Fungal Trunk Pathogens Associated With *Juglans regia* in the Czech Republic

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Abstract

Juglans regia L. (English walnut) trees with cankers and dieback symptoms were observed in two regions in the Czech Republic. Isolations were made from diseased branches. In total, 138 fungal isolates representing 10 fungal species were obtained from wood samples and identified based on morphological characteristics and molecular methods: *Cadophora novi-eboraci*, *Cadophora spadici*, *Cryptovalsa ampelina*, *Diaporthe eres*, *Diplodia seriata*, *Dothiorella omnivora*, *Eutypa lata*, *Eutypella* sp., *Peroneutypa scoparia*, and *Phaeoacremonium sicilianum*.

Pathogenicity tests conducted under field conditions with all species using the mycelium-plug method indicated that *Eutypa lata* and *Cadophora* spp. were highly virulent to woody stems of walnut. This is the first study to detect and identify fungal trunk pathogens associated with diseased walnut trees in Europe.

Keywords: Botryosphaeriaceae, *Cadophora*, *Diaporthe*, Diatrypeaceae, phylogenetic analysis, sequencing, trunk pathogens, walnut trees

Juglans regia L. (English walnut) originated in Central Asia and is the most important walnut species. This tree is grown for its seed, which is a great source of nutrients, especially essential fatty acids and proteins (Badenes and Byrne 2012). The world production of walnut seed accounted for 3.7 million tons in 2016, with China being the world's largest producer (1.78 million tons) followed by the United States (607,814 tons) and Iran (405,281 tons) (FAO 2018). Walnut seed production in the Czech Republic is still in its infancy and presently occupies 175 ha and yielded 91 tons in 2016 (FAO 2018). Approximately 80% of walnuts are estimated to grow in yards and close to village buildings, 18.8% in tree avenues, and only 1.2% in commercial orchards in the Czech Republic (Avanzato et al. 2014).

In the past 10 years, cankers and dieback symptoms such as leaf chlorosis, dead branches, and bud and shoot dieback have become frequent on walnut trees in the Czech Republic. Internal wood symptoms on affected trees range from black spots or circular discoloration of the xylem vessels to wedge-shaped necrosis. These symptoms are characteristic of trunk disease pathogens, which have been reported on grapevine and fruit trees worldwide (Gramaje et al. 2016). These fungi primarily invade woody hosts through openings such as sucker wounds (Makatini 2014) or pruning wounds (English and Davis 1978; van Niekerk et al. 2011). Fungal trunk pathogen spores are aerially dispersed, but arthropods were also found to carry them in vineyards (Moyo et al. 2014). The abnormalities caused by these pathogens lead to lower quality of fruits and reduced yield, which ultimately affects the amount of income for growers.

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Research has revealed that walnut represents a rich catch crop for several fungal trunk pathogens. Species belonging to the families Botryosphaeriaceae (Agustí-Brisach et al. 2019; Chen et al. 2013, 2014; Dervis et al. 2019; Díaz et al. 2018; Inderbitzin et al. 2010; Li et al. 2015; Linaldeddu et al. 2016; Zhang et al. 2017), Diaporthaceae (Agustí-Brisach et al. 2019; Chen et al. 2014; Fan et al. 2018; Meng et al. 2018), and the genera *Cytospora* (Fan et al. 2015; Zhao et al. 2018) and *Phaeoacremonium* (Spies et al. 2018) have been reported on walnut trees.

Although many fungal species belonging to a number of genera are well-recognized pathogens of walnut trees, the etiology of the severe decline of walnut trees in the Czech Republic is still unknown. Therefore, the aim of this study was to identify fungal trunk pathogens associated with wood necrosis of English walnut in the Czech Republic and to evaluate their status as pathogens on this host by conducting pathogenicity tests under field conditions.

Materials and Methods

Sampling and collection of fungal isolates. In 2016, field surveys were conducted in six localities in the Moravia region: namely, Rajhrad, Pouzdřany, Boleradice, and Perná in South Moravia and Choryně and Lešná in the Moravian-Silesian region (Fig. 1). Symptomatic wood from branches and shoots showing cankers and dieback were collected from six walnut orchards (one orchard per locality), aged between 30 and 40 years. In each orchard, 10 symptomatic branches were collected from 10 different trees, taken to the laboratory, and processed as follows. Branches were debarked and transversely cut to 1-cm chips. These wood fragments were first surface-sterilized in 75% ethanol for 1 min followed by flaming. From each wood fragment, five pieces of approximately 2 × 2 mm were plated on malt extract agar (MEA) (Sigma-Aldrich Laboratories, St. Louis, MO) supplemented with 0.5 g/liter of streptomycin sulfate (MEAS) (Biosynth, Staad, Switzerland). A total of five plates were used per wood sample, 25 pieces in total. Plates were incubated at 25°C in the dark for up to 4 weeks. To prevent the overgrowing of fast-spreading mycelia, the plates were checked every day and growing mycelia of potential fungal pathogens were transferred to potato dextrose agar (PDA) (HiMedia, Einhausen, Germany) plates for further identification and characterization. Pure cultures of hyphal tipped or single spore isolates were maintained in the culture collection of the Mendeleum Institute of Genetics.

Morphological identification of fungal isolates. All fungal isolates were initially characterized and classified on the basis of their cultural appearance on PDA and microscopic structures. Species of Botryosphaeriaceae, Diaporthaceae, and Diatrypaceae families were identified based on colony morphology, colony color and growth, and conidial color and shape according to Phillips et al. (2013), Marin-Felix et al. (2019), and Dissanayake et al. (2017), respectively. To enhance sporulation, six mycelial plugs (5-mm diameter) of each Botryosphaeriaceae and Diaporthaceae isolate were placed in plates containing 2% water agar (Merck, Kenilworth, NJ) supplemented with sterilized pine needles. Plates of each isolate were incubated under continuous light at 24°C until pycnidia were produced on the pine needles. Culture characteristics and pigment production on PDA, MEA (Merck), and oatmeal agar (30 g of oatmeal and 15 g of agar; Merck) and the main microscopic structures (phialide type and shape, conidiophore morphology and hyphal wart size, and conidial shape and size) from aerial mycelium were used to identify of *Phaeoacremonium* isolates (Marin-Felix et al. 2019). The main morphological characteristics such as conidiophore, phialide, collarettes, and conidial morphology were used to identify *Cadophora* isolates (Harrington and McNew 2003). All of the main microscopic structures were mounted on glass microscope slides and studied in more detail under a digital microscope (VHX-6000; Keyence, Osaka, Japan).

DNA isolation, PCR amplification, and sequencing. For each isolate, 50 mg of fungal mycelium was scraped from the culture surface and mechanically disrupted by grinding to a fine powder under liquid nitrogen using a mortar and pestle. Total DNA was extracted using a NucleoSpin Tissue DNA extraction kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The internal transcribed spacers (ITS1 and ITS2) including the 5.8S ribosomal RNA gene were amplified to identify all fungal trunk pathogen isolates. For the Botryosphaeriaceae spp., a partial sequence of translation elongation factor 1- α (*tef1*) was also amplified and sequenced to confirm species identity. The ITS region and *tef1* gene were amplified using primer pairs ITS1/ITS4 (White et al. 1990) and EF1-688F/EF1-1251R (Alves et al. 2008), respectively. For the *Diaporthe* spp., a multilocus identification was used based on five genomic loci (ITS, *tef1*, calmodulin [*cal*], histone H3 [*his3*], and partial β -tubulin [*tub2*]) (Guarnaccia et al. 2018). The primers

EF1-728F/EF1-986R and CAL-228F/CAL-737R (Carbone and Kohn 1999) were used to amplify part of the *tef1* and *cal* genes, respectively. The *tub2* region was amplified using the primers Bt2a/Bt2b (Glass and Donaldson 1995), and the *his3* region was amplified using the primers CYLH3F/H3-1b (Crous et al. 2004; Glass and Donaldson 1995). The *Phaeoacremonium* spp. were also identified by sequence analysis of *tub2* and actin (*act*) genes, using the primer pairs T1 (O'Donnell and Cigelnik 1997) and Bt2b (Glass and Donaldson 1995) and ACT-512F/ACT-783R (Carbone and Kohn 1999). The *Cadophora* spp. were confirmed by sequence analysis of the *tub2* and *tef1* genes using the primer pairs BTCadF/BTCadR (Travadon et al. 2015) and EF1-728F/EF1-986R (Carbone and Kohn 1999). For the Diatrypaceae spp., the ITS region was amplified using primer pairs ITS1/ITS4 (White et al. 1990). PCR was performed utilizing G2 Flexi DNA polymerase (Promega, Madison, WI) with primers targeting the mentioned gene sequences using respective amplification conditions according to the authors of the primers. PCR amplicons were sequenced as described in Eichmeier et al. (2010). Fungal species were initially identified using the NCBI MegaBLAST algorithm (<https://www.ncbi.nlm.nih.gov>).

Phylogenetic analyses. Fungal sequences from walnuts from the Czech Republic were aligned with available sequences in GenBank/NCBI, including ex-type specimens from several hosts for comparison using the MAFFT sequence alignment program version 6 (Katoh and Toh 2008) (Supplementary Table S1). Alignments were inspected in Sequence Alignment Editor version 2.0a11 (Rambaut 2002). Phylogenetic analyses were performed in MEGA version 6 (Tamura et al. 2013) for groups of fungi with more than one fungal species. The maximum likelihood (ML) estimation was applied on six separate data sets of the fungal families Botryosphaeriaceae (ITS and *tef1*) and Diatrypaceae (ITS) and the genus *Cadophora* (ITS, *tub2*, and *tef1*). All resulting tree topologies (*data not shown*) were visually compared for congruence to combine two Botryosphaeriaceae data sets (ITS and *tef1*) and three *Cadophora* data sets (ITS, *tub2*, and *tef1*) for multilocus analyses. ML analyses were performed in MEGA using the best-fit model as estimated with the Bayesian information criterion (BIC) in jModelTest 2.1.10 (Darriba et al. 2012). Branch support was calculated from 1,000 bootstrap replicates for the single and concatenated data sets.

Fig. 1. Czech Republic map indicating the fungal species detected in each region surveyed. *Cdn* = *Cadophora novi-eboraci*, *Cds* = *Cadophora spadici*, *Dee* = *Diaporthe eres*, *Pms* = *Phaeoacremonium sicilianum*, *Das* = *Diplodia seriata*, *Caa* = *Cryptovalsa ampelina*, *Doo* = *Dothiorella omnivora*, *Eul* = *Eutypa lata*, *Eus* = *Eutypella* sp., and *Pas* = *Peroneutypa scoparia*.

The ITS sequence of *Xylaria hypoxylon* (CBS 122620) was used as an outgroup in phylogenetic analysis of Diatrypaceae, whereas the ITS and *tef* sequences of *Botryosphaeria dothidea* (CMW8000) and *Neoscytalidium dimidiatum* (CBS 499.66), respectively, were used as outgroups in Botryosphaeriaceae. For *Cadophora* spp., sequences of *Cadophora finlandica* (CBS 44486) served as the outgroup taxon. Representative sequences of fungal trunk disease isolates derived in this study were deposited in GenBank/NCBI (Table 1) and the alignments in TreeBASE under the study numbers 24575 (Botryosphaeriaceae spp. and Diatrypaceae spp.) and 24491 (*Cadophora* spp.) (<https://treebase.org>).

Pathogenicity tests. One isolate of each of the following species was used in the field pathogenicity test: *Diplodia seriata*, *Dothiorella omnivora*, *Eutypella* sp., *Eutypa lata*, *Cryptovalsa ampelina*, *Diaporthe eres*, *Cadophora novi-eboraci*, *Cadophora spadicea*, *Peroneutypa scoparia*, and *Phaeoacremonium sicilianum* (Supplementary Table S2). In April 2017, healthy 1-year-old dormant grafted seedlings of walnut trees (Apollo cultivar grafted onto *J. regia* L. rootstock) were obtained from a commercial nursery and planted in rows spaced 0.4 m apart, with an in-row spacing of 0.5 m. Wounds were made on the woody stems of the walnut trees using a 6-mm cork borer. Agar plugs 6 mm in diameter were taken from the margin of a growing colony on PDA (7 to 20 days old), placed into the fresh wound and immediately covered by moist cotton, and wrapped with parafilm and aluminum foil to prevent desiccation. Experiments were laid down following a completely randomized design with seven replications for each fungal isolate. In addition, seven plants were wounded and inoculated in a similar manner with sterile PDA plugs, to serve as controls. The experiment was repeated twice. Lengths of wood lesions induced by inoculated isolates were evaluated 6 months after inoculation. The extent of vascular discoloration was measured upward and downward from the inoculation point. Fungal reisolations were carried out from the edges of lesions on MEAS, and fungi were identified as described previously fulfilling Koch's postulates.

Statistical analysis. Homogeneity of variance across treatments was evaluated using the Levene test (Box et al. 1978). To meet parametric assumptions, a square root transformation to total lesion length was applied. Lesion length was then analyzed using the analysis of variance. Data from all experiments were analyzed using Statistix 10 software (Analytical Software, Tallahassee, FL).

Transformed data means were compared using Tukey's honestly significant difference test at $P = 0.05$.

Results

Sampling and collection of fungal isolates. Both internal and external disease symptoms were observed on the surveyed trees and included cankers on trunks and branches and branch dieback (Fig. 2). The most common symptoms in cross-sections were wedge-shaped necrosis, irregular wood necrosis, central necrosis, and black spots (Fig. 2).

Based on colony morphology, phylogenetic analyses, and conidial characteristics (see below), 138 fungal isolates belonging to five families were recovered from English walnut (Supplementary Table S2). One species of the Togniniaceae family (*Phaeoacremonium sicilianum*), two species of the Botryosphaeriaceae family (*Diplodia seriata* and *Dothiorella omnivora*), four species of the Diatrypaceae family (*Cryptovalsa ampelina*, *Eutypa lata*, *Peroneutypa scoparia*, and *Eutypella* sp.), one species of the Diaportheaceae family (*Diaporthe eres*), and two species of the genus *Cadophora* (*Cadophora novi-eboraci* and *Cadophora spadicea*) were isolated from symptomatic branches of walnut trees. Species of *Cadophora* (66.7%) were the prevalent fungi associated with wood symptoms from which isolations were made, followed by species in families Diatrypaceae and Botryosphaeriaceae, both with 14.5% of the fungi isolated. In general, there was not a regional effect on fungal distribution (Fig. 1), but species of Diatrypaceae were only found in the South Moravian region, which is 1.2°C warmer on average than the Moravian-Silesian region (<http://portal.chmi.cz/historicka-data/pocasi/uzemni-teploty#>).

Fungal trunk pathogens were isolated in 34 of the 60 samples (56.7%) collected (Table 2). More than one fungal species was isolated from a single sample in 11 of the 34 samples (32.3%) that tested positive for fungal trunk pathogens. Most of the fungal isolates were recovered from black spots (42.0%) and central necrosis (34.1%) (Table 3). The black discoloration of the xylem vessels and central necrosis were mostly colonized by *Cadophora* spp., with 96.5 and 68.1% of the total number of isolates, respectively. The irregular wood necrosis was mostly colonized by Botryosphaeriaceae spp. (56.2%), followed by *Cadophora novi-eboraci* (25%). The wedge-shaped wood necrosis was mostly colonized by Diatrypaceae spp. (72.2%), followed by *Dothiorella omnivora* (27.8%).

Table 1. Origin and GenBank accession numbers of trunk disease fungal isolates obtained from walnut (used in the phylogenetic studies)

Species	Code	Locality	GenBank accession number ^z		
			ITS	BT	TEF
<i>Cadophora novi-eboraci</i>	OCR1	Pouzdrány	MK431098	MK431099	MK993408
<i>Cadophora novi-eboraci</i>	OCR7	Lešná	MK431101	MK431102	MK993409
<i>Cadophora novi-eboraci</i>	OCR12	Lešná	MK431103	MK431104	MK993410
<i>Cadophora spadicea</i>	OCR32	Lešná	MK431118	MK431119	MK993411
<i>Cadophora spadicea</i>	OCR36	Pouzdrány	MK431106	MK993414	MK993412
<i>Cadophora spadicea</i>	OCR38	Boleradice	MK993416	MK993415	MK993413
<i>Cryptovalsa ampelina</i>	OCR93	Boleradice	MK431120	—	—
<i>Cryptovalsa ampelina</i>	OCR94	Boleradice	MK993417	—	—
<i>Diplodia seriata</i>	OCR120	Boleradice	MK431136	—	MK431137
<i>Diplodia seriata</i>	OCR123	Pouzdrány	MK993418	—	MK431135
<i>Diplodia seriata</i>	OCR124	Choryně	MK993419	—	MK431138
<i>Dothiorella omnivora</i>	OCR127	Boleradice	MK431141	—	MK431142
<i>Dothiorella omnivora</i>	OCR128	Rajhrad	MK431139	—	MK431140
<i>Dothiorella omnivora</i>	OCR129	Boleradice	MK431143	—	MK431144
<i>Eutypa lata</i>	OCR142	Boleradice	MK431147	—	—
<i>Eutypa lata</i>	OCR149	Rajhrad	MK431146	—	—
<i>Eutypa lata</i>	OCR150	Rajhrad	MK993420	—	—
<i>Peroneutypa scoparia</i>	OCR155	Rajhrad	MK431149	—	—
<i>Peroneutypa scoparia</i>	OCR156	Rajhrad	MK993421	—	—
<i>Peroneutypa scoparia</i>	OCR157	Rajhrad	MK993422	—	—
<i>Eutypella</i> sp.	OCR159	Pouzdrány	MK431148	—	—

^z ITS = internal transcribed spacer, BT = β -tubulin, and TEF = translation elongation factor 1- α . Dashes indicate sequences not available.

Morphological identification and characterization of fungal isolates. Based on morphological criteria (microscopic and culture characterization), the fungal isolates obtained from walnut were classified into *Cadophora*, *Diaporthe*, *Diplodia*, *Dothiorella*, and *Phaeoacremonium* genera and several genera belonging to the Diatrypaceae family. Two isolates were characterized by pale brown to brown, flat, slow-growing cultures on PDA and MEA, abundant sporulation, and aseptate and hyaline conidia. Septate hyphae were fasciculate or single. Three types of phialides, variable in shape and size, were observed in these fungal isolates. These morphological characteristics corresponded to the genus *Phaeoacremonium* (Marin-Felix et al. 2019).

Twenty isolates formed white, dark green, or gray fast-growing colonies on PDA. These isolates also produced globose and black pycnidia on pine needles after 25 days. Conidia were hyaline or pigmented. All morphological and cultural characteristics corresponded to the Botryosphaeriaceae family (Phillips et al. 2013). At the genus level, isolates were assigned to two genera: *Diplodia* and *Dothiorella*.

Twenty-four isolates were characterized by having white-cream cottony slow-growing mycelium on PDA, lacking fruiting structures after an incubation time of 3 to 4 weeks in the dark. After 4 weeks under continuous fluorescent light, small black pycnidia were formed on the agar. Conidia developing in the fruiting bodies were hyaline and slightly curved, which corresponds to descriptions of species in the Diatrypaceae family (Glawe and Rogers 1984).

Five isolates were characterized by having white, cottony, slow-growing mycelium. Dark brown or black, eustromatic pycnidia released a mucilaginous cream drop containing the two characteristic spore type: alpha and beta conidia. These morphological characteristics were similar to those described for *Diaporthe* species (Marin-Felix et al. 2019). Ninety-two isolates formed white to pale yellow or vinaceous buff, felty, flat colonies on PDA. Conidia were elongate or ellipsoid. Prominent flask-shaped phialides and collarettes were frequently observed. Morphological and cultural characteristics of

these isolates resembled those of *Cadophora* spp. (Harrington and McNew 2003; Travadon et al. 2015).

Molecular characterization and phylogenetic analyses. The BIC best-fit nucleotide substitution model identified by jModelTest was the Hasegawa-Kishino-Yano model with gamma distributed with invariant sites rates (G+I) for the *Cadophora* multilocus analysis. Alignment of 25 *Cadophora* sequences resulted in a 1,260-character data set. Three isolates clustered strongly (100%) with the type specimen of *Cadophora spadici*s (CBS 111743), and three isolates were identified as *Cadophora novi-eboraci* (100%, isolate NYC13) (Fig. 3).

Table 2. The diversity of trunk disease pathogens isolated from wood samples collected from *Juglans regia* orchards in the Czech Republic

Pathogen isolated	Samples (n)
<i>Cadophora novi-eboraci</i>	2
<i>Cadophora spadici</i> s	9
<i>Diaporthe eres</i>	2
<i>Diplodia seriata</i>	4
<i>Dothiorella omnivora</i>	3
<i>Eutypa lata</i>	2
<i>Peroneutypa scoparia</i>	1
<i>Cadophora novi-eboraci</i> + <i>Cadophora spadici</i> s	5
<i>Cadophora novi-eboraci</i> + <i>Phaeoacremonium sicilianum</i>	1
<i>Cadophora spadici</i> s + <i>Eutypella</i> sp.	1
<i>Cadophora spadici</i> s + <i>Dothiorella omnivora</i>	2
<i>Diplodia spadici</i> s + <i>Dothiorella omnivora</i>	1
<i>Cadophora novi-eboraci</i> + <i>Cryptovalsa ampelina</i> + <i>Diaporthe eres</i>	1
Total number of samples tested positive to trunk disease fungi	34

Fig. 2. Disease symptoms associated with fungal trunk pathogens on walnut trees in the Czech Republic. **a and b**, Dieback and wilting of branches. **c, d, e, and f**, Internal symptoms visible when transversal and longitudinal cuts were made in branches used for fungal isolation: central and irregular necrosis (c, d, and e) and black spots (f).

The ITS sequences of the nine representative diatrypeous isolates from walnut were aligned with 29 reference strains and the outgroup. The selection of the reference strains was based on the phylogenetic closeness with the walnut isolates and the clade classification of Senwanna et al. (2017): there were 10 strains from clade H

(*Eutypella sensu lato*), six from clade D (*Eutypa sensu stricto*), nine from clade I (genus *Peroneutypa*), and two from each of the clades L and M (*Cryptovalsa sensu lato* and genus *Quaternaria*, respectively). The resulting alignment consisted of 588 characters including gaps and the ML analysis was performed using the Kimura

Table 3. Number of isolates, locality, and disease symptoms associated with trunk disease fungi isolated from *Juglans regia*

Isolate family	Species	Locality	Total	Isolates (n)			
				Internal wood lesion type ^z			
				BS	CN	IWN	WSN
Diatrypeaceae	<i>Cryptovalsa ampelina</i>	Boleradice	2	–	1	–	1
	<i>Eutypa lata</i>	Boleradice, Rajhrad	13	–	4	–	9
	<i>Peroneutypa scoparia</i>	Rajhrad	4	–	1	1	2
	<i>Eutypella</i> sp.	Pouzďřany	1	–	–	–	1
Diaporthaceae	<i>Diaporthe eres</i>	Lešná, Boleradice, Pouzďřany	4	–	2	2	–
Botryosphaeriaceae	<i>Diplodia seriata</i>	Boleradice, Pouzďřany, Choryně	7	–	2	5	–
	<i>Dothiorella omnivora</i>	Boleradice, Perná	13	–	4	4	5
Togniniaceae	<i>Phaeoacremonium sicilianum</i>	Lešná	2	2	–	–	–
Incertae sedis	<i>Cadophora novi-eboraci</i>	Pouzďřany, Boleradice, Choryně, Lešná, Perná	31	17	10	4	–
	<i>Cadophora spadicis</i>	Pouzďřany, Boleradice, Choryně, Lešná, Perná	61	39	22	–	–
Total			138	58	47	16	–

^z Various internal wood lesion types from which isolates were collected in a cross-section of affected wood samples: BS = black spot, CN = central necrosis, IWN = irregular wood necrosis, and WSN = wedge-shaped necrosis. Dashes indicate symptoms not available.

Fig. 3. Maximum likelihood phylogeny of *Cadophora* spp. as estimated from concatenated alignments of a three-gene data set (internal transcribed spacer, partial β -tubulin, and translation elongation factor 1- α). Maximum likelihood bootstrap percentages are indicated at the nodes. Support values <70% bootstrap are omitted. The scale bar indicates 0.05 expected changes per site. Fungal species isolated in this study are indicated with an asterisk.

2-parameter model with gamma distribution. The walnut isolates clustered with high support with the reference sequences of *Cryptovalsa ampelina* (isolates OCR93 and OCR94; bootstrap 98%), *Eutypa lata* (isolates OCR142, OCR149, and OCR150; bootstrap 98%), *Eutypella* sp. (isolate OCR159; bootstrap 100%), and *Peroneutypa scoparia* (isolates OCR155, OCR156, and OCR157; bootstrap 97%) (Fig. 4).

The ITS and *tef* sequences of six botryosphaeriaceous isolates were aligned with 10 and nine reference strains of *Diplodia* and *Dothiorella*, respectively, and the outgroups. The multilocus alignment consisted of 772 characters including gaps (470 from ITS and 302 from *tef*), and the Tamura 3-parameter model, with gamma distribution, was used for ML analysis. Isolates OCR127, OCR128, and OCR129 clustered with the *Dothiorella omnivora* reference

Fig. 4. Maximum likelihood phylogeny of Diatrypeaceae spp. as estimated from concatenated alignments of a single gene data set (internal transcribed spacer). Maximum likelihood bootstrap percentages are indicated at the nodes. Support values <70% bootstrap are omitted. The scale bar indicates 0.10 expected changes per site. Fungal species isolated in this study are indicated with an asterisk.

strains with 95% bootstrap support, whereas isolates OCR120, OCR123, and OCR124 clustered with the reference strains of *Diplodia seriata* with 88% support (Fig. 5).

Two isolates, OCR 160 (*tub2* accession MN013372 and *act* accession MN013374) and OCR 161 (*tub2* accession MN013373 and *act* accession MN013375) clustered (98%) with sequences of the ex-type of *Phaeoacremonium sicilianum* (CBS 123034). Two isolates, OCR 95 (ITS accession MK431127, *tefl* accession MK468701, *cal* accession MK431128, *his3* accession MK468702, and *tub2* accession MK431127) and OCR 96 (ITS accession MK431121, *tefl* accession MN052813, *cal* accession MN052814, *his3* accession MN017709, and *tub2* accession MN017710) clustered (99%) with sequences of the ex-type of *Diaporthe eres* (CBS 791.68).

Pathogenicity tests. There were no significant differences in lesion lengths between the two field trials in which seedlings were inoculated with fungal mycelia ($P = 0.6325$); therefore, data from both trials were combined. All isolates caused wood necrosis that developed upward and downward from the point of inoculation. *Cadophora novi-eboraci*, *Cadophora spadicea*, and *Eutypa lata* caused

vascular discoloration that was significantly longer than control plants (Fig. 6). The mean lesion lengths were 54.9 mm for *Cadophora novi-eboraci*, 49.8 mm for *Cadophora spadicea*, and 41.5 mm for *Eutypa lata*. Although mean lesion lengths caused by *Diaporthe eres* (24.0 mm), *Phaeoacremonium sicilianum* (22.7 mm), and *Cryptovalsa ampelina* (22.1 mm) were not significantly different from those caused by *Cadophora novi-eboraci*, *Cadophora spadicea*, and *Eutypa lata*, they also statistically overlapped with the control treatment (8.2 mm). Percent recovery on MEAS was >80% for all treatments, and reisolated fungi were morphologically identical with those previously inoculated, thereby confirming Koch's postulates. None of these trunk disease pathogens were isolated from control plants.

Discussion

This study represents the first comprehensive characterization of trunk disease fungi recovered from wood of English walnut exhibiting typical symptoms of trunk diseases in Europe. Ten species belonging to five fungal families were isolated from black spots in

Fig. 5. Maximum likelihood phylogeny of Botryosphaeriaceae spp. as estimated from concatenated alignments of a two-gene data set (internal transcribed spacer and translation elongation factor 1- α). Maximum likelihood bootstrap percentages are indicated at the nodes. Support values <70% bootstrap are omitted. The scale bar indicates 0.05 expected changes per site. Fungal species isolated in this study are indicated with an asterisk.

the xylem vessels, as well as central, irregular, and wedge-shaped necrosis. Different fungal species often co-occurred in a single symptom, highlighting the complexity of the etiology of the symptoms observed.

The most predominant fungal taxa isolated from symptomatic wood of English walnut in this study belonged to the genus *Cadophora*. Species of *Cadophora* are involved in Petri disease and esca, which occur on young and mature grapevines, respectively, and are caused by a complex of fungi, often including *Phaeoconiella chlamydospora* and multiple species of *Phaeoacremonium* (Gramaje et al. 2018). Two *Cadophora* species, namely, *Cadophora novi-eboraci* and *Cadophora spadicea*, were associated mainly with two wood lesion types: black spots in the xylem vessels and central necrosis. These fungal species were described by Travadon et al. (2015), based on isolates collected from wood cankers or discolored wood of grapevine in North America and from previously misidentified *Cadophora* spp. isolates associated with discolored wood of kiwi fruit trees in Italy (Di Marco et al. 2004; Prodi et al. 2008). *Cadophora novi-eboraci* was isolated from necrotic wood of *Malus domestica* L. in Germany in 2017 (Gierl and Fischer 2017). The relationship between environmental conditions and *Cadophora* spp. distribution has been suggested by Blanchette et al. (2010) and Travadon et al. (2015). *Cadophora luteo-olivacea* has been mainly isolated from the warm, Mediterranean climate found in California (Rooney-Latham 2005; Travadon et al. 2015), South Africa

(Gramaje et al. 2014; Halleen et al. 2007), and Spain (Gramaje et al. 2011). By contrast, other species that have been isolated from cooler climates, such as *Cadophora malorum* in Antarctica (Blanchette et al. 2010) or *Cadophora novi-eboraci* in New York (Travadon et al. 2015), Germany (Gierl and Fischer 2017) and the Czech Republic (Eichmeier et al. 2018), might then be better adapted to them. This study represents the first record of *Cadophora novi-eboraci* and *Cadophora spadicea* on English walnut worldwide.

Botryosphaeriaceae and Diatrypaceae species were the second most predominant groups of fungi associated with three types of wood necrosis of English walnut in this study. Two Botryosphaeriaceae spp. were identified: namely, *Diplodia seriata* and *Dothiorella omnivora*. The role of Botryosphaeriaceae species as pathogens causing stem cankers and twig and branch dieback in English walnut has been extensively studied in California, with the description of up to 10 species belonging to six genera (Chen et al. 2013, 2014; Inderbitzin et al. 2010). *Diplodia seriata* has been isolated in affected English walnut trees in California (Chen et al. 2014) and China (Zhang et al. 2017). The low incidence of *Diplodia seriata* in our study agrees with the results of Inderbitzin et al. (2010) from peach and almond trees in California, Gramaje et al. (2012) from almond trees in Spain, Moyo et al. (2016) from persimmon trees in South Africa, and Panahandeh et al. (2019) from *Syzygium cumini* (L.) Skeels in Iran. In contrast, this species was the dominant Botryosphaeriaceae species on stone and pome fruit trees in several studies carried out in South

Fig. 6. Box plots illustrating the distribution of the length of wood discoloration measured in walnut trees at 6 months after inoculations with 10 fungal species. Results are ordered according to the mean. Solid lines and circles within the boxes correspond to the median and the mean, respectively. The top and bottom lines of the box correspond to the 25th and 75th percentiles of the data, respectively. Error bars represent the 10th and 90th percentiles, and circles represent the fifth and 95th percentiles. Mean lengths of wood discoloration of walnut trees with different letters are significantly different at $P < 0.05$ (Tukey test). *Pa.* = *Peroneutypa*, *Do.* = *Dothiorella*, *Da.* = *Diplodia*, *Ea.* = *Eutypella*, *Ca.* = *Cryptovalsa*, *Pm.* = *Phaeoacremonium*, *De.* = *Diaporthe*, *Eu.* = *Eutypa*, and *Cd.* = *Cadophora*.

Africa (Cloete et al. 2011; Damm et al. 2007; Slippers et al. 2007). *Dothiorella omnivora* affecting walnut trees in France and Iran was reported in 2016 (Linaldeddu et al. 2016). Other hosts for this species include grapevine in Hungary (Váczy et al. 2018) and Australia (Linaldeddu et al. 2016), hazelnut and the European hop-hornbeam in Italy (Linaldeddu et al. 2016), the northern white-cedar and the Lawson cypress in Serbia (Linaldeddu et al. 2016), and dogwood in Italy (Dissanayake et al. 2017).

The majority of walnut isolates in the Diatrypaceae family belonged to *Eutypa lata*, whereas a minority of isolates were *Cryptovalsa ampelina*, *Eutypella* sp., and *Peroneutypa scoparia*. Studies of Diatrypaceae spp. on English walnut are scarce, as only *Eutypa lata* and *Cryptovalsa ampelina* have been reported to affect this crop in Greece (Rumbos 1988) and California (Trouillas et al. 2010), respectively. To the authors' knowledge, *Eutypella* sp. and *Peroneutypa scoparia* have not been reported on English walnut before this study. Thus, this is the first report of *Eutypella* sp. and *Peroneutypa scoparia* on English walnut worldwide. Members of the family Diatrypaceae are widespread globally, but the involvement of up to 17 species of this family in cankers and wood necrosis of grapevines worldwide led to researchers' interest in studying them (Moyo et al. 2018). In the Czech Republic, *Eutypella lata* is considered the most common species associated with grapevine trunk diseases (Baránek et al. 2018). The overlap of Diatrypaceae spp. between vineyards and natural ecosystems was demonstrated in South Africa in 2019 (Moyo et al. 2019).

Additional fungal trunk pathogens isolated in low numbers in this study include fungi belonging to the families Diaporthaceae (*Diaporthe eres*) and Togniniaceae (*Phaeoacremonium sicilianum*). The species criterion concept in the *Diaporthe eres* complex was revised in 2018 based on a multilocus sequence approach (Fan et al. 2018). *Diaporthe eres* has often been reported as a plant pathogen in a wide range of woody hosts, including English walnut in China (Fan et al. 2018; Yang et al. 2018) and Italy (Gomes et al. 2013). *Phaeoacremonium sicilianum* was recorded on *Juglans* sp. in South Africa in 2018 (Spies et al. 2018). Other additional hosts for this species include fig in South Africa (Spies et al. 2018); grapevine in Italy (Essakhi et al. 2008), South Africa (Spies et al. 2018; White et al. 2011), and Spain (Gramaje et al. 2009); and olive in Italy (Carlucci et al. 2015).

Results of the field pathogenicity test showed significant differences in the degree of virulence among fungal species inoculated into English walnut woody stems. Of the 10 inoculated species, *Cadophora novi-eboraci*, *Cadophora spadicis*, and *Eutypa lata* caused lesions significantly longer than the control. *Cadophora* spp. have been traditionally considered as slow colonizing fungi in several pathogenicity tests conducted on grapevine (Navarrete et al. 2011; Travadon et al. 2015; Úrbez-Torres et al. 2014). In particular, *Cadophora novi-eboraci* caused wood discoloration in young vines ranging from 1.5 to 2.6 cm after a long 24-month incubation period in California (Travadon et al. 2015). In contrast, *Cadophora luteo-olivacea* produced lesions of up to 4.4 cm in grapevine trunks after 14 months of incubation under field conditions in South Africa (Halleen et al. 2007), or up to 9.2 cm in grapevine rootstock cuttings after 14 weeks of incubation under controlled conditions in Spain (Gramaje et al. 2011). Although a wide degree of variability in lesion development was found among individual trees, species of *Cadophora* were shown to be highly virulent in English walnut in the Czech Republic, with necrosis reaching up to 11.1 cm after 6 months of incubation under field conditions. This finding, along with the high frequency of *Cadophora* spp. isolates collected from affected trees, demonstrates that English walnut should be considered as a susceptible host for these fungi. Inoculation tests with *Eutypa lata* on walnut also yielded characteristic disease cankers significantly longer than the control in Greece (Rumbos 1988). It is interesting to note that other species considered as pathogens in other woody hosts, such as *Peroneutypa scoparia* on kiwifruit in Chile (Castilla-Cayuman et al. 2019) or *Dothiorella omnivora*, *Cryptovalsa ampelina*, and *Phaeoacremonium sicilianum* on grapevine in Hungary (Váczy et al. 2018), California (Trouillas and Gubler

2010) and Spain (Gramaje et al. 2009), respectively, produced short lesions in inoculated English walnut trees in the present study. Conflicting reports on the virulence of several fungal trunk pathogens exist in the literature. For example, *Diplodia seriata* was also considered a weak pathogen on walnut (Chen et al. 2014), as well as on other woody hosts such as olive (Moral et al. 2010) or grapevine (Úrbez-Torres and Gubler 2009). However, other studies have confirmed the pathogenicity of this fungal species by artificial inoculation on grapevine (Auger et al. 2004; Rovesti and Montermini 1987; van Niekerk et al. 2006). Elena et al. (2015) proved the existence of different virulence levels in this fungal species on grapevine. *Cryptovalsa ampelina* has also been classified as weakly pathogenic based on the relatively short canker extension after grapevine inoculations in Spain (Luque et al. 2006). In the case of *Cryptovalsa ampelina*, the low frequencies of both mycelium reisolation and wound canker extension on grapevines suggested a low virulence for this fungus in Spain (Luque et al. 2006), compared with the significant dark brown stem discoloration caused by the pathogen in South Africa (Mostert et al. 2004).

The information obtained from this research provides the local walnut industry with knowledge on the occurrence of fungal trunk disease pathogens and forms a baseline for further research in this pathosystem worldwide.

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