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FIRST REPORT OF *Diaporthe eres* ASSOCIATED WITH STEM AND BRANCH CANKER ON *Quercus robur* L. IN CROATIA

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SUMMARY

In June 2020, stem and branch cankers were observed on young *Quercus robur* trees growing in a floodplain forest near the Sava River in the eastern part of Croatia. Samples of affected stems and branches were randomly collected, and fungi present in the symptomatic tissue were isolated on agar media. The molecular and morphological identification of the obtained cultures revealed 29 fungal isolates belonging to 12 different taxa. The most frequently isolated species was *Diaporthe eres*, found in 77% of sampled trees. The ability of *D. eres* to cause cankers was tested in a pathogenicity trial on 3-year-old *Q. robur* saplings. Bark and wood necroses developed on all inoculated saplings, and *D. eres* was successfully re-isolated and identified using molecular tools. None of the control saplings revealed any symptoms during the trial. Therefore, Koch's postulates were fulfilled, and *D. eres* was verified as a causative agent of cankers on *Q. robur*.

KEY WORDS: pedunculate oak, bark necrosis, wood necrosis, pathogenicity, Sava River

INTRODUCTION

Quercus robur L. (pedunculate oak) is a widespread European tree species, growing in forest ecosystems and urban areas, where it provides multiple economic, social, and ecological benefits (Eaton et al. 2016, Mölder et al. 2019). In Croatia, it is the second most abundant forest tree species, covering approximately 15% of the total forest area (350 000 ha), mostly in lowland and floodplain regions, where it is greatly valued for its ecological services and high-quality timber (Županić et al. 2009, Čavlović 2010). Unfortunately, the decline of this valuable tree species has been reported in several European countries during the last few decades, caused by the synergistic activity of different abiotic and biotic factors (Thomas et al. 2002). Since *Q. robur* has shown to be highly susceptible to changes in water availability and climate changes, these factors are considered to be crucial or at least very important drivers for its decline (Ugarković et al. 2016, Macháčová et al. 2022). Besides a direct impact on the species, the aforementioned changes can also lead to a proliferation of weak and opportunistic pathogens, such as different endophytic and saprobic fungi, otherwise harmless organisms which are present

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in the healthy plant tissue or are widespread in the environment (Marçais and Breda 2006, Macháčová et al. 2022).

During June 2020, stem and branch cankers were observed on young *Q. robur* trees growing in a floodplain forest near the Sava River in the eastern part of Croatia. It was hypothesized that fungal pathogens might have caused these symptoms. To test this hypothesis, the following aims were set in this research: (1) to isolate and identify fungi present in the necrotic tissue, and (2) to test the pathogenicity of the most frequently isolated fungal species on *Q. robur* saplings.

MATERIALS AND METHODS

Study area and recorded symptoms

Reddish brown sunken canker lesions and associated chlorosis of leaves were observed during June 2020 on stems and branches of young *Q. robur* trees in the natural six-year-old floodplain forest stand in the Sava River basin in Croatia (44°55'09.4"N, 18°50'08.1"E). The forest stand was established by natural regeneration. Approximately 10% of *Q. robur* trees throughout the entire forest stand area (66.36 ha) were affected. On the less affected trees, cankers were observed only on the branches in the crown, and on the more affected trees, canker lesions have spread from branches to the stem. Necrotic bark was sunken in comparison to adjacent healthy tissue and reddish-brown to black in colour. Phloem, cambium, and xylem (sapwood) underneath the cankers were darkly discoloured and necrotic as well. Leaves on the affected branches displayed chlorosis and wilting and were shed prematurely (Figure 1).

Fungal isolation and identification

Samples of affected stems and branches were randomly collected from 13 trees in July 2020 (1 branch or stem per tree, 13 samples in total). Fungal isolations were made from the edges of symptomatic stem and branch bark (phloem) and wood (xylem) tissue. After the surface sterilization for 1 min in 96% ethanol, followed by a rinse in sterile distilled water, four small pieces of tissue (5 x 5 mm) per sample were plated on potato dextrose agar (PDA, Oxoid) supplemented with streptomycin sulphate (200 mg/L, Sigma-Aldrich) in 90 mm Petri dishes. The Petri dishes were then incubated in the dark at 19–20°C for one month and emerging mycelia were subcultured onto PDA. The obtained pure fungal cultures were grouped into morphotypes according to their morphological characteristics. At least one mycelial culture from each morphotype was subjected to molecular identification.

DNA was extracted from mycelia cultured in malt extract broth (MEB, Liofilchem) in 2 ml microtubes for 7 days by the salting-out method according to Cenis (1992). Minor modifications were made to this method according to Kranjec Orlović et al. (2024). The internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was amplified in polymerase chain reaction (PCR) with primers ITS1-F (Gardes and Bruns 1993) and ITS 4 (White et al. 1990), according to the established protocol (Kranjec Orlović et al. 2021). For two isolates belonging to the genus *Diaporthe* (QUE15 and QUE17), the partial translation elongation factor 1-α (TEF1- α), calmodulin (CAL) and β-tubulin (TUB) genes of the isolates were additionally amplified using the primers EF1-728F/EF1-986R, CAL-228F/CAL-737R (Carbone and Kohn 1999), and Bt2a/Bt2b (Glass and Donaldson 1995), following the protocols of Udayanga et al. (2014). The obtained PCR products were sequenced in both directions with primers used for amplification at the DNA sequencing facility of Macrogen Europe (Amsterdam, Netherlands). The obtained raw sequences were edited using BioEdit

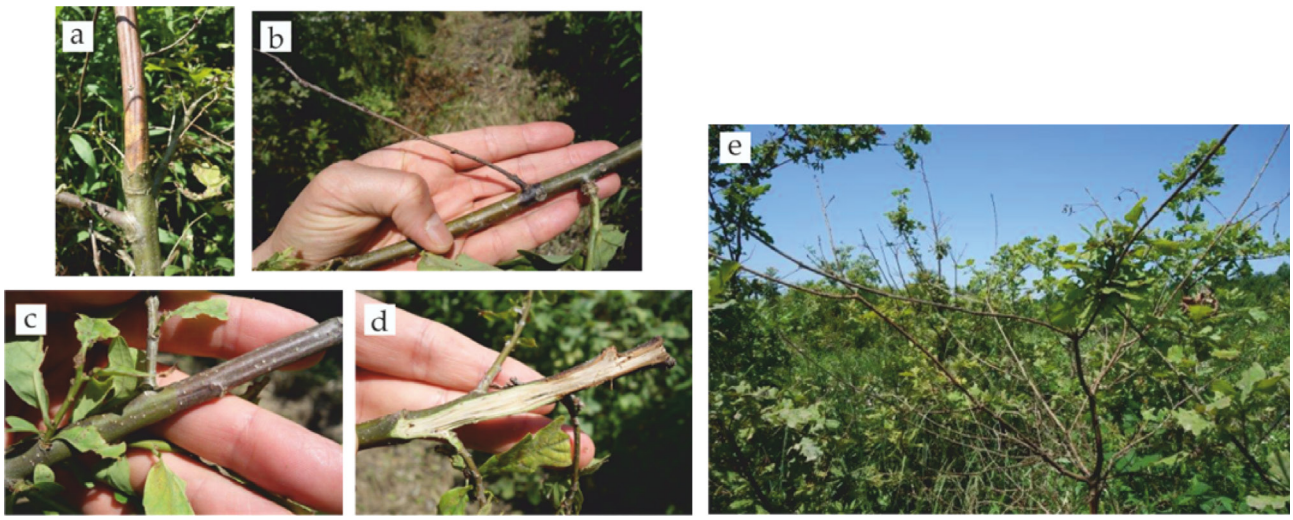

Figure 1 Symptoms observed on *Q. robur* trees: (a–c) reddish brown to black sunken canker lesions on stem and branches; (d) discoloration and necrosis of phloem, cambium, and xylem (sapwood) underneath the cankers; (e) premature shedding of leaves on affected branches.

Sequence Alignment Editor v.7.2.5 software (Hall 1999) and then compared with reference sequences deposited in the National Center for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST + 2.15.0) (Altschul et al. 1990). Sequences with 99.0%–100% and 95.0%–98.99% similarity to the reference ones were identified to the species level and to the genus level, respectively. Morphological characteristics of the obtained cultures grown on PDA for 30 days were used to confirm the results of molecular identification. Spores were harvested from pycnidia which developed in cultures, and were visualised using the Olympus DP-23 camera mounted on a microscope (Olympus BX-41). The spores were measured using the associated PRECiV Core software (Olympus, version 2.1) and their shape and dimensions were compared to the description of *D. eres* in Udayanga et al. (2014). In total, 100 spores were measured (length and width).

Pathogenicity test

Pathogenicity of the most frequently isolated fungus in this research, *Diaporthe eres* Nitschke, was tested on 3-year-old *Q. robur* saplings grown in a ground in an open field trial conducted in the nursery "Šumski vrt i arboretum" located at the University of Zagreb Faculty of Forestry and Wood Technology (45.8202° N, 16.0228° E). Saplings were on average 182 cm (± 22 cm) high and with an average root collar diameter of 1.8 cm (± 0.3 cm). During the experiment, which was conducted from 24 May to 5 July 2021, an average daily temperature ranged from 10 to 30°C (10–20°C in May, 15–30°C in June and 20–27°C in July), and total monthly rainfall amounted to 124 mm, 13.2 mm and 74.5 mm in May, June and July, respectively. Ten saplings were inoculated with the *D. eres* isolate QUE15. The upper part of a stem (30 cm from the top) was surface disinfected with 96% ethanol and then a small piece of outer bark (7 mm × 7 mm) was cut with a sterile scalpel. A PDA-mycelium plug was taken from the margin of a seven-day-old culture with a sterile steel cork borer (inner diameter 7 mm). The plug was placed in an inflicted wound (mycelium facing the inner bark), the cut bark was re-attached on top of the plug, and the stem was wrapped with Parafilm (Bemis Company Inc.) and with a piece of aluminium foil. Ten saplings were used as controls and inoculated with a sterile PDA plug applied as described above. The saplings were checked for symptoms on the outer bark around the inoculation point weekly. The re-isolation of *D. eres* was attempted six weeks after the inoculation. Stem sections 5–8 cm long with an inoculation point were submerged into 0.5% sodium hypochlorite for 15 seconds, 96% ethanol for one minute, rinsed in sterile distilled water and dried under the laminar flow hood, before transferring five pieces of inner bark or xylem (5 mm × 5 mm) taken around the margin of the observed necroses onto PDA. The obtained isolates which morphologically belonged to the genus *Diaporthe* were subjected to identification by molecular analysis of ITS region of DNA, using the already described protocol (one representative mycelial culture per inoculated sapling).

RESULTS

Fungi isolated from stem and branch cankers on *Q. robur*

In total, 29 fungal isolates belonging to 12 different taxa (Table 1) were obtained from 52 plated pieces of tissue taken from the affected *Q. robur* in the Sava River basin (four pieces from each of the 13 sampled trees).

Table 1 Parameters of multiple linear regression model for productivity.

Phylum	Fungal taxon	GenBank accession no. [1]	Isolation frequency (% of colonized samples)
Ascomycota	*Diaporthe eres* Nitschke	PQ475913 / 497657 / 497661 / 497659 PQ475914 / 497658 / 497662 / 497660	77
	Neocucurbitaria sp.	PP496181	23
	Dendrostoma leiphaemia (Fr.) Senan. & K.D. Hyde	PP496182	15
	Monochaetia sp.	PP496183	15
	Alternaria sp. 1	PP496184	8
	Alternaria sp. 2	PP496185	8
	Cladosporium cladosporioides (Fresen.) G.A. de Vries	PP496186	8
	Didymellaceae sp.	PP496187	8
	Paraconiothyrium brasiliense Verkley	PP496188	8
	Paraphaeosphaeria sp.	PP496189	8
	Sordaria fimicola (Roberge ex Desm.) Ces. & De Not.	PP496190	8
	Valsa ceratophora Tul. & C. Tul.	PP496191	8

[1] For two *D. eres* isolates (QUE15 and QUE17), GenBank accession numbers are provided for the ITS, TEF, CAL and TUB DNA region, respectively, and for other isolates only for the ITS region.

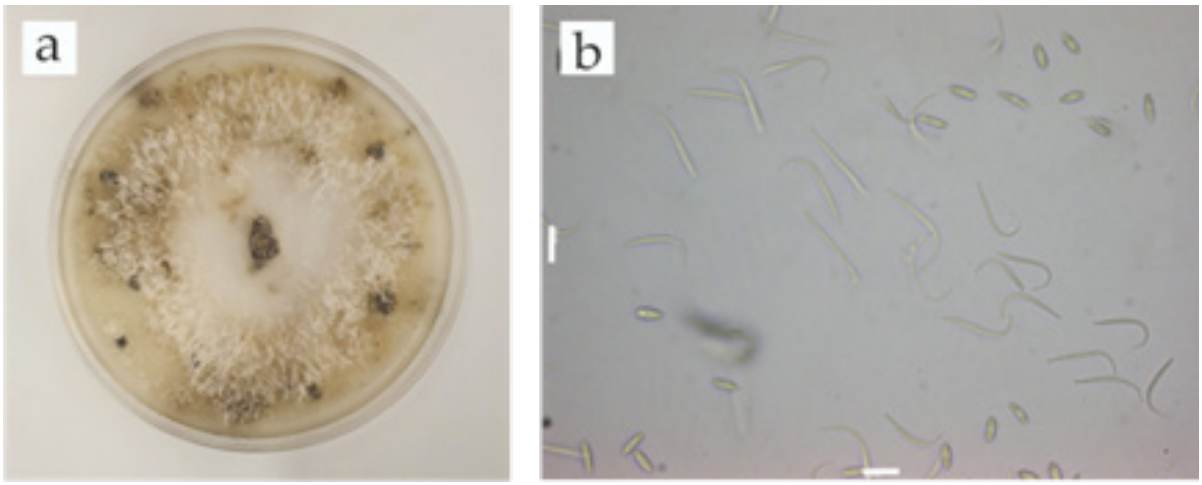

Figure 2 Morphological characteristics of *D. eres* isolate QUE15: (a) mycelium culture on PDA at 21°C after 30 days, (b) alpha and beta conidia, white scale bars: 10 μm.

The most frequently isolated species was *D. eres*, isolated from 77% of samples. Pure cultures consisted of white aerial mycelium, which turned gray after 3–4 weeks and produced dark pycnidia. Alpha conidia were hyaline, aseptate, biguttulate, fusiform to ellipsoid, measuring 5.7 to 7.9 × 2.3 to 3.5 µm (n = 50). Beta conidia were hyaline, aseptate, linear to curved, measuring 17.2 to 29.7 × 0.9 to 2.4 µm (n = 50) (Figure 2). BLAST searches confirmed isolates QUE15 and QUE17 as *D. eres*, showing the similarity of both with the *D. eres* CBS 101742 (GenBank accession numbers: KC343073/KC343799/KC343315/KC344041). QUE15 had 100% (549/549 nt), 100% (342/342 nt), 99.74% (389/390 nt) and 99.82% (549/550 nt) homology, whereas QUE17 had 100% (549/549 nt), 99.42% (342/344 nt), 99.23% (388/391 nt) and 99.82% (552/553 nt) homology with *D. eres* CBS 101742, for ITS, TEF, CAL and TUB region, respectively.

Pathogenicity of *D. eres* on *Q. robur* saplings

The first symptoms on inoculated saplings appeared one week after the inoculation, in the form of bark discoloration extending only few millimetres around the inoculation point. During the next few weeks bark discoloration extended further around the inoculation point and became darker in colour, indicating tissue necrosis. Six weeks after the inoculation, brown to black necroses extending 2–3 cm around the inoculation point were visible. Slight cracking of the bark was observed on some saplings (Figure 3a). Bark removal revealed that these necroses extended into sapwood, and only in a few cases into the heartwood as well (Figures 3b to 3d). In control saplings, callus was formed around the inoculation point and there were not any visible discolorations or necroses on bark or wood (Figures 3e and 3f).

Mycelia cultures morphologically resembling the genus *Diaporthe* were isolated from all ten inoculated saplings. ITS analysis confirmed that these isolates share 100% identity with the *D. eres* isolate QUE15. Their sequences were uploaded into the NCBI GenBank online database (accession numbers: PQ475915-24). No *Diaporthe* resembling isolates were obtained from the control saplings.

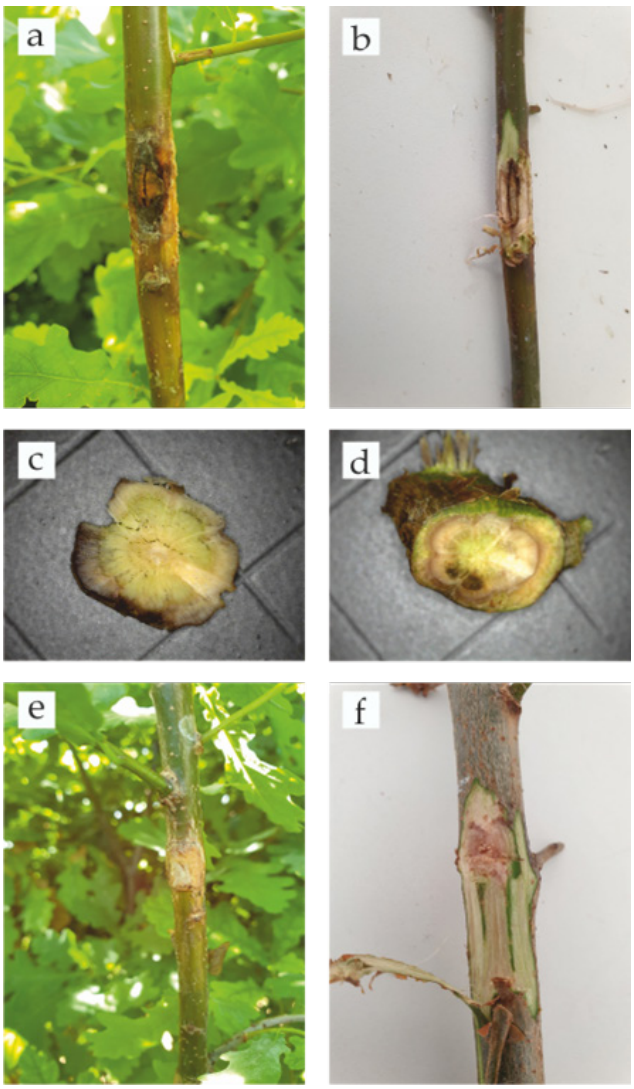

Figure 3 The appearance of *Q. robur* saplings six weeks after the inoculation: (a–d) symptoms on saplings inoculated with *D. eres* isolate QUE15; (e–f) lack of symptoms on control saplings.

DISCUSSION AND CONCLUSIONS

Diaporthe eres was frequently isolated from stem and branch cankers on oak trees from the natural floodplain forest stand in the Sava River basin in 2020, and the ability of the fungus to cause bark and wood necroses on *Q. robur* was confirmed by a pathogenicity test, where Koch's postulates were fulfilled on all inoculated saplings. Most of the other fungal taxa isolated from cankers on *Q. robur* in this study were represented with only one isolate. According to the available literature, some of the identified genera and species have been previously associated with *Q. robur* as endophytes or opportunistic pathogens, such as some members of the genera *Monochaetia* (Gennaro et al. 2003, Akdeniz and Hacer 2023) and *Neocucurbitaria* (Jaklitsch et al. 2018, Costa et al. 2020), and the species *Dendrostoma leiphaemia* (Fr.) Senan. & K.D. Hyde (Jaklitsch and Voglmayr 2019). Due to limited time and resources, *D. eres* was the only species tested for its pathogenicity towards *Q. robur* in this study.

In literature *D. eres* has been described as an opportunistic pathogen which colonizes many woody plant species, causing leaf spots, shoot blight, stem and branch cankers and other various disease symptoms. It was reported on *Juglans cinerea* L. in the USA (Anagnostakis 2007), *Prunus persica* (L.) Batsch in Greece and Italy (Thomidis and Michailides 2009, Prencipe et al. 2017), *Vitis vinifera* L. in the USA (Baumgartner et al. 2013), *Vaccinium* species in Europe (Lombard et al. 2014), *Ziziphus jujuba* Mill. in China (Zhang et al. 2018), *Corylus avellana* L. in the USA (Wiman et al. 2019), *Fraxinus excelsior* L. in Montenegro (Vemić et al. 2019), *Malus domestica* (Suckow) Borkh. in Canada (Ali et al. 2020), *Cinnamomum camphora* (L.) J.Presl in China (Li et al. 2021), *Prunus amygdalus* Batsch (*P. dulcis* (Mill.) D.A.Webb) in the USA (Holland et al. 2021), *Juglans regia* L. in Hungary (Zabiák et al. 2023), and *Prunus avium* L. in China (Chen et al. 2023). In Croatia, the fungus was reported to cause cane blight on *Rubus* sp. (Vrandečić et al. 2011), wood canker of *V. vinifera* (Kaliterna et al. 2012), and twig blight and canker of *Vaccinium corymbosum* L. (Ivić et al. 2018). In this study, necroses obtained in a pathogenicity trial were limited to a relatively small circumference around the inoculation point on stems of *Q. robur* saplings. This is consistent with the results of previous research on oaks, where the fungus was isolated from the wood and bark necroses on *Quercus suber* L. in Portugal and *Q. robur* in Poland and described as a weak pathogen on these hosts based on the relatively small lesion development in inoculation trials (Lopes et al. 2021, Jankowiak et al. 2022). Based on the obtained results in this study, the same conclusion could be drawn.

The endophytic lifestyle of *D. eres* in different plant tissues was also reported by some authors, e.g. in shoots of *Prunus domestica* L. (Abramczyk et al. 2022), branches of *Fagus sylvatica* L. (Kowalski and Kehr 1992), leaves of *Acer macrophyllum* Pursh (Sieber and Dorworth 1994), bark of *Taxus chinensis* (Pilg.) Rehder (Liu et al. 2009), leaves and branches of *Quercus cerris* L. (Moricca et al. 2012), needles of *Larix kaempferi* (Lamb.) Carrière and *Pinus densiflora* Siebold et Zucc. (Kim et al. 2013), and twigs and branches of *Citrus* sp. (Huang et al. 2015). In the light of the above, there is a possibility that the fungus was already present in the healthy wood or bark of *Q. robur* in the studied floodplain forest as an endophyte, and that it shifted to a pathogenic lifestyle and caused cankers on those plants which experienced a loss of vitality due to other factors. Although isolations from healthy control saplings in this study did not corroborate this (no *D. eres* found in the healthy *Q. robur* tissue), isolation of the fungus from the healthy wood or bark tissue of *Q. robur* growing in natural habitats should be attempted in future studies.

The studied *Q. robur* trees with cankers were removed during the usual sanitary activities by foresters (personal communication) and the remaining trees have not exhibited similar symptoms on a larger scale so far (to the day of publishing this article). *Q. robur* in eastern Croatia has shown to be highly susceptible to climate change and availability of water (Ugarković et al. 2016), and it is often exposed to severe attacks by an invasive pest, *Corythuca arcuata* (Say, 1832) (Csóka et al. 2020). It is possible that the young oak trees in this study underwent the loss of vitality caused by some of these factors, and that *D. eres*, and possibly some other fungi, shifted from endophytic to parasitic lifestyle and contributed to the development of cankers. In the case of another outbreak of similar symptoms on oaks in Croatia, the pathogenicity of other fungi and other *D. eres* isolates towards *Q. robur* should be tested in inoculation trials, to verify the results obtained in this study.

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