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Identification of *Botryosphaeria dothidea* and *Diaporthe eres* from rotted walnut fruits and other plant parts in different phenological stages

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RESEARCH ARTICLE

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ABSTRACT

The acreage of English walnut (*Juglans regia* L.) is constantly expanding in Hungary, due to the favorable climatic conditions and economic importance. Last years, serious damage was reported from several orchards with high percentage of rotted, moldy kernels. The aim of this research was to identify the pathogens at different growth stages. Fungi were cultured from the spotty, shriveled and rotted kernels, and monosporic isolates were identified based on morphological characters and molecular markers (ITS region and *tef1* locus sequences). *Botryosphaeria dothidea* and *Diaporthe eres* were identified in high proportion from symptomatic kernels. These species were also isolated from different parts of walnut trees in different seasons. *D. eres* was detected in a high proportion from asymptomatic buds in March, while the presence of

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both species was observed in symptomatic husks with Overnight Freezing-Incubation Technique (ONFIT) in June. Their optimal growth temperature defined to be between 20–25 °C, and the growth of *D. eres* isolates was completely inhibited at 35 °C.

KEYWORDS

bud infection, green nut infection, walnut latent infection, optimal growth temperature

INTRODUCTION

Juglans regia L. is an ancient species originated from Central Asia (Fernández-López et al., 2001). Walnut kernels are famous for their exceptional nutritional properties, especially due to their high protein and essential fatty acids content (Boriss et al., 2006). Walnut is an economically important crop, cultivated on 1.1 million hectares, where 3.5 million tonnes nut were harvested in 2021. 31% of the world's nuts were produced in China, while 19% in the United States and 10% in Europe, where Ukraine is the dominant nuts producer. In Hungary, 5,950 tonnes from 6,440 ha of orchards were harvested in 2021 (FAOSTAT, 2021).

While black walnut (*Juglans nigra*) is grown for edible purposes in the USA, there is only English walnut (*J. regia* L.) in European orchards, where the Mediterranean Region and Central Europe are the best areas for walnut production. In Hungary, the region alongside the river Tisza provides particularly favorable environmental conditions for walnut growth and nut production (Szentiványi, 1980).

Walnut production is threatened by pathogens and insects worldwide, which can cause serious yield losses. *Xanthomonas* species cause bacterial walnut blight that appears in the form of brown spots on the husk and the nut (Moragrega et al., 2011; Catara et al., 2021). This bacteria plays a role in brown apical necrosis together with *Alternaria* and *Fusarium* spp. as well (Belisario et al., 2002; Moragrega et al., 2011). Walnut anthracnose is a widespread disease caused by *Ophiognomonia leptostyla*. Symptoms are observable on most parts of the walnut tree as growing brown patches and the disease results in rot on the end of the husks (Belisario et al., 2008; Pollegioni et al., 2010), hence causing premature fruit drop and has an effect on the quality of the kernel as well (Holb et al., 2002). Nut crops are more infected by fungal pathogens in recent years, and the damage affects not the woody plant parts only but the kernels, as well (Chen et al., 2014; Michailides and Morgan, 2016; Moral et al., 2019). *Botryosphaeria* spp. are widely distributed pathogens, and colonize woody tissues of numerous plants (Sutton, 1981; Michailides et al., 1998; Garibaldi et al., 2012). The infection of nut trees results in different symptoms, like lesions, cankers and rot on woody tissues and kernels (Chen et al., 2014; Wiman et al., 2019; Gusella et al., 2020; López-Moral et al., 2020; Zabiák et al., 2023b). Fungi of this genus are able to infect healthy tissues and cause symptoms after onset stress of the host (Maresi et al., 2007; Pérez et al., 2010). Climate change can alter the environmental factors, which may provide more favorable conditions for these pathogens both for infection and disease development (Desprez-Loustau et al., 2006).

Diaporthe species distribute in large geographical area and can colonize wide range of hosts, not only as pathogens, but also as saprobes or endophytes (Gomes et al., 2013). This genus has

been isolated from rotted hazelnut (Battilani et al., 2018), and from numerous plant parts of walnut trees previously (Chen et al., 2014). *Diaporthe* genus is a complex group, which taxonomy has been modified recently, based on molecular markers (Udayanga et al., 2014). Earlier morphological characters and hosts were determined to distinguish species, which method resulted in the description of several hidden cryptic species (Wehmeyer, 1933; Udayanga et al., 2014).

Botryosphaeria and *Diaporthe* spp. are the main pathogens of walnut trees in the USA causing serious losses because of the diseased branches, shoots and fruits (Michailides, 1991). These fungi produce stroma for overwinter and oversummer, which makes them more resistant against environmental impacts and fungicides, and they are able to survive two to six years in cankered tissues (Michailides, 1991). Pycnidiospores have been proven to play the most important role in the infection, which is highly associated with the weather conditions (Michailides and Morgan, 1992). The pycnidiospores of these pathogen genera spread especially by splashing rain or irrigation water (Ahimera et al., 2004). Germination of spores may be postponed on susceptible plant tissues in case of inadequate environmental conditions such as temperature, humidity or antagonistic effects (Chen et al., 2003). The pathogens produce various enzymes and toxins to support their penetration into the plant cells (Andolfi et al., 2011; Esteves et al., 2014). Colonization may also occur through natural openings or wounds (Michailides, 1991). Environmental conditions, determine disease the development, too. Beyond high humidity and precipitation, drought stress of the plant also contributes to the development of the disease (Ma et al., 2001).

Walnut cultivation is significant in Hungary, because of the favorable environmental conditions for this tree nut. However, in recent years, rotted and moldy kernels caused increasing problems (Zabiák et al., 2023b). The symptoms were visible often only on the kernel, in the form of rotted, black spotted, browned, moldy and shriveled nuts. The high ratio of rotted walnut is an urgent problem that needs to be solved. Our aims were to (i) identify the casual agents of this destructive disease, (ii) to survey the occurrence of *Botryosphaeria* and *Diaporthe* genera on the nut trees at different phenological phases, and to (iii) determine their optimal growth temperature.

MATERIAL AND METHODS

Isolation of kernel infecting fungi

Three North-Eastern Hungarian commercial walnut orchards in Hajdúdorog, Jánkmajtis, and Tarpa were selected for survey. 200 nuts were collected during harvest, in September 2018. Walnuts both with black and healthy shells were collected. Each sample was shelled, and classified based on disease severity, similarly to the previously described four class scale (Zabiák et al., 2023b). Class 0 marked healthy samples, while nuts with small spots and hyphae belonged to class 1. Partially shriveled, completely brown kernels were registered in class 2. Totally shriveled and rotted nuts were registered with class 3 symptoms (Fig. 1).

To determine the severity of symptoms caused by fungi, the McKinney index (Imc) (McKinney, 1923) was calculated by using the following formula:

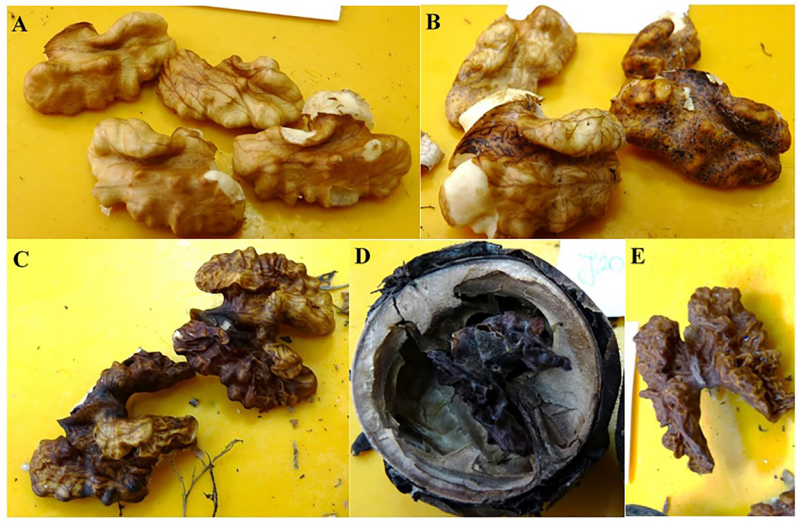

Fig. 1. Symptom severity on walnuts and their classification. A: Class 0, symptomless nuts; B: Class 1, spotty kernel with hyphae; C: Class 2, partly shrivelled walnut; D–E: Class 3, rotted kernel in shell or fully shrivelled nut

$$Imc\ (\%) = \frac{Sum\ of\ all\ disease\ rating}{total\ number\ of\ rating \times maximum\ disease\ grade} \times 100$$

Four pieces of kernel samples were disinfected with 10% chlorogen-sesquihydrate (Neomagnol; Parma Produkt Ltd., Hungary)-Tween20 (Sigma-Aldrich, Germany) solution (50 mL/L) and rinsed in sterile distilled water twice. The small tissue pieces were then plated on potato dextrose agar (PDA, Biolab, Hungary) containing streptomycin-sulphate (0.1 g L^{-1}) (Sigma-Aldrich, Germany) antibiotic. Plates were incubated for 7 days in dark, at room temperature, then pure cultures were grown on PDA (Kovács et al., 2021).

Normality of data was evaluated based on Q-Q plots and homogeneity of variances was tested with Levene's-test. Statistical analysis of scale values was performed with Mann-Whitney U tests as pairwise comparisons.

Bud monitoring

The state of infection and the incidence of walnut rot caused by Botryosphaeriaceae and *Diaporthe* species was define with bud monitoring (BUDMON) technique (Michailides et al., 2014). In early March 2019, ten randomly chosen, asymptomatic buds (catkins) per tree were collected from 15 trees in a commercial orchard near Jánkmajtis. The surface of the buds was sterilized with 5% sodium-hypochlorite and washed twice in sterile water. After drying, maximum five cut-in-half buds per plate were placed on PDA amended with streptomycin-sulphate (0.1 g L^{-1}). After 7 days of incubation on 25 °C, pathogens were recorded (Michailides et al., 2014).

ONFIT assay

Green walnuts were studied by ONFIT (Overnight Freezing-Incubation Technique) method (Michailides et al., 2010) to determine latent infection of walnuts. In early June, 38 symptomatic and 32 asymptomatic walnuts were collected from Jánkmajtis and 50 symptomatic green walnuts from Alsószentiván. Symptoms included deep brown lesions and spots on the surface of the green husks (Fig. 2).

First, samples were disinfected with the same method as described for the kernel above. Then the green walnuts were incubated at −16 °C for 15 h. After freezing, the nuts were placed separately in disinfected plastic containers, and incubated under 95% relative humidity for 14 days at 25 °C. Specific mycelia and pycnidia were observed on the surface of the husks and some of them were isolated for further analysis.

Morphological and molecular identification

During morphological, genus-level identification, the characteristics of colonies were observed. Morphology of the isolates was studied on PDA after four and ten days of incubation at 25 °C in dark. Hyphae and conidia (when produced) were analyzed, but not measured with microscope, and isolates were grouped based on these characteristics. Microscopic properties were analyzed with optical microscope (Zeiss AxioImager phase-contrast microscope, equipped with AxioCam MRc5 camera) at 400× magnification (Jacobs and Rehner, 1998; Andersen et al., 2002; Summerell et al., 2003; Gomes et al., 2013; Visagie et al., 2014).

Fig. 2. Immature symptomatic walnuts during soaking procedure

Genomic DNA was extracted with NucleoSpin Plant II Kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany) following the manufacturer's protocol. 24 pathogen isolates were cultured on PDA and 7 days old mycelia were placed into 2 mL ZR BashingBead Lysis Tubes (Zymo Research Corp, Irvine, CA, UCA) that contained 0.7 mL of 2 mm bashing beads and 500 µL Lysis Buffer 1, and vortexed to disrupt fungal cells and release DNA. It was followed by PCR reaction to amplify the ITS region and *tef1* gene, using primer pairs ITS1-4 (IDT, Leuven, Belgium) (White et al., 1990), and EF1-728F and EF1-986R (IDT), respectively (Carbone and Kohn, 1999). The final volume of PCR mixture was 25 µL, and contained 12.5 µL 2× DreamTaq Green Master Mix (Thermo Fisher Scientific, Germany), 0.5 µL of each primer (10 pmol μL^{-1}), 10.5 µL nuclease-free water, and 1 µL of DNA solution (10 ng μL^{-1}). Amplification started with an initial denaturation step of 3 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 45 s at 56 °C for ITS or 55 °C for *tef1* products, 60 s at 72 °C and a final extension at 72 °C for 5 min. PCR products were separated in 1% agarose gel (Bioline, Memphis, TN, USA) stained with GelRed (Biotium, Fremont, CA, USA) for 60 min at 100V, in order to verify successful amplification. PCR fragment purification was performed with NucleoSpin Gel, PCR Clean Up Kit (Macherey-Nagel GmbH and Co., KG, Düren, Germany). Isolated fragments were sent for sequencing to automatic Sanger equencing at the Microsynth GmbH (Vienna, Austria). Sequences were edited manually, were aligned first with Clustal X 2.1 (Larkin et al., 2007) and adjusted using Genedoc (Nicholas et al., 1997), when it was necessary. Phylogenetic tree was created with the MEGA 7.0 software (Kumar et al., 2016) and nearest-neighbor interchange (NNI) method with 1000 bootstrapped replications. The maximum likelihood method, based on the cpREV + F model was used for all sequences. Positions containing gaps and missing data were not considered. For maximum likelihood analyses, the nearest-neighbor interchange was used as the heuristic method for tree inference. Support for internal branches was assessed by 1000 bootstrapped pseudoreplicates of data.

Growth temperature test

The optimal temperature for growth of four *Diaporthe eres* (J2034, J2028, JT2024, JT2050) and two *Botryosphaeria dothidea* (JT2015, T2016) isolates was determined. 10 mm mycelial plugs were cut with sterile cork borer from the actively growing part of the 7-day-old fungal colonies. Plugs of each isolate were plated on PDA in three replicates and incubated at 15, 20, 25, 30, and 35 °C. Colony diameters were measured for 7 days.

RESULTS

Culturable fungal population of symptomatic and asymptomatic kernels

Alternaria (31%), *Diaporthe* (26%), *Penicillium* (22%), *Botryosphaeria* (13%) and *Fusarium* (9%) isolates were present in the collected symptomatic and asymptomatic walnut kernels, based on the morphological characters of pure cultures.

Isolates belonging to the *Alternaria* genus produced woolly, olive green colonies with rapid growth. Conidia were septate in vertical and horizontal directions, oval-shaped and created chains. For *Botryosphaeria* spp., initially white fluffy colonies showed rapid growth producing plenty of white aerial mycelia and turned grey in five days. In case of *Diaporthe* spp., mycelial

growth was slower than that of the *Botryhospaheria* isolates under the same conditions. Colonies were first white with darker color in the centre and produced concentric rings with irregular margin and white hyphae. White, pink or purple colonies of *Fusarium* isolates were fast growing, and cottony with lot of aerial mycelia. Conidia were sickle-shaped and septate. *Penicillium* spp. produced dark green and felty colonies with white margin, which appeared scattered on the media as well. Conidia were spherical and produced on branched brush-like conidiophores.

In several cases, more than one genus was cultured from one kernel. Connection between disease scale values of the kernels and the different detected fungi were also analyzed (Fig. 3). Mostly *Diaporthe* and *Botryosphaeria* were associated with severe symptoms. The Imc was 74%, when *Botryosphaeria* spp. was present, and 62% in the case of *Diaporthe* sp. The Imc of fruits, infected with *Alternaria*, *Fusarium* and *Penicillium* genera was lower, 53%, 38% and 48% respectively.

ITS and *tef1* sequences of some pathogen isolates cultured from kernels were amplified and sequenced (Table 1). Sequences that had 99% similarity to the ITS (AY259092) and *tef1*

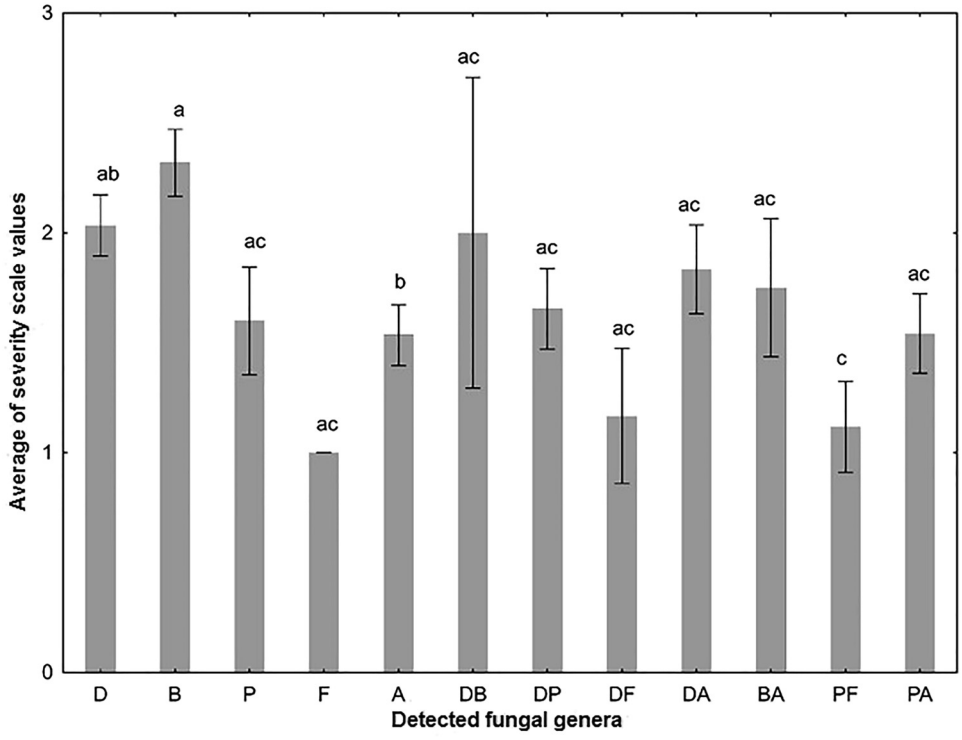

Fig. 3. Disease severity scale values of rotted kernels, naturally infected by different genera. D: *Diaporthe* spp., B: *Botryosphaeria* spp., P: *Penicillium* spp., F: *Fusarium* spp., A: *Alternaria* spp. Two letters indicate mixed infection. Different letters above columns indicate significant differences based on of Mann-Whitney U test. Error bars mark ±SE

Table 1. Botryosphaeria dothidea and *Diaporthe eres* isolates from walnut kernels, their origin and GenBank accession numbers of the ITS and *tef1* sequences

Species	Strain	Habitat	Locality	ITS	*tef1*
B. dothidea	J2015	Rotted walnut kernel	Jánkmajtis, Hungary	–	MT152102
	J2026	Rotted walnut kernel	Jánkmajtis, Hungary	–	MT152106
	JT2015	Rotted walnut kernel	Jánkmajtis, Hungary	MN706192	MT152104
	JT2035	Rotted walnut kernel	Jánkmajtis, Hungary	MT111097	MT152105
	T2016	Rotted walnut kernel	Jánkmajtis, Hungary	MT111096	MT152103
D. eres	J2008	Rotted walnut kernel	Jánkmajtis, Hungary	MT111104	MT152114
	J2010	Rotted walnut kernel	Jánkmajtis, Hungary	–	MT152113
	J2012	Rotted walnut kernel	Jánkmajtis, Hungary	MT111099	MT152108
	J2023	Rotted walnut kernel	Jánkmajtis, Hungary	MT111101	MT152110
	J2028	Rotted walnut kernel	Jánkmajtis, Hungary	MT111105	MT152115
	J2034	Rotted walnut kernel	Jánkmajtis, Hungary	MT111103	MT152112
	J2037	Rotted walnut kernel	Jánkmajtis, Hungary	MT111106	MT152116
	J2050	Rotted walnut kernel	Jánkmajtis, Hungary	MT111100	MT152109
	JT2024	Rotted walnut kernel	Jánkmajtis, Hungary	MT111102	MT152111
	JT2050	Rotted walnut kernel	Jánkmajtis, Hungary	MT111098	MT152107
	JT2036	Rotted walnut kernel	Jánkmajtis, Hungary	MN706193	–
	J3001	Asymptomatic bud	Jánkmajtis, Hungary	MT111109	MT152118
	J3009	Asymptomatic bud	Jánkmajtis, Hungary	MT111112	MT152121
	J3013	Asymptomatic bud	Jánkmajtis, Hungary	MT111114	MT152123
	J3015	Asymptomatic bud	Jánkmajtis, Hungary	MT111110	MT152119
	J3017	Asymptomatic bud	Jánkmajtis, Hungary	MT111107	MT152117
	J3024/1	Asymptomatic bud	Jánkmajtis, Hungary	MT111115	MT152124
	J3024/2	Asymptomatic bud	Jánkmajtis, Hungary	MT111111	MT152120
	J3031	Asymptomatic bud	Jánkmajtis, Hungary	MT111113	MT152122

(AY573218) sequences of the CBS110302 type strain (Alves et al., 2004) were identified as *B. dothidea* based on the phylogenetic analysis of merged ITS and tef1 sequences (Fig. 4).

D. eres species had 99–100% similarity to the ITS (OM698848) and *tef1* (OM752197) sequences of the CBS 138594 epitype culture (Udayanga et al., 2014), and this finding was supported by phylogenetic analysis as well (Fig. 5.)

Culturable fungi of walnut buds

During bud monitoring, fungi were grown from each symptomless bud. Mostly *Alternaria* spp. (88 pcs, 59%) and *Diaporthe* spp. (66 pcs, 44%) grew from the samples (Fig. 6). *Botryosphaeria* spp. was found in 4 sampled buds, while *Epicoccum* spp. was present in 4 buds (3%). ITS and *tef1* sequences of eight *Diaporthe* isolates cultured from catkins were amplified, sequenced and deponated in GeneBank (Table 1). Sequences had 99% similarity to the ITS (KC343075) and *tef1* (KC343801) sequences of the CBS109767 type strain (Gomes et al., 2013), and were identified as *D. eres*.

Result of ONFIT analysis

For ONFIT analysis, 32 healthy and 85 symptomatic green walnuts were collected in June. Symptoms were observed on the husk surface as dry brown lesions and spots. After the samples

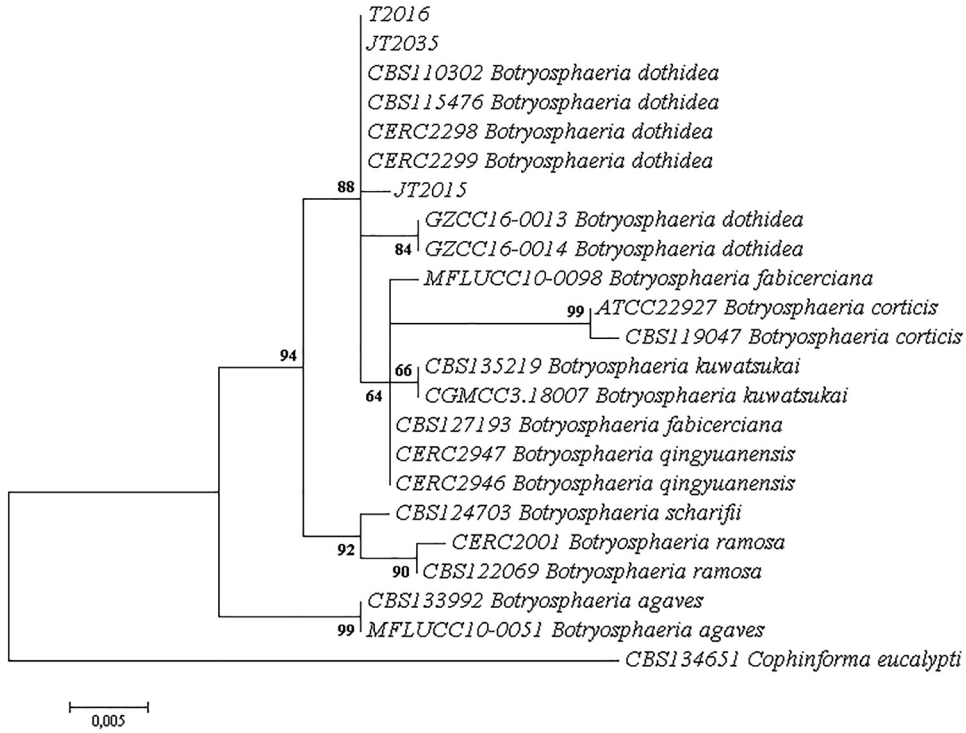

Fig. 4. Maximum likelihood phylogeny tree for the *Botryoshpaeria* species for the concatenated ITS and tef1 molecular taxonomy markers. The publicly accessible sequences of the analyzed strains from Zhang et al. (2021). The three monoclonal isolates (JT2015, JT2035 and T2016) from this study are in Table 1. Strain CBS110302 is a culture of the type *B. dothidea*. Branch length is proportional to the number of nucleotide changes per site; the scale bar is shown underneath the tree. Relative bootstrap values resulting from 1000 iterative replicates are given beside the relevant nodes, where they exceed 50%. The tree is rooted on *Cophinforma eucalypti* strains CBS 112549 and CBS134651) orthologous sequences, respectively

were cut in half, the initialization of discoloration on immature kernel was detected in several cases (Fig. 7A and B). Following freezing and incubation procedure, pathogen colonies were recorded on husks only with brown spots and lesions. *Botryosphaeria* and *Diaporthe* species were identified on 59 green walnuts based on their morphological characteristics. Botryosphaeriaceae was observed on 34 green husks, while colonies that showed characters of *Diaporthe* spp. were identified on 50 samples. Both genera were observed on the same husk in 25 cases (Fig. 7C and D).

Growth temperature characteristics of D. eres *and* B. dothidea

The result of this test showed that the temperature had significant effect on mycelial growth, and the studied fungal isolates of the two species had different optimal growth temperatures. Differences were observed even between *D. eres* isolates (Fig. 8). *D. eres* JT2050 and JT2024 isolates showed similar growth rate at all temperatures, while the other two *D. eres* isolates (J2034 and

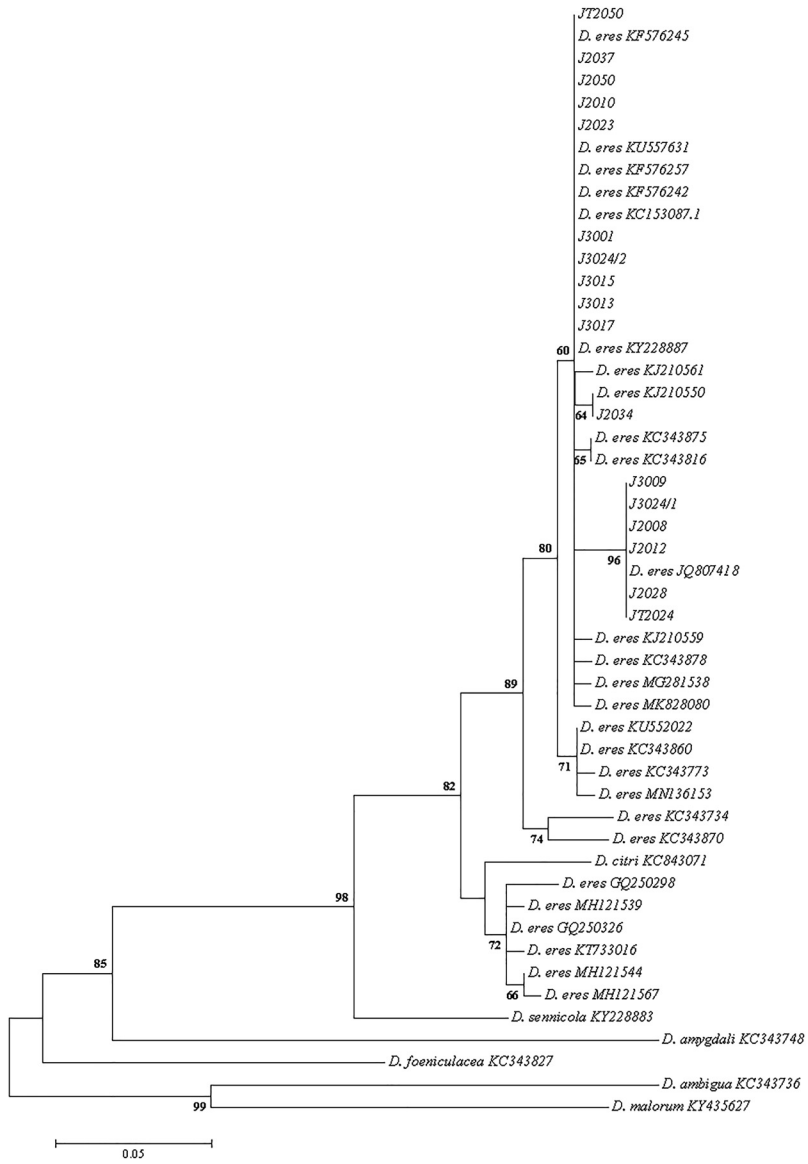

Fig. 5. Maximum likelihood phylogenetic trees of the *Diaporthe eres* species complex *tef1* sequences. Sequences deposited at the NCBI's webservers with an accession number following species name are from Hilário et al. (2021). The *tef1* sequences of strains from this study are in Table 1. Strain CBS138594 is a culture of the epitype *D. eres*. The length of branches is proportional to the number of nucleotide differences per site, and the scale is shown under the dendrogram. Branch support values (>50%) resulting from 1000 iterative bootstrap replicates are given at the nodes. The trees are rooted on *Diaporthe ambigua* CBS 114015 and *Diaporthe malorum* CAA734

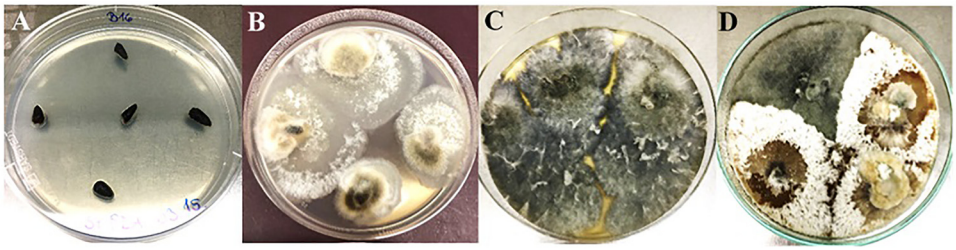

Fig. 6. A, Half-cut buds placed on PDA; B, *Diaporthe* with white structures and green *Alternaria* colonies.; C, *Botryosphaeria* spp. grown from buds with plenty of aerial mycelia; D, *Alternaria* spp. formed grey colony and *Diaporthe* colonies with characteristic white mycelia

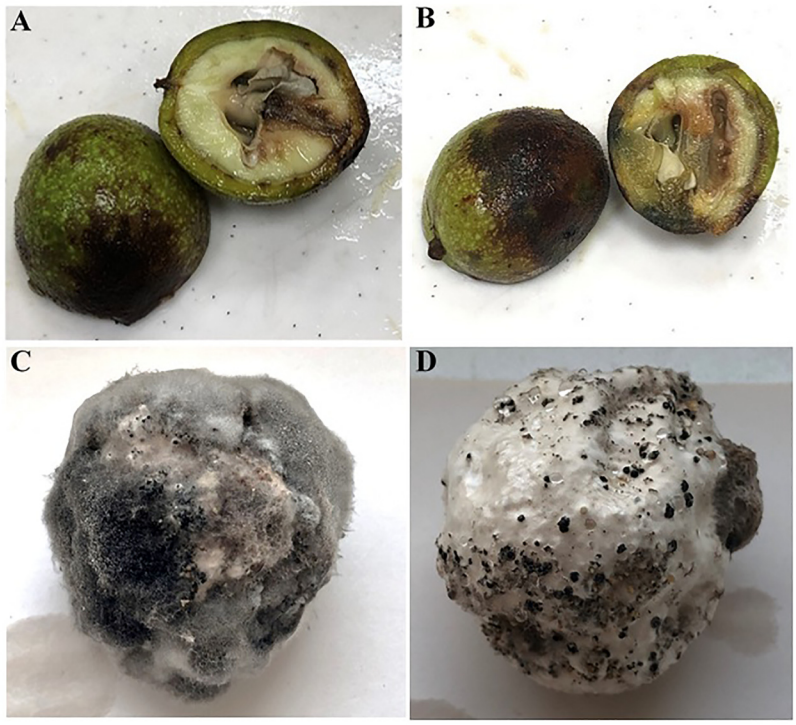

Fig. 7. A and B: Browned husk and kernel. C: *Botryosphaeria* with grey fluffy colonies after 14 day incubation and D: *Diaporthe* colony produced black pycnidia on husk

J2028) had slower colony growth, particularly at 30 °C. The *B. dothidea* isolates (JT2015 and T2016) grew at 35 °C, while the development of *D. eres* isolates was inhibited at this higher temperature. There were significant differences between the daily growth of the two species and between *D. eres* isolates (Fig. 9). Two *D. eres* isolates (J2034 and JT2024) showed the fastest

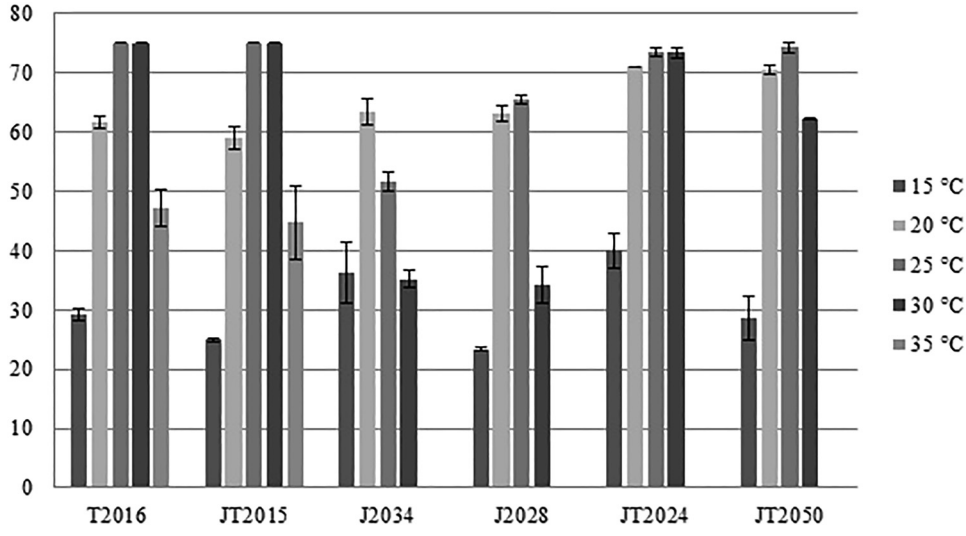

Fig. 8. Colony size of *B. dothidea* (T2016, JT2015) and *D. eres* (J2028, JT2024, JT2050) after 7 day incubation according to different temperatures. Error bars on columns indicate ±SE

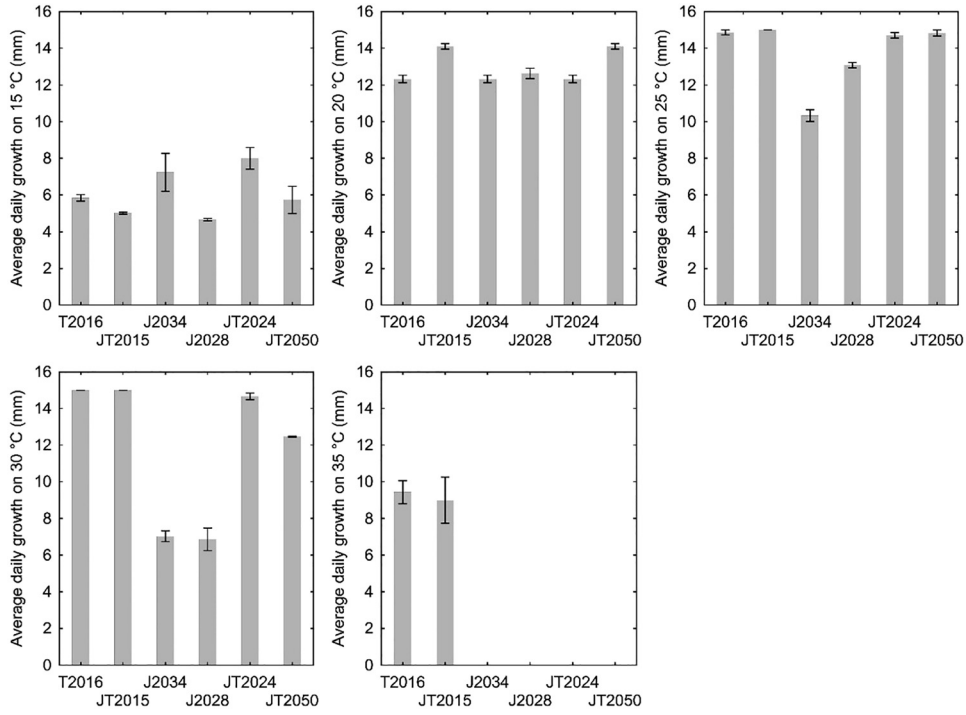

Fig. 9. Daily growth of the analysed isolates on different temperatures. Error bars indicate ±SE

growth at 15 °C, while the other two *D. eres* and the two *B. dothidea* isolates grew at a similarly slower rate. Daily growth rate of the JT2024 (*D. eres*) isolate continuously increased as the temperature was raised to 30 °C, while the growth rate of the other three *D. eres* isolates decreased above 25 °C. The two *B. dothidea* isolates preferred higher temperature, as their growth rate was higher on 35 °C than on 15 °C.

DISCUSSION

Last years, Hungarian growers recorded significant losses in their walnut orchards because of rotted, inedible nuts. Walnuts have a wide range of pests and pathogens, however producers have faced new ones recently, to which phenomenon changing climate conditions may contribute to (Desprez-Loustau et al., 2006). This work aimed to identify the pathogens of walnut kernel rot and analyze their development. Presence of *Botryosphaeria* and *Diaporthe* genera in symptomatic nuts pointed to their potential role in kernel rot in the studied samples from Hungarian commercial orchards, and the McKinney index supported this finding, as well.

Diaporthaeae and Botryosphaerieaceae are present in walnut orchards worldwide, affecting mostly branches and shoots. Members of the *D. eres* complex caused disease in walnut in China, where symptomatic branches were sampled (Fan et al., 2018), as in Czech Republic, where *D. eres* was also detected in necrotic branches (Eichmeier et al., 2020). In Turkey, species belonging to the Botrysphaeriaceae family, including *B. dothidea* were cultured from necrotic leaves, stems, shoots and blighted walnut fruits (Yildiz et al., 2022). Recently, *B. dothidea* caused canker and dieback on English walnut in Italy (Gusella et al., 2020). In Spain, *B. dothidea* and numerous of Botryosphaeriaceae and *Diaporthe* species caused shoot blight and branch dieback (López-Moral et al., 2020). Recently, green walnut brown rot caused by *B. dothidea* was recorded in China (Li et al., 2023). Moldy and black walnut caused by Botryosphaeriaceae (incl. *B. dothidea*) and Diaporthaceae were detected in the last decades in USA (Chen et al., 2014).

Our results showed the connection between these pathogens and the severe symptoms, as *Botryophaeria* (74%) and *Diaporthe* (62%) isolates had the highest index. Isolates were identified as *B. dothidea* and *D. eres* based on molecular markers. Both pathogens has wide host range. *B. dothidea* has been described to colonize *Pinus* spp. and *Eucalyptus* spp. (Smith et al., 1996), apple (Parker and Sutton 1993), pistachio (Michailides et al., 1991) blueberry (Milholland, 1972) and walnut (Zhang et al., 2022) as well. Recently several previously described *Botryosphaeria* spp., isolated from different plants were re-defined as *B. dothidea* (Zhang et al., 2021). *D. eres* was recorded on peach (Thomidis and Michailides, 2009), hazelnut (Arciuolo et al., 2021), apple (Ali et al., 2019) and blackberry (Vrandečić et al., 2011). The number of its host has been largely increased by integrating different *Diaporthe* species into the *D. eres* species complex (Hilário et al., 2021). *B. dothidea* was recorded first on giant sequoia (Vajna and Schwarczinger, 1998), and reported from walnut (Zabiák et al., 2023a) in Hungary. *D. eres* was isolated from grapevine (Kovács et al., 2014; Guarnaccia et al., 2018) and walnut (Zabiák et al., 2023a, 2023b) in Hungary.

Both fungi have been described as latent pathogens. In apple white rot, caused by *B. dothidea*, infection happed within 7 weeks after blooming (Parker and Sutton, 1993), while symptoms appeared only 6–8 weeks before harvest (Sutton, 1990), thus the pathogen is present in the immature apple fruit asymptomatically for weeks, which is the result of fungitoxic compounds

in the fruit, that inhibit the fungal growth (Kohn, 1983; Brown, 1984). Hilário et al. (2020) described blueberry twigs infection with latency for *Diaporthe* spp.

Bud monitoring (BUDMON) is a technique that can be used to estimate the current infestation of an orchard, as overwintered pathogens can initiate early infections (Moral et al., 2019). In early March, culturable microbiome of buds was surveyed in the Jánkmajtis growing area, to determine disease risk and identify the time and the potential ways of the infection routes. *D. eres* was cultured from 44% of the collected asymptomatic buds, while *B. dothidea* was present in 2.7% of samples. This analysis may serve as a pre-season prediction of walnut disease and identify the expected invasion of pathogens that are responsible for nut rot at harvest (Michailides and Morgan, 2004).

Overnight Freezing-Incubation Technique (ONFIT) (Michailides et al., 2010) indicates the microbiome of immature walnut fruit in early summer. In contrast to BUDMON, *B. dothidea* was detected in high rates on the green fruits in June. In addition, both genera were culturable, but only from husks with lesions, which may indicate the damage of walnut husk fly (*Rhagoletis completa* Cresson); however, its larvae were not found inside the nuts and the swarming of the fly usually begins in mid-July (Tóth et al., 2021). Consequently, these lesions occurred for a different reason that made green walnuts susceptible to fungal infection.

The low presence of *B. dothidea* during bud monitoring raised a question whether the lower spring temperature and slightly different life cycle was responsible for the overwhelming presence of *D. eres*. To answer this, the mycelial growth of six isolates were studied. Two *B. dothidea* and four *D. eres* isolates from rotted walnut kernel were incubated at different temperatures (15–35°C). The optimal temperature for growth of the two *B. dothidea* isolates were higher, supporting our hypothesis. Moreover, heat tolerance of these isolates is also an important information (Sánchez et al., 2003). Growth of *D. eres* isolates was inhibited at 35 °C, however, vegetative survival was not excluded (Abramczyk et al., 2020). The optimal growth temperature of the different *D. eres* strains varied between 20 and 30 °C providing rapid growth and disease development among broad temperature range.

Effective protection against walnut rot is only possible with complex techniques preferring prevention. Methods demonstrated in this work (BUDMON, ONFIT) may be suitable for infection or risk assessment in Hungarian walnut orchards, which contribute to manage the disease caused by *Botryosphaeria* and *Diaporthe* species. *B. dothidea*, with higher optimal temperature, was rarely detected in buds. Both pathogens could be detected in green walnuts with ONFIT method in high proportion from symptomatic samples. This method is suitable for the detection of latent infection, providing data for the farmers in decision-making for disease management actions (Moral et al., 2019). In addition to careful hygiene and proper horticultural techniques, it is important to schedule the spraying, including early spring infection, as pathogens could be detected in asymptomatic buds (catkins) and to pay special attention to avoid scars and wounds which may initiate the secondary, latent infection of these pathogens.

Overall, it can therefore be declared based on our results, that *B. dothidea* and *D. eres* species play an important role in defecting walnut kernels in Hungarian orchards. Their different appearance in phenological phases can be attributed to their different environmental requirements and biology, though both species were present in all studied period. *D. eres* overwintering on the twigs may initiate bud infection, while walnut rot may develop from the susceptible green walnuts colonized by fungi under favourable conditions. Therefore the monitoring of the symptoms on the different plant parts (buds, twigs, green nuts) from dormancy is necessary through

the whole year. Orchard sanitation eliminating diseased twigs and rotted nuts is essential for prevention. The timing of fungicide application is crucial, and so is the development of effective plant protection technologies. Chemical agents can be used for plant protection against the disease. Cyprodinil and fludioxonil, tebuconazole and in combination with fluopyram were effectively inhibited the mycelial growth of both pathogens in the *in vitro* tests (Zabiák et al., 2023a). Tebuconazole was used in emergency by aerial application last years in Hungary (NÉBIH 2020, 2021); however, this fungicide is no longer allowed to be used, and so more emphasis should be placed on prevention that is difficult due to the size of the walnut tree. Biological control agents are attractive alternatives to chemical fungicides. *Trichoderma afroharzianum* TR04 and *Trichoderma simmonsii* TR05 showed effective *in vitro* biocontrol activity against walnut pathogen *B. dothidea* and *D. eres* (Kovács et al., 2021). The supernatant of *Bacillus amyloliquefaciens* RD.006 was also able to inhibit the mycelial growth of *B. dothidea* isolated from rotted walnut (Zhang et al., 2022).

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