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Short Communication

First Report of *Diplodia fraxini* and *Diplodia subglobosa* Causing Canker and Dieback of *Fraxinus excelsior* in Slovenia

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Abstract

In recent decades the vitality and productivity of European ash trees in Slovenia have been reduced by the onset of canker and dieback disease symptoms on young and old trees, identified primarily as ash dieback caused by *Hymenoscyphus fraxineus*. Given the limited information available about the etiology of this emerging disease, a study was carried out to isolate, identify, and characterize the fungal species involved in the observed ash symptoms. Field surveys were conducted in five forest sites where 50 symptomatic branch samples were collected. All samples were inspected and used for fungal isolation. Based on morphology, colony appearance, and DNA sequence data of the internal transcribed spacer region, 125 fungal colonies belonging to five species were isolated and identified. Only a few symptomatic ash samples yielded colonies of *H. fraxineus*, whereas Botryosphaeriaceae species were isolated with a high frequency, with *Diplodia fraxini* as the dominant species. A pathogenicity test proved that all isolated species were pathogenic on European ash, causing bark lesions and wood discoloration. All Botryosphaeriaceae species isolated in this study are reported for the first time on European ash in Slovenia.

Keywords: Botryosphaeriaceae, emerging disease, invasive species, pathogenicity

European ash (*Fraxinus excelsior* L.) is an ecologically and economically important tree species in Slovenia, with a recorded wood stock of 3.8 million m^3 in 2019 (Slovenia Forest Service 2019a). Since 2006, severe ash dieback symptoms such as leaf necrotic spots; shoot, twig, and branch dieback; necrotic bark; sunken canker; and gray to brown discoloration of the wood have been observed throughout the country. The amount of felled ash trees due to the disease has risen exponentially in recent years, and in 2019, it reached 47,827 m^3 (Ogris 2018; Slovenia Forest Service 2019b).

Ash dieback is an emerging and devastating disease widespread in Central Europe. The causal agent was initially identified as the invasive fungus *Hymenoscyphus fraxineus* (T. Kowalski) Baral, Queloz & Hosoya (Bakys et al. 2009; Giongo et al. 2017; Kowalski 2006).

In Slovenia, *H. fraxineus* was first detected in the northeast in 2006, and in a few years it became widespread throughout the country (Ogris et al. 2009). In particular, *H. fraxineus* was most frequently isolated from the leading edge of discolored wood and necrotic leaf petioles and rarely from necrotic bark. In addition to *H. fraxineus*, other fungal pathogens and pests, such as *Armillaria* spp., *Diplodia mutila* (Fr.: Fr.) Fr., and the ash bark beetle (*Leperesinus fraxini* Panzer, 1799), have been associated with the disease in different Slovenian ash stands (Hauptman et al. 2012, 2016). Given the complex symptomatology and etiology of this emerging disease in Slovenia, a thorough study was conducted to isolate, identify, and characterize the main causal agents.

Field surveys were conducted in five young European ash forest stands with close-to-nature management where European ash is present naturally. The stands are distributed in the central part of Slovenia, where ash dieback symptoms have been observed (Table 1). In August 2018, a circular monitoring plot (MP) 10 m in diameter was established at each site and geographic coordinates recorded with a portable GPS. All European ash trees inside the MP were visually inspected for the presence of ash dieback symptoms according to the method of Linaldeddu et al. (2020). To ascertain the causal agents of the main symptoms observed, at each MP 10 symptomatic European ash trees were randomly chosen for sampling. A single branch showing dark brown outer and inner bark discoloration or sunken cankers was taken from each tree. Estimated tree heights were 2 to 6 m, estimated breast height diameter of trunks ranged from 2 to 8 cm, estimated diameter of branches collected for sampling ranged from 1.5 to 5 cm. Samples were stored at 4°C and processed in the laboratory within 72 h.

In the laboratory, all branch samples were visually inspected and superficially disinfected with 90% ethanol for 1 min, and the outer bark was removed with a sterile scalpel. Longitudinal and transverse cuts were made to observe any internal symptoms. Isolations were made from approximately 5-mm^2 chips of inner bark and xylem cut aseptically from the margin of necrotic lesions (Linaldeddu et al. 2020). All chips were placed in 90-mm Petri dishes containing 3.9% (wt/vol) potato dextrose agar (PDA, Oxoid Ltd.). After incubation at 25 ± 1°C for 5 to 10 days in the dark, the emerging fungal colonies were subcultured onto PDA and incubated at 25°C in the dark.

All isolates were initially grouped into morphotypes based on micromorphological features and colony growth patterns, including surface and reverse colony appearance observed after 7 days of incubation on PDA at 25°C in the dark. Molecular analysis was used to identify all isolates at species level. The InstaGene Matrix (BioRad Laboratories, Hercules, CA) was used to extract genomic DNA from the mycelium of 5-day-old colonies. The primers ITS1 and ITS4 (White et al. 1990) were used to amplify and sequence the internal transcribed spacer (ITS) regions, including the complete 5.8S gene.

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PCR mixtures and amplification conditions were as described by Linaldeddu et al. (2016a). The PCR products were purified with a EUROGOLD gel extraction kit (EuroClone S.p.A.) according to the manufacturer's instructions. The ITS regions were sequenced by the BMR Genomics DNA sequencing service (www.bmr-genomics.it), in both directions, with the primers used for amplification. The nucleotide sequences were read and edited with FinchTV version 1.4.0 (Geospiza, Inc.; https://digitalworldbiology.com/FinchTV) and then compared with reference sequences (type material) retrieved from the GenBank database via the BLASTn algorithm. ITS sequences from representative isolates obtained in this study were deposited in GenBank (Table 2).

The pathogenicity of eight isolates representing the five species isolated in this study was assessed on 3-year-old European ash seedlings in spring (May to July). Six seedlings grown in plastic pots (12 cm diameter and 2 liters in volume) were inoculated with each of the eight selected isolates, and six seedlings were used as controls. A total of 54 seedlings were included in the experiment. The fungal isolates were inoculated in the lower part of the main stem (about 1.2 cm in diameter). The inoculation point was initially surface disinfected with 90% ethanol, and then a small wound (5 mm diameter) made with a flamed cork borer was inoculated with an agar–mycelium plug of the same size, taken from the margin of an actively growing colony on PDA. The inoculation site was covered with cotton wool soaked in sterile water and wrapped in a piece of aluminum foil secured with masking tape. Controls were inoculated with a sterile PDA plug applied as described previously. All inoculated plants were kept in field conditions (outdoors) at 12 to 38°C and watered regularly for 60 days. Reisolation of inoculated species was attempted by transferring 10 pieces of inner bark and wood tissues taken from the margin of each lesion onto PDA. The stem was surface disinfected with 90% ethanol before fungal reisolation. The cultures obtained were grown in daylight at room temperature and then identified by morphological and molecular analysis (ITS region). The data (wood discoloration lengths) from the pathogenicity test were first checked for normality and then subjected to analysis of variance. Significant differences between mean values were determined via the least significant difference multiple range test ($P = 0.05$) after a one-way analysis of variance. Statistical analyses were performed in XLSTAT 2008 software (Addinsoft, France).

Field surveys conducted in five European ash forest stands in Slovenia revealed the presence of severe branch cankers and dieback symptoms in all sampling sites (Fig. 1). Other common external symptoms included inner bark lesions, epicormic shoots often associated with top dieback and the death of branches, and wilting foliage. Internal wood symptoms on branches ranged from brown vascular streaking visible as spots in cross-sections and characteristic "V" shaped sectors (Fig. 1).

The highest incidence of symptomatic European ash trees was observed at sites 1 and 2 (66.4 and 59.2% of monitored trees, respectively). In contrast, only 26.9% of surveyed trees in site 5 exhibited typical canker or dieback symptoms. The highest tree mortality rate was detected at sites 4 and 5 (29.9 and 26.9%, respectively). The highest percentage of visually asymptomatic trees (63.1%) was observed at site 3.

Isolations performed from 50 symptomatic branch samples (19 branches with inner bark discoloration and 31 with sunken cankers) yielded a total of 125 fungal colonies that, on the basis of colony appearance and conidial features, were grouped into five morphotypes. BLAST analysis of ITS sequences confirmed the identity of isolates that belong to the species *Diplodia fraxini* (Fr.: Fr.) Fr. (37 isolates), *Diaporthe eres* Nitschke (36), *Diplodia subglobosa* A.J.L. Phillips, Deidda & Linaldeddu (26), *Diplodia seriata* De Not. (16), and *H. fraxineus* (10) (Table 2). Botryosphaeriaceae species showed a wide geographic distribution across the monitored ash forest stands. In particular, *D. fraxini* was the dominant species and was recorded in all sites and from both types of symptoms, although with a higher incidence from sunken cankers with the characteristic V-shaped necrotic sector (Table 2). *Diaporthe eres* was the second most common species and was often found in association with *Diplodia* spp.

In the artificial inoculation trial, selected isolates representing *D. fraxini*, *D. subglobosa*, *D. seriata*, *Diaporthe eres*, and *H. fraxineus* proved to be pathogenic to European ash, although with different levels of aggressiveness (Fig. 1). *D. fraxini* was the most aggressive species, whereas no significant differences in lesion size were detected between the isolates of the other four species (Table 3). All inoculated fungal species were successfully reisolated from necrotic stem tissues, although *H. fraxineus* was reisolated at a low frequency (Table 3). Control seedlings inoculated with sterile PDA plugs remained symptomless.

This study is the most comprehensive investigation of ash dieback–causing agents to date in Slovenia. Several studies have investigated the possible roles played by different fungi and oomycetes in the ash dieback etiology in Europe, demonstrating that, besides *H. fraxineus*, other species, such as *D. mutila*, *Diaporthe eres*, and *Phytophthora* spp., are also associated with the disease (Kowalski et al. 2017; Orlikowski et al. 2011; Przybyl 2002a, b). Moreover, in a recent study conducted in Italy, *D. fraxini* and *D. subglobosa* were found to be the main species involved in the ash dieback etiology (Linaldeddu et al. 2020). The results obtained in this study confirm that Botryosphaeriaceae species are the dominant component associated with branch canker and dieback symptoms, suggesting that *D. fraxini* plays a primary role in the

Table 1. Information on study sites

Study site	Elevation (m a.s.l.)	Geographic coordinates		No. of monitored trees
1	291	46°01′19″ N	14°28′08″ E	125
2	354	45°59′04″ N	14°15′03″ E	49
3	319	46°08′45″ N	14°22′56″ E	344
4	303	46°03′09″ N	14°29′32″ E	97
5	311	46°03′17″ N	14°28′53″ E	67

Table 2. Internal transcribed spacer (ITS) accession numbers deposited in GenBank and number of isolates of each species obtained from branches with brown inner bark discolorations and branches with sunken canker

Fungal species	Accession no.	Number of isolates		Number of sites
		Bark lesions	Sunken canker	
Diaporthe eres	MZ008006	12	24	5
Diplodia fraxini	MZ008008	8	29	5
Diplodia seriata	MZ008010	5	11	5
Diplodia subglobosa	MZ008011	3	23	5
Hymenoshyphus fraxineus	MZ008013	7	3	4

pathogenic process. *D. fraxini* has recently been identified as the main canker-causing agent on narrow-leaved ash (*Fraxinus angustifolia* Vahl) in Spain (Elena et al. 2018). *D. subglobosa* has been isolated in all study sites, confirming it to be an emerging species directly involved in the ash dieback etiology. The Botryosphaeriaceae family, encompassing a range of invasive pathogens, represents a growing threat to agricultural, forestry, and urban ecosystems in temperate, subtropical, and tropical areas (Kazemzadeh Chakusary et al. 2019; Linaldeddu et al. 2016b; Mahamedi et al. 2020; Pereira et al. 2009; Urbez-Torres 2011). All Botryosphaeriaceae species isolated in this study are reported for the first time on European ash in Slovenia.

Another interesting result of this study is related both to the low isolation frequency of *H. fraxineus* and its presence in only four of the five sites monitored. All Botryosphaeriaceae species isolated were more frequently related to branches with sunken canker and *H. fraxineus* with bark lesions (Table 2). In addition, as also found by other studies (Vemić et al. 2019), the isolation frequency of *H. fraxineus* from artificially inoculated tissues was low.

Diaporthe eres has been detected as the second most common species. This finding is in agreement with other studies where this pathogen was isolated from the twigs and branches of European ash with dieback symptoms (Linaldeddu et al. 2020; Vemić et al. 2019). Vemić et al. (2019) reported *Diaporthe eres* as a weak pathogen on 1-year-old common ash plants and emphasized that this species poses an additional risk to the natural regeneration of forest stands affected by ash dieback.

In consideration of the new insight into the etiology of this disease, a study is in progress to elucidate the possible detrimental

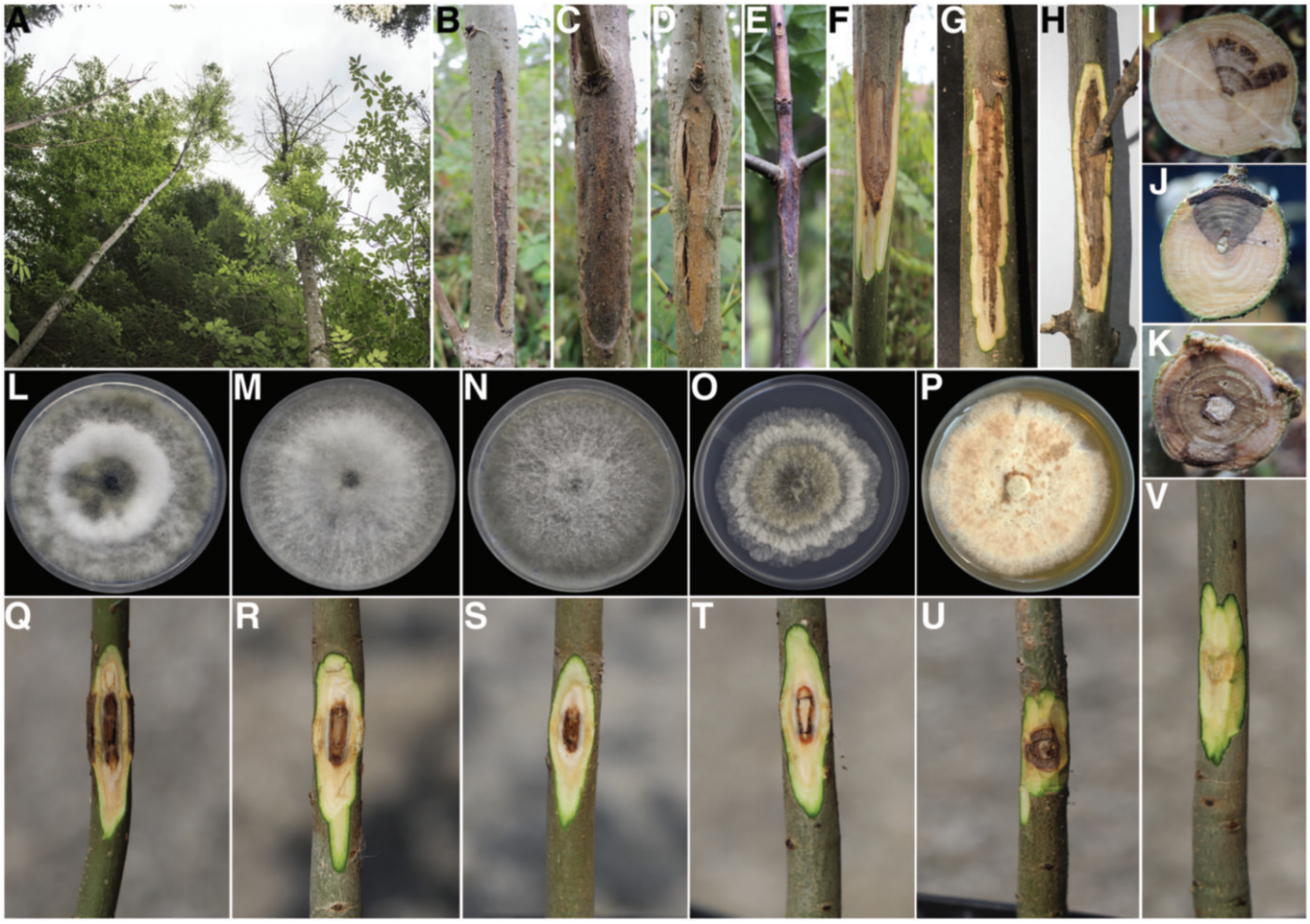

Fig. 1. Main disease symptoms observed on European ash trees. **A,** Extensive canopy dieback. **B to H,** Branches with active sunken cankers and inner bark discolorations. **I to K,** Cross-sections of branches showing wood necrotic sector. Colony morphology of **L,** *Diplodia fraxini*; **M,** *Diplodia subglobosa*; **N,** *Diplodia seriata*; **O,** *Diaporthe eres*; and **P,** *Hymenoscyphus fraxineus* after 7 days of growth at 25°C on PDA in the dark (after 30 days on 60 mm Petri dish for *H. fraxineus*). Wood symptoms observed on European ash seedlings 60 days after inoculation with **Q,** *D. fraxini*, **R,** *D. subglobosa*, **S,** *D. seriata*, **T,** *D. eres*, and **U,** *H. fraxineus*. **V,** Control.

Table 3. Mean lesion length (± standard deviation) caused by isolates of the five fungal species on European ash seedlings and percentage of positive reisolations

Species	Isolate code	Lesion length (mm)[z]	Reisolation frequency (%)
Diaporthe eres	SL2	10 ± 0.1 b	100
Diaporthe eres	SL27	10 ± 0.2 b	83
Diplodia fraxini	SL1	18.1 ± 0.3 a	100
Diplodia fraxini	SL13	17.3 ± 0.8 a	100
Diplodia seriata	A1	8.5 ± 0.1 b	100
Diplodia subglobosa	SL8	11.7 ± 0.2 b	100
Diplodia subglobosa	SL12	11.5 ± 0.3 b	100
Hymenoscyphus fraxineus	SL54	10 ± 0.2 b	50

[z]Values with the same letter do not differ significantly at $P = 0.05$, according to the least significant difference multiple range test.

interaction between *D. fraxini*, *D. subglobosa, H. fraxineus*, *Diaporthe eres*, and some *Phytophthora* species detected recently in declining ash stands (B. T. Linaldeddu, *unpublished data*). In conclusion, this study allowed us to expand knowledge on the etiology of ash dieback in Europe, suggesting that it is a complex disease caused by coinfections by multiple pathogens. Therefore, to properly manage declining ash stands, the remarkable diversity of the pathogens involved should be given due consideration.

Acknowledgments

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