonsporium pyriforme	CBS 120522	OP819934	Austria, Vienna, 2006

Fungal identification

For all sequences generated, BLAST algorithm searches were performed on the NCBI GenBank (www.ncbi.nlm.nih.gov, Altschul et al. 1997) database and genus or species determination of the sequenced isolates was conducted using the method of Bien and Damm (2020). Identifications were based primarily on an evaluation of nucleotide differences in the respective ITS alignments to selected reference sequences. Reference sequences were chosen based on close matches with strains identified to species level, preferably of ex-type strains or strains deposited in reputable culture collections, such as the collection of the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands. Species are identified with high certainty, if their respective ITS sequence show ≤ 4 nucleotide differences to the reference sequences of not more than one species. Low certainty identifications are indicated with "cf.", if the respective ITS sequences differ in 5–10 nucleotides to reference sequences of not more than one species. Isolates are identified to genus level only, if the respective sequences differ in > 10 nucleotides from the closest named reference strain or matched with sequences of more than one species. Appropriate family or order names are applied in case a strain matches with no named reference strain or with reference strains belonging to more than one genus. All identifications were verified through microscopic observation of strain morphology. Morphotypes for which the sequencing procedure failed were identified based on their morphology only, following adequate identification literature and keys.

Table 2 List of taxa isolated from symptomless woody stem tissue of *Acer pseudoplatanus*, with number of isolates and positive trees, representative strains and GenBank accession numbers. [N] New report for the host *A. pseudoplatanus*

Taxon	No. of isolates	No. of positive trees	Strain No. NW-FVA	GenBank accession
Ascomycota				
*Angustimassarin*a sp.	3	3	5635	OP819917
Aureobasidium sp.	3	2	5637	OP819919
Biscogniauxia mediterranea[N]	2	2	5631	OP819914
Biscogniauxia nummularia	1	1	5628	OP819920
Cladosporium sp.	7	6	5612	OP819902
Coniochaeta velutina[N]	6	6	5623	OP819909
Cytospora sp.	1	1	5632	OP819915
Diaporthe pustulata	10	5	5620	OP819906
Diaporthe cf. *eres*	1	1	5645	OP819925
Diaporthe cf. *nobilis* A	16	5	5615	OP819904
Diaporthe cf. *nobilis* B	2	1	5616	OP819905
Diaporthe cf. *rudis*	95	10	5613	OP819903
Gibellulopsis catenata[N]	1	1	5736	OP819927
Morchella sp.	1	1	5643	OP819924
Neocucurbitaria quercina[N]	2	1	5636	OP819918
Paraphaeosphaeria neglecta	12	5	5624	OP819910
Petrakia irregularis	37	10	5622	OP819908
Pezicula sp.	15	5	5630	OP819913
Plagiostoma sp.	1	1	5738	OP819929
Pseudopithomyces sp.	1	1	5737	OP819928
Stegonsporium pyriforme s.l.	17	7	5627	OP819912
Tangerinosporium thalictricola[N]	1	1	5640	OP819921
Diatrypaceae sp.	2	1	5743	OP819933
Didymellaceae sp.	1	1	5621	OP819907
Pleosporales sp. 1	2	2	5625	OP819911
Pleosporales sp. 2	5	3	5642	OP819923
Xylariales sp.	3	3	5641	OP819922
coelomycete 1	3	2	5742	OP819932
coelomycete 2	1	1	5735	OP819926
Basidiomycota				
Coprinellus sp.	1	1	5740	OP819931
Peniophora sp.	1	1	5739	OP819930
Phanerochaete sp.	1	1	5633	OP819916
Mucoromycota				
Mucor sp.	1	1	5644	–

Pathogenicity tests and under bark inoculation test

In total, 80 SSS were selected for the pathogenicity tests (Fig. 1a) in vivo according to Henle-Koch postulates (Evans 1976), using strains of seven different Ascomycota (Table 1). The phytopathogenic species were selected because they have been associated with dieback symptoms in sycamore maples in unpublished, investigated cases in north-western Germany, or because they are considered to be pathogenic to sycamore (Langer et al. 2013).

Six of the fungal strains used (*C. corticale*, *D. mutila*, *Dothiorella* sp., *N. cinnabarina*, *Neo. coccinea*, and *Neo. punicea*) were isolated from diseased *A. pseudoplatanus* between 2009 and 2019 in Germany and stored in the culture collection of the NW-FVA. One strain of *Stegonsporium pyriforme* (Hoffm.) Corda = *Prosthecium pyriforme* Jaklitsch & Voglmayr (CBS 120,522) was retrieved from the Westerdijk Fungal Biodiversity Institute (CBS), Utrecht, the Netherlands. Identity and origin of all strains used for pathogenicity testing is listed in Table 1.

After surface sterilisation with 70% ethanol the SSS were wounded at 70 cm height from the basal end through the phloem and cortex tissue with a sterilised 5 mm diameter cork-borer and inoculated with a colonised agar plug (5 mm diameter, Fig. 1b) grown for one week on MYP medium. The infection side was covered with the removed bark piece and wrapped with Parafilm® (Fig. 1c). The tops of the SSS were sealed by double dipping in liquid paraffin. Ten SSS were inoculated with each fungal strain, respectively. For mock controls, ten stems were treated with uncolonized MYP plugs. A further ten SSS were left untreated as negative controls. All SSS were stored in 10 l plastic containers filled with tap water (exchanged weekly, Fig. 1a). Every second to third day, basal ends of the incubated SSS were re-trimmed diagonally to increase water uptake. The temperature profile during storage was recorded using a HOBO Pro v2 (Onset, Bourne, MA, US) data logger. Arithmetic mean air temperature during the experiment in greenhouse was 21 °C (min 12 °C, max 30 °C).

After three weeks, the bark of the stems was peeled off and lesions visible in phloem or the xylem tissue were measured horizontally and vertically. Re-isolations were made from the developing necroses and the resulting cultures identified by ITS, according to the procedures described above.

Pathogenicity tests with a *C. corticale* spore suspension

Conidial spores of *C. corticale*, collected from conidial masses of a recent disease outbreak (Germany, Hesse, UTM 32U 502,572 5,582,007) on *A. pseudoplatanus*,

Fig. 1 *Acer pseudoplatanus*-sapling stem sticks (SSS) stored in 10 l plastic containers filled with tap water (**a**), wounded through the phloem and cortex tissue with a cork-borer and inoculated with a colonized agar plug (**b**) and wrapped with Parafilm® (**c**)

were suspended in 1% Tween solution filtered twice through sterilised cotton wool, and the solution filled into a 100 ml squirt bottle. The approximate spore density of the suspension, measured spectrophotometrically, was 8.24×10^8 conidia per ml suspension.

Sycamore SSS were wounded at 70 cm height using a sterilised 5 mm diameter cork-borer. Ten wounded as well as ten unwounded sample SSS were inoculated through two spray pumps with the spore suspension and the sprayed area was covered with Parafilm for one week. For negative controls each ten wounded and unwounded SSS were sprayed with a 1% Tween solution and the sprayed area also covered with Parafilm for one week. Subsequent treatment of all trees and lesion analyses as were conducted as described above and re-isolations of fungi were according the method described in the paragraph "Isolation of fungal endophytes".

Statistical analysis

The significance of differences in necrosis length associated with differences in fungal taxa were assessed using ANOVA and the Tuckey HSD post hoc test. R version 3.6.2 (R Core Team 2019) was used for all data analyses.

Results

Isolation and identification of endophytic fungi

In total, 262 fungal strains were isolated from 450 chips (74% colonized) of symptomless wood and bark of *Acer pseudoplatanus* originating from 10 different saplings and grouped into 33 morphotypes (Table 3). In 26% of the wood chips no fungal growth was observed and yeasts grew out of 20% of the samples. Sequencing of the ITS sequence region was successful for 28 morphotypes (Table 3). In total 26 morphotypes could be identified to genus or species level. Nine of these were identified to species level with high certainty, while one morphotype was identified without certainty ("cf."). In 13 cases genus names were applied, as the respective ITS sequences showed none or only few differences to more than one reference sequence. In a further three cases, no ITS sequence data were available and morphological observation only allowed identification to genus level as morphological features are not sufficient for discrimination between species in the respective genera. In five cases family (2) or order (3) names were applied based on BLAST searches, while two morphotypes could not be identified, as no DNA sequence data was available and no informative morphological structures were produced in culture (coelomycete 1 and 2).

Table 3 Fungal isolates used for the pathogenicity tests in this study; NW-FVA Number and necrosis length (arithmetic mean of ten studied sapling stem sticks (SSS) in mm); Re-isolation: strains were isolated from woody tissue, after 3 weeks of incubation

Taxon	Strain No. NWFVA	Inoculation method	Necroses length in mm and standard deviation	Re-isolation
Cryptostroma corticale	4962	Mycelium	96.3 / 87,0	***Cryptostroma corticale***, *Sordaria* sp., *Penicillium* spp.
Diplodia mutila	4947	Mycelium	18.8 / 13.2	***Diplodia mutila***, Fungus sp.
Dothiorella sp.	5344	Mycelium	22 / 15.5	***Dothiorella*sp.**, *Xylaria longipes*
Nectria cinnabarina	0007	Mycelium	25.9 / 11.3	***Nectria cinnabarina***
Neonectria coccinea	2478	Mycelium	43 / 30.5	***Neonectria coccinea***, *Sordaria* sp.
Neonectria punicea	3096	Mycelium	29.4 / 21.1	***Neonectria punicea***, *Sordaria* sp., *Trichoderma* sp.
Stegonsporium pyriforme	5542; CBS 120522	Mycelium	29 / 16.9	*Diaporthe* sp., *Xylaria* sp., *Sordaria* sp.
control		MYP	24.1 / 18	*Diaporthe* sp., *Trichoderma* sp.
control		untreated	0	*Biscogniauxia nummularia*, *Stegonsporium pyriforme* s.l.

Fig. 2 Sapling stem sticks (SSS) treated with MYP (**a**), with *C. corticale* (**b**) and *S. pyriforme* (**c**) 21 days after treatment

The majority of isolated taxa belong to Ascomycota (30 taxa, 90.9%), while two taxa belong to Basidiomycota (0.6%) and one to Mucoromycota (0.3%). The most abundant taxa were *Diaporthe* cf. *rudis* (95 times isolated) and *Petrakia irregularis* Aa (37 times isolated), both isolated from all ten sample trees. Seven species have been isolated ten times or more (*Diaporthe* cf. *rudis*, *D. pustulata* Sacc., *Diaporthe* cf. *nobilis* B, *Paraphaeosphaeria neglecta* Verkley, Riccioni & Stielow, *Petrakia irregularis*, *Pezicula* sp., and *Stegonsporium pyriforme* s.l.). Fourteen species were isolated only once (Table 3*). Biscogniauxia mediterranea* (De Not.) Kuntze, *Coniochaeta velutina* (Fuckel) Cooke, *Gibellulopsis catenata* Giraldo López & Crous, *Neocucurbitaria quercina* (Kabát & Bubák) Wanas., E.B.G. Jones & K.D. Hyde, and *Tangerinosporium thalictricola* L. Lombard & Crous are reported for the first time for *A. pseudoplatanus* (Table 3).

Underbark inoculation test

All previously inoculated fungi, except for *S. pyriforme*, were re-isolated after completion of the experiment (Table 2). *Stegonsporium pyriforme* s.l. was isolated from untreated controls (Table 2).

All untreated control shoots remained healthy and symptomless. All inoculated shoots showed symptoms of infection (discolouration). Stems treated with MYP also showed discolouration (Fig. 2a). The longest necroses were induced by *C. corticale* (Table 2; Figs. 2b and 3). The length of necroses evoked by inoculation with *C. corticale* differed significantly from all other necroses (except *N. coccinea*; Table 2; Fig. 3). The necroses caused by strains of the *Botryosphaeria* family (*D. mutila* and *Dothiorella* sp.) were on average smaller than those of the control (inoculated with MYP-medium). There were also differences in the depth/quality of the necroses: when inoculated with *C. corticale*, some entire stem cross-sections showed dark discolouration. The necroses due to *D. mutila* and *Dothiorella* sp. were not only superficial, but showed deeper black discolouration, while the necroses caused by the inoculation with *S. pyriforme* also showed discolouration of a darker colour (Fig. 2c).

Pathogenicity tests with a *C. corticale* spore suspension

The longest necroses were induced with the combination of wounding and inoculation with *C. corticale* (Table 4;

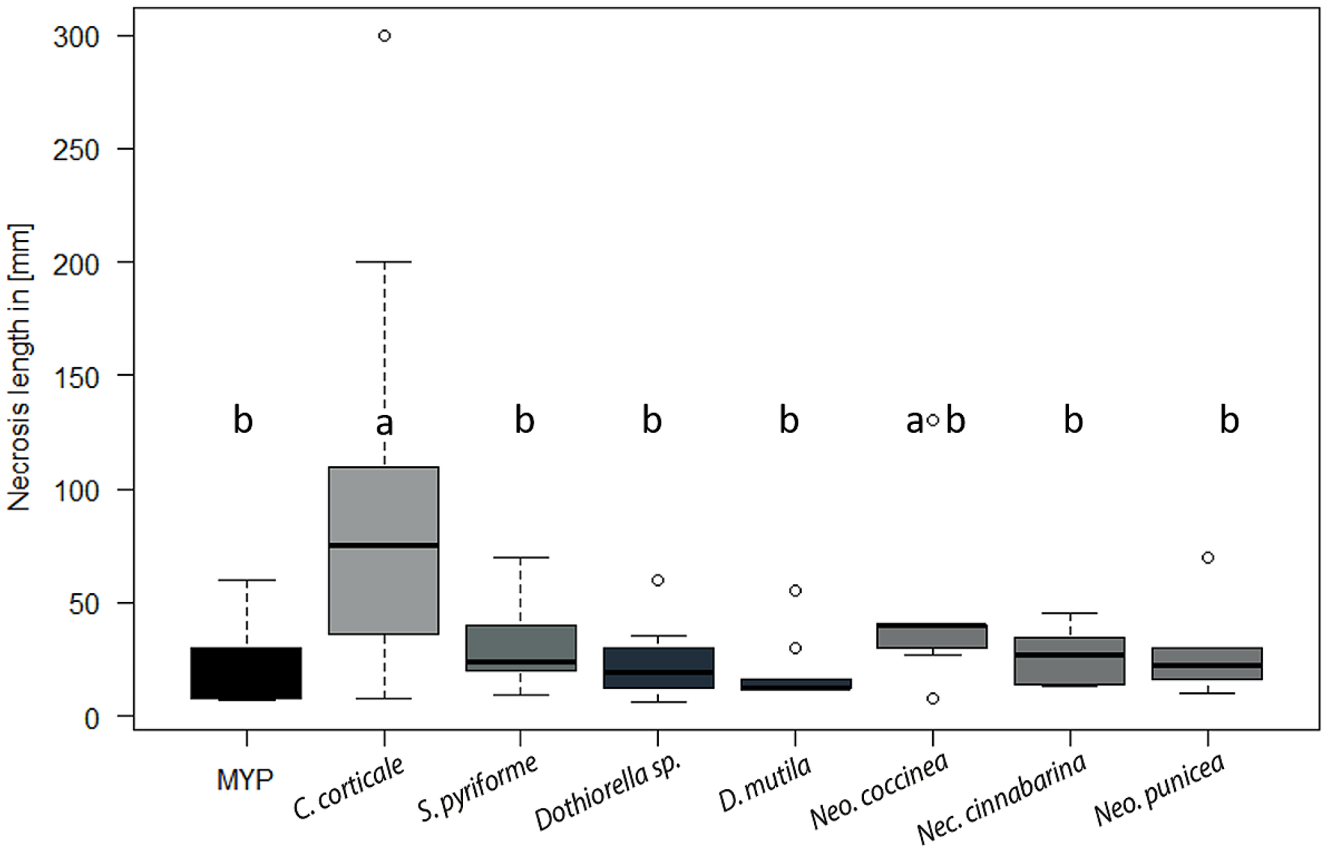

Fig. 3 Necrosis caused on *Acer pseudoplatanus* twigs by different species within 21 days. Boxplot of necrosis length n = 10 sapling stem sticks (SSS) per strain analysed. Different letters (**a-b**) indicate significant differences at the 0.01 probability level

Table 4 Necrosis length after tests with a *C. corticale* spore suspension (arithmetic mean and standard deviation of ten studied sapling stem sticks (SSS) in mm); Re-isolation: strains were isolated from woody tissue, after 3 weeks of incubation

Taxon	Inoculation method	Necroses length in mm and standard deviation	Re-isolation
Crypto-stroma corticale	Conidia, wounded	65.4 / 66	***Cryptostroma corticale***, *Biscogniauxia nummularia*
Crypto-stroma corticale	Conidia, not wounded	0	***Cryptostroma corticale***, *Biscogniauxia nummularia, Sordaria* sp., *Trichoderma* sp.
control	Tween, wounded	10.8 / 2.9	*Jackrogersella cohaerens, Fusarium* sp.
control	Tween, not wounded	0	*Xylaria* sp., Fungus sp.

Fig. 4). Minor wounds were induced by wounding and tween treatment. No necrosis/discolouration was observed in the treatment with *C. corticale* without prior wounding. Both *C. corticale* and *Biscogniauxia nummularia* were isolated from samples treated with the *C. corticale* spore suspension, irrespective of the wounding procedure. *Jackrogersella cohaerens* and a *Fusarium* sp. (with wounding) as well as a *Xylaria* sp. and an unidentified fungus (without wounding) were isolated from samples treated with the tween solution (Table 4).

Discussion

Since the first record of *C. corticale* in Germany in 1964 (Plate and Schneider 1965), the causal agent of sooty bark disease has spread throughout German forests (Schlößer et al. 2023). It is now known that *C. corticale* can also be detected latently in woody tissues of adult healthy sycamore (Schlößer et al. 2023). The question of whether this pathogen also occurs in juvenile sycamores was the aim of our investigations and can be answered in the negative, at least for the sycamore saplings examined.

Endophytic fungal community in *A. pseudoplatanus* saplings

In this study, 33 fungal taxa were isolated from symptomless woody tissue of juvenile *Acer pseudoplatanus* trees, which corresponds with previous investigations, in which 10 52

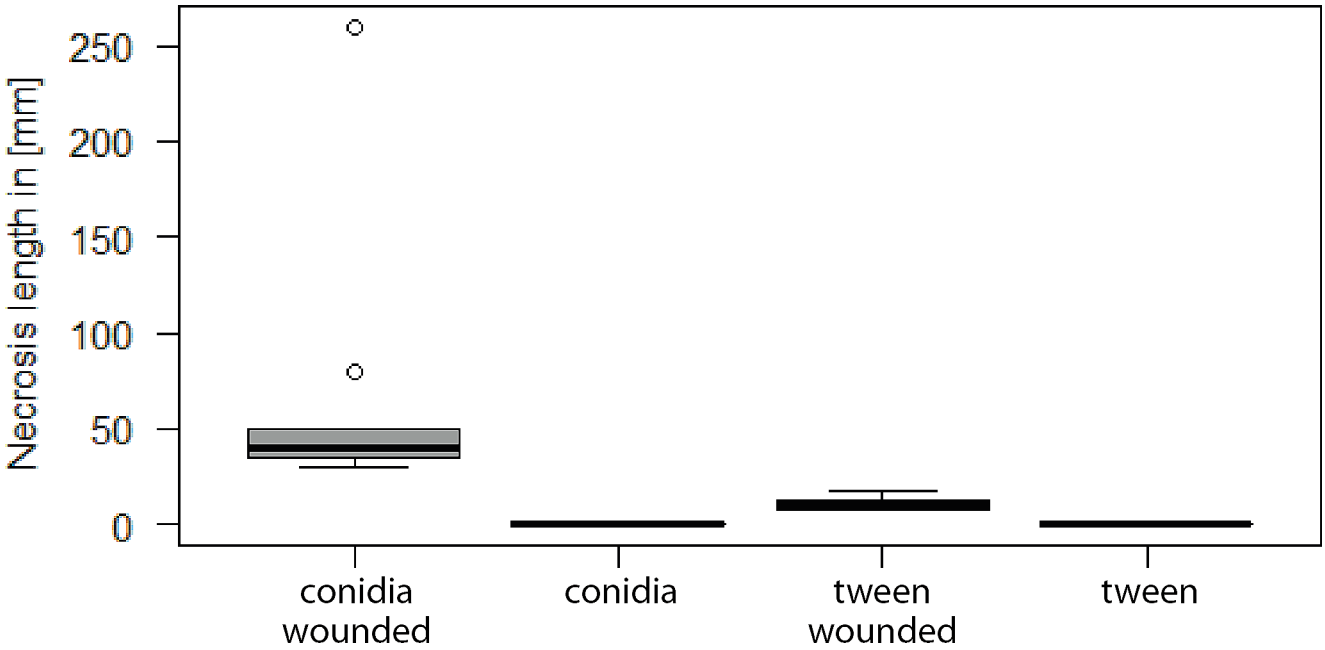

Fig. 4 Necrosis caused on *Acer pseudoplatanus* sapling stem sticks (SSS) by *Cryptostroma corticale* conidial solution on wounded and unwounded bark tissue within 21 days. Boxplot of necrosis length n = 10 SSS per treatment analysed

different taxa were identified (Butin and Kowalski 1986; Kowalski and Kehr 1992; Unterseher et al. 2005; Brglez et al. 2020a). These results illustrate that even very young *A. pseudoplatanus* trees are inhabited by a diverse endophytic community. In comparison to the findings of Schlößer et al. (2023) from woody stem tissues of older sycamore trees (30–65 years old), which revealed 91 endophytic fungal species, the endophytic community of juvenile trees investigated here has fewer taxa. This difference in diversity can be explained by the larger sampling size and the higher number of sample sites studied by Schlößer et al. (2023). However, the 33 taxa found, can only express a small picture of the whole potential endophytic mycobiome, as the endophytic diversity in this tree species can be expected to rise with increasing plant age (Qi et al. 2012).

It can be assumed that as the sample size and number of sample plots increases, more taxa, particularly those with lower frequency, should be detected in young stands (Bien and Damm 2020). This assumption is further supported by the results of this study, which included the detection of additional five fungal taxa (*Sordaria* sp., *X. longipes*, *Trichoderma* sp., *Jackrogersella cohaerens, Fusarium* sp.) through the process of re-isolations during the pathogenicity testing.

Also Schlößer et al. (2023) have found that the composition of the sycamore endophytes differed significantly between the sampled forest plots, with very little overlap between the sites. In contrast to the results of Schlößer et al. (2023), C. *corticale* could not be isolated as endophyte from the studied woody tissues of the sycamore saplings. Several factors could explain its absence. In case of low frequency of the fungus, it could have been overlooked due to a comparatively small sample size. The chance of colonization of the trees by *C. corticale* increases with time and, accordingly, host age. Additionally, colonization requires an inoculum source and suitable conditions for spore release in the vicinity. The trees sampled, however, were purposefully chosen at a very young age, in order to ensure that they were completely symptom-free. Given the short life span of the studied trees, said conditions might not have occurred within this time frame.

Remarks on the identification process and selected endophytic species

The taxa isolated in this study could be identified to a different extent. For fungal identification, searches with ITS sequences were conducted as this is common practice in similar studies aiming for fungal community elucidation (Hofstetter et al. 2019). Results were then confirmed through morphological observation. Although the ITS region is considered to be the universal barcode region for fungi and the most commonly sequenced locus in mycology, it is not suitable for species delimitation in each genus due to different inter- and intraspecific variability (Hughes et al. 2009; Kiss 2012; Schoch et al. 2012). Morphological identification of fungal species, on the other hand, is in many cases limited due to a lack of identification-relevant features in general or in the cultures studied, or due to phenotypic plasticity or cryptic speciation (Slepecky and Starmer 2009; Muggia

et al. 2014; Chethana et al. 2021). In order to provide clear information about the level of certainty on the reported identifications, taxon designation based on the procedure of Bien and Damm (2020) was utilized. In twelve cases, identification was limited to genus level due to ITS concordance with more than one species and a lack of clear discriminating morphological features in the culture.

Isolates of *Stegonsporium* could be assigned to the species complex of *S. pyriforme* s.l. as comparisons with ITS sequences alone and morphological features do not allow a further distinction between *S. protopyriforme*, *S. pseudopyriforme* and *S. pyriforme* (Voglmayr and Jaklitsch 2014).

The most diverse taxon isolated in this study is represented by strains of *Diaporthe*. Five different species were distinguished, with *Diaporthe* cf. *nobilis* A, *Diaporthe pustulata* and *Diaporthe* cf. *rudis* being most abundant. Species of *Diaporthe* show wide geographic distribution and host range, however exact distribution and host specificity for most is unknown (Sun et al. 2011; Udayanga et al. 2011). They are frequently reported as phytopathogens, endophytes in leaves and stems, saprobes on decaying wood and leaf litter or as parasites in humans and other mammals (Udayanga et al. 2011, 2012). *Diaporthe pustulata* was originally described from *A. pseudoplatanus* (Gomes et al. 2013) and was further found associated with this species, however isolated from dead twigs and branches (Brglez et al. 2020; Butin and Kowalski 1986). Due to the high diversity of this genus and in part unclear species differentiation in many cases, multi-locus DNA examination is required for explicit species identification (Gao et al. 2017). This fact is illustrated in this study, as three out of four taxa for which ITS sequence data is available could not be assigned to one particular species with certainty, due to unclear placement within insufficiently discriminated species complexes. Particularly, as revealed through a preliminary phylogenetic analysis containing strains of *Diaporthe* species retrieved from GenBank (data not shown), the strains NW-FVA 5615 and NW-FVA 5616 express an affinity with a number of different reference sequences assigned to *Diaporthe nobilis* Sacc. & Speg. However, the ITS sequences of these strains show considerable nucleotide differences (13 nucleotide difference, 97.6% identity). As a consequence, both strains are distinguished here as *Diaporthe* cf. *nobilis* A and B, respectively. The reference strain of *Diaporthe* cf. *nobilis* B, shows high concordance with strains isolated from dead branches of *A. pseudoplatanus* in Slovenia designated as *Diaporthe* sp. 1 (Brglez et al. 2020).

Several taxa identified to genus level in this study correspond to the findings of Schlößer et al. (2023), which allows direct ITS comparison. For example, the ITS sequences of strains NW-FVA 5637 (*Aureobasidium* sp.) and NW-FVA5632 (*Cytospora* sp.) isolated in this study, are identical with the strains NW-FVA6586 (*Aureobasidium* sp.) and NW-FVA 6237 (*Cytospora* cf. *populina*), respectively, isolated by Schlößer et al. (2023). On the other hand, strain NW-FVA 5635 isolated here and assigned to *Angustimassarina* sp., differs in 8 nucleotides (98.3% identity) to the strain NW-FVA 6253 (*Angustimassarina* sp.) isolated by Schlößer et al. (2023). The *Diaporthe* strains NW-FVA 5632, NW-FVA 5645, and NW-FVA 5620 differ in two (99.6% identity), five (99.1% identity), and one (99.8% identity) nucleotides to the reference strains of *Diaporthe* cf. *rudis*, *Diaporthe* cf. *eres* and *Diaporthe* cf. *pustulata*, isolated by Schlößer et al. (2023). The variability of the ITS region in each of the relevant taxa determines whether the aforementioned strains belong to concordant species, whose elucidation would necessitate more in-depth research.

Taxa identified with certainty to species level were subjected to a literature search for reported fungus-host associations with *Acer* or *A. pseudoplatanus*. *Biscogniauxia mediterranea* has been reported from *Acer rubrum* L., *Acer* sp. or *Acer* spp., however, *A. pseudoplatanus* has not been mentioned specifically (Ju et al. 1998; Nugent et al. 2005; Ragazzi et al. 2011). *Coniochaeta velutina* has been reported from different woody host plants worldwide (Damm et al. 2010; Johnová 2009; Shamoun and Sieber 2000), including *A. saccharum* in Canada (Basham et al. 1969). In Germany, the fungus was identified from *Alnus viridis* (Schmid-Heckel 1988). *Gibellulopsis catenata* was described from the cervical swab of a mare in Germany (Giraldo and Crous 2019). On the GenBank database, one further record is provided from the dust of a mattress in Belgium (OW984154; P Becker unpubl. data). *Neocucurbitaria quercina* is typically reported from *Quercus* spp., but also from *Fraxinus pennsylvanica Marshall*, *Olea europaea* L., and sea water (Bilański et al. 2022; de Gruyter et al. 2010; Nigro and Ippolito 2000). Wanasinghe et al. (2017) discuss a possible anamorph-teleomorph connection of this species with *Neoc. acerina* Wanas., Camporesi, E.B.G. Jones & K.D. Hyde described from *Acer campestre*. However, the authors stress that the basis for this assumption is an unverified sequence of *Neoc. quercina*, which does not originate from type material. No report of *Neoc. quercina* or its basionym *Pyrenochaeta quercina* Kabát & Bubá could be found connecting the species to the host *A. pseudoplatanus*. *Tangerinosporium thalictricola* was described in 2016, isolated from *Thalictrum flavum* L. (*Ranunculaceae*) in the UK (Lombard et al. 2016). The only other record of this fungus derives from soil samples collected from a volcanic crater in China (Wang and Pecoraro 2021). Consequently, these five fungal species are reported here from the host plant *A. pseudoplatanus* for the first time. Furthermore, for the first time *T. thalictricola* is reported from the host genus *Acer* and *G. catenata* is reported from a plant host in general.

For most of the fungal species recognized in this study, their particular role within the wood-inhabiting community of sycamore or during community succession remains unclear. For a few fungal taxa isolated in this study, the ability for a lifestyle change from endophytic to pathogenic is known, for example for species of *Botryosphaeriaceae* (Desprez-Loustau et al. 2006; Slippers and Wingfield 2007) or *Diaporthe* (Gai et al. 2021; Gomes et al. 2013; Udayanga et al. 2014). For none of the fungal species recognized, connections to distinct disease outbreaks on sycamore maple are known, which indicates that the presence of the isolated fungi poses no current risk for sycamore maples in Germany. Only after carrying out individual pathogenicity testing would it be possible to make statements about the potential hazards of specific agents. However, the risk of disease development depends on multiple factors such as presence of pathogens and antagonists, environmental factors, plant vigour or stress. It is therefore difficult to assess the risk posed by the individual fungi, especially in the context of a changing environment.

Pathogenicity tests

Except for *S. pyriforme*, re-isolation of the inoculated pathogens according to the Henle-Koch postulates (Evans 1976) could be fulfilled for all tested fungal strains. Out of the seven tested fungal taxa, only *C. corticale* caused significant wounds and necroses. On average, five fungi (*Dothiorella* sp., *N. cinnabarina*, *Neo. coccinea*, *Neo. punicea*, *S. pyriforme*) caused slightly larger necroses than the control, although not statistically significant. One reason for this result could be the relatively short experimental time (21 days) for fungi to spread, establish and cause damage in wood. The comparably large necroses that developed in the control plants could be caused by a *Diaporthe* species that was isolated from the same tissues. Pathogenic behavior of the *Diaporthe* species, already growing endophytically (Gomes et al. 2013) inside the *Acer* tree before (the experiment, could have been triggered through the wounding procedure. *Cryptostroma corticale* shows a much higher virulence and speed of spread than the other pathogens tested, which may explain the apparent ability of the fungus to kill whole trees in a relatively short periods of time following the appearance of the first symptoms (see Enderle 2020). However, test conditions potentially more favorable for *C. corticale* than for the other pathogens tested (esp. temperature) may have caused the observed difference in virulence. The average air temperature in this experiment was 21 °C, while the in vitro optimum growing temperature for *C. corticale* is around 25 °C (Dickenson 1980).

Ogris et al. (2021) isolated *C. corticale*, together with other fungal species, from necroses in inoculated saplings representing 19 morphotypes in total. 49.2% of the 445 cultures obtained were identified as *C. corticale*. In comparison, in our study 22% of the re-isolates were identified as *C. corticale*. Additionally, Ogris et al. (2021) isolated *Fusarium* sp. (13.7%) and *Alternaria* sp. (10.1%) from the margins of the developed lesions in the bark. Other isolated strains belonged to the genera *Didymella*, *Trichocladium*, *Paraphoma*, *Chaetomium*, *Phomopsis*, *Paraphaeosphaeria*, *Pestalotiopsis*, *Cladosporium*, and *Arthriunum*. In our study, besides *C. corticale*, only one *Sordaria* species, *Penicillium* spp., *Trichoderma* sp. and *B. nummularia* were isolated from the necroses.

Neonectria coccinea caused the second largest necroses in our experiments. This fungus is primarily known as a beech pathogen. However, Kowalski and Materniak (2007) isolated the species, together with *N. cinnabarina*, from cankers on branches and trunks from dieback-infected sycamore trees in Poland. Additionally, after weather-related bark damage, *N. coccinea* appeared as a pathogen on sycamore maple in Austria, in which *S. pyriforme* was detected at the edge of the necroses (Cech 1995). Since *Neo. coccinea* could cause wounds in artificially inoculated sycamores, it was classified as a weak pathogen of sycamore, causing damage to pre-damaged sycamores (Gregory 1982).

In this experiment, only small necroses were observed on samples inoculated with *Stegonsporium pyriforme.* Although it could not be re-isolated, the type of necroses (deep, rather than superficial) indicates successful infection of this fungus. Re-isolation of *S. pyriforme* might not have been successful because the fungus grows very slowly on the culture medium. *Stegonsporium pyriforme* is characterized by Voglmayr and Jaklitsch (2008) as an opportunistic, moderately phytopathogenic fungus involved in branch dieback or twig blight on species of *Acer*. Tomiczek et al. (2005) described a dieback of branches and twigs by *S. pyriforme*, which is limited to a few centimetre, but can lead to the death of young plants. In Nageleisen (1994) *S. pyriforme* (as *Prosthecium pyriforme*) is mentioned on sycamore in France, whereas also branch dieback can be caused by. Branch dieback can be caused by *Prosthecium* spp. (Voglmayr and Jaklitsch 2008). *Neonectria punicea* and *S. pyriforme* have already been found to cause wounds on maple trees located in forest stands in North-western Germany (own observations).

In the current study *C. corticale* was re-isolated from wounded and unwounded SSS, which had been sprayed with a *C. corticale* spore suspension. In contrast, *C. corticale* was neither isolated from wood of symptomless SSS, nor from wood of wounded or unwounded SSS sprayed on with a non-*C. corticale* spore suspension. It is remarkable, that *C. corticale* was re-isolated when sprayed on uninjured SSS where no necroses occurred. This could mean that *C.*

corticale does not need wounds to penetrate the host and is able to remain as endophyte for a certain time. It is likely that an external host damage or loss of host vigour could trigger a lifestyle change to pathogenic behaviour of *C. corticale*. Bevercombe and Rayner (1984) suggested that stromal formation after a latent phase is promoted by access of air to the tissues in the context of bark and wood death.

In the past, it was assumed that *C. corticale* infects the tree through fresh wounds but not through intact bark and only occurs within necroses (Dickenson 1980). Unless *C. corticale* was already present in the branches before the experiment was carried out, this theory seems to be disproved. Other authors have also detected *C. corticale* in healthy tree tissue: Townrow (1953) reports about frequent isolation of *C. corticale* together with *Trichoderma viride* Pers and *Fusarium* spp. from discoloured wood, as well as from non-necrotised tissue, "about 2 inches away from the stain". The results of Kelnarová et al. (2017) indicate an endophytic life stage of *C. corticale*, as it could be detected from healthy wood extracted from trees showing external symptoms such as wood discoloration or defoliation. Probably the strongest evidence for endophytic behaviour of *C. corticale* was reported by Schlößer et al. (2023), who found *C. corticale* non-symptomatically in 26% of all observed, apparently healthy sycamores in different areas in Germany, some of which were at a considerable distance from diseased trees. The results of the experiment presented here serve as additional evidence of the ability of *C. corticale* to establish endophytically within the host and, in particular, of the ability of the fungus to establish a successful infection/entrance through intact bark.

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