J Plant Dis Prot (2025) 132:100
https://doi.org/10.1007/s41348-025-01096-z

SHORT COMMUNICATION

First report of various *Diplodia* spp. causing stem cankers of *Ulmus* sp., *Platanus* x *hispanica*, *Aesculus hippocastanum*, *Fagus sylvatica*, *Sorbus domestica*, *Carpinus betulus* and *Crataegus persimilis* in central Europe

Kathrin Blumenstein[1] · **Yasin Korkmaz**[1]

Received: 22 October 2024 / Accepted: 29 April 2025 / Published online: 24 May 2025

Abstract

This report documents the first identification of *Diplodia* species on atypical tree hosts, isolated from stem cankers. *Diplodia* is part of the Botryosphaeriaceae family, which includes plant pathogens that predominantly infect conifer species. However, *Diplodia* species are expanding their host range and geographic distribution worldwide. Typically associated with tip blight, the fungi described in this study were isolated from stem necroses in urban trees across various German cities, representing distinct geographical regions. *Diplodia mutila* was identified for the first time on *Carpinus betulus*, *Fagus sylvatica*, *Crataegus persimilis*, and *Platanus x hispanica*. Additionally, *Diplodia juglandis* was isolated from *Aesculus hippocastanum* and *Platanus x hispanica*, marking a first record for these hosts. *Diplodia seriata* was detected as a first finding on *Aesculus hippocastanum*, *Crataegus persimilis*, *Platanus* x *hispanica*, and *Ulmus* species, while *Diplodia malorum* was isolated for the first time from *Sorbus domestica*, and *Diplodia sapinea* on *Ulmus* species. The taxonomy of these fungi was verified through sequencing with multiple primers, including ITSu1/4, LSU/LR6, EF1a 526/1567R, Beta-tubulin T1/T22, and species-specific *Diplodia* primers where applicable. The pathogenicity of these fungi was confirmed by fulfilling Koch's postulates. Stem infection trials were conducted on the corresponding tree hosts using the isolated fungi, and the resulting symptoms confirmed their pathogenic role. The growing incidence of *Diplodia* species on non-coniferous hosts in urban environments raises significant concerns not only for forestry but also for urban management. This underscores the need for further research and heightened awareness of these pathogens.

Keywords Urban tree health · Host range expansion · Pathogenicity · Botryosphaeriaceae · Molecular primers · Stem necrosis

Introduction

Members of the Botryosphaeriaceae family are well-known as tree saprotrophs and opportunistic pathogens that can cause serious diseases such as needle blight, stem cankers, and tip blight (Slippers and Wingfield 2007). Traditionally, *Diplodia* spp. have been associated with conifers, particularly in forest ecosystems. However, *Diplodia* species can shift from an endophytic lifestyle, where they reside in plants without causing harm, to a pathogenic state under favorable conditions (Bußkamp et al. 2020). This lifestyle flexibility enhances their survival and ability to infect trees, especially those already weakened by environmental stressors.

The expansion of *Diplodia*'s host range is also increasingly recognized as a serious threat to global forestry. *Diplodia* spp. were known to primarily affect conifer species such as *Pinus* and *Picea*, where they have caused significant economic and ecological losses. This is particularly evident in Northern European countries, where the natural distribution range of *Pinus* spp. has been severely impacted by these pathogens (Terhonen et al. 2021; Adamson et al. 2021). However, emerging research has demonstrated that *Diplodia* species are not confined to conifers. Instead, they are now increasingly found on deciduous trees as well, indicating a broadening of their host preferences. For example, *Diplodia sapinea* (Fr.) Fuckel, has recently been documented on *Fagus sylvatica*

✉ Kathrin Blumenstein
kathrin.blumenstein@forpath.uni-freiburg.de

[1] Faculty of Environment and Natural Resources, Chair of Pathology of Trees, University of Freiburg, Bertoldstr. 17, 79098 Freiburg, Germany

L. (European beech), a deciduous species, underscoring its ability to infect a wider variety of hosts than previously recognized (Langer and Bußkamp 2021). This host jump represents a growing concern for urban forestry and natural ecosystems alike, as it suggests that *Diplodia* spp. are highly adaptable and capable of exploiting diverse environments, including those outside their traditional ecological niches (Blumenstein et al. 2022).

Urban trees, which are a vital part of city landscapes, providing crucial ecological, aesthetic, and recreational benefits (Sousa-Silva et al. 2024). Unlike trees in forests, urban trees face a multitude of additional stress factors, such as drought, pollution, poor soil conditions, and mechanical injuries from pruning and construction. These stresses can make them more susceptible to infections by opportunistic pathogens like *Diplodia* spp. Previous studies on these fungi have primarily focused on their impact on conifers in forests and timber plantations, but little is known about their behavior on non-coniferous hosts, especially in urban environments. The recent identification of *Diplodia* spp. on deciduous trees suggests that these fungi are expanding their ecological and geographical range, posing new challenges for tree health management in both urban and rural areas. This study addresses an important knowledge gap by reporting the first identification of *Diplodia* species on atypical tree hosts isolated from stem cankers in urban environments across various German cities. Understanding the pathogenic potential of these fungi on a wider range of tree hosts is essential for developing effective management strategies and mitigating the future risks posed by these emerging tree pathogens (Franić et al. 2023).

Materials and methods

Sample collection

Inventories were conducted in urban areas where various tree species are widespread, selected to include diverse geographical conditions in the south, central, and west of Germany (see cities in Table 1). Multiple tree species showed signs of similar necrotic canker tissue symptoms in different parts of these cities, including city parks and streets: *Ulmus* sp. (elm hybrid), *Platanus* × *hispanica* Münchh. (London plane), *Aesculus hippocastanum* L. (European horse chestnut), *Fagus sylvatica* (European beech), *Sorbus domestica* L. (service tree), *Carpinus betulus* L. (hornbeam) and *Crataegus persimilis* L. (plumleaf hawthorn). Visually detectable, partially bleeding cankers were observed on the stems and branches (both freshly pruned and unpruned) of urban trees (Fig. 1a–h). Samples were then collected for microbiological analysis to assess the presence of microorganisms associated with the cankers. Two types of samples were collected: (1) canker tissue samples, and (2) wood chip samples.

(1) *Canker tissue samples*: The necrotic area underneath the upper microbial canker surface was wiped using sterile cotton swabs (Fig. 1d). These swabs were then placed into 2.0 ml Eppendorf tubes containing 1 ml of 1 × TAE buffer. The swabbing process aimed to collect microbial communities, including any microorganisms present in the necrotic tissue. The swabs were immersed in the buffer to allow microorganisms to be suspended in the liquid for further analysis. Later the same day, half of the tubes containing the swabs and buffer were poured onto MEA (Malt Extract Agar) Petri dishes to allow microbial growth. The remaining

Table 1 Primer characteristics used for the taxonomical identification of the fungal isolates

Locus	Primer	Forward/reverse	Annealing temperature	References
ITS	ITSu 1 / ITSu 4	5'-GGA AGK ARA AGT CGT AAC AAG G-3' 5'-RGT TTC TTT TCC TCC GCT TA-3'	57 °C	(Cheng et al. 2016)
LSU	[1]LSU / [2]LR 6	5'-GAC CCG AAA GAT GGT GAA-3' 5'-CGC CAG TTC TGC TTA CC-3'	55 °C	[1]Blumenstein and Korkmaz (this study) [2](Vilgalys and Hester 1990)
tef	[3]EF1a 526 / [4]EF1a 1567R	5'-GTC GTY GTY ATY GGH CAY GT-3' 5'-ACH GTR CCR ATA CCA CCR ATC TT-3'	52 °C	[3]Blumenstein and Korkmaz (this study) [4](Rehner and Buckley 2005)
TUB2	Beta-Tubulin T1 / Beta-Tubulin T22	5'-AAC ATG CGT GAG ATT GTA AGT-3' 5'-TCT GGA TGT TGT TGG GAA TCC-3'	52 °C	(O'Donnell and Cigelnik 1997)
mtSSU	DiSapi-F / Diplo-R	5'-CCC TTA TAT ATC AAA CTA TGC TTT GT-3' 5'-TTA CAT AGA GGA TTG CCT TCG-3'	61 °C	(Adamson et al. 2021)
SCAR	DS3.8S3 F/ DS3.8 R6	5'-ATC CTC ATA CTA CGG CAC GG-3' 5'-AAC GGT GAC CCA TTC CAC-3'	60 °C	(Martín et al. 2014)

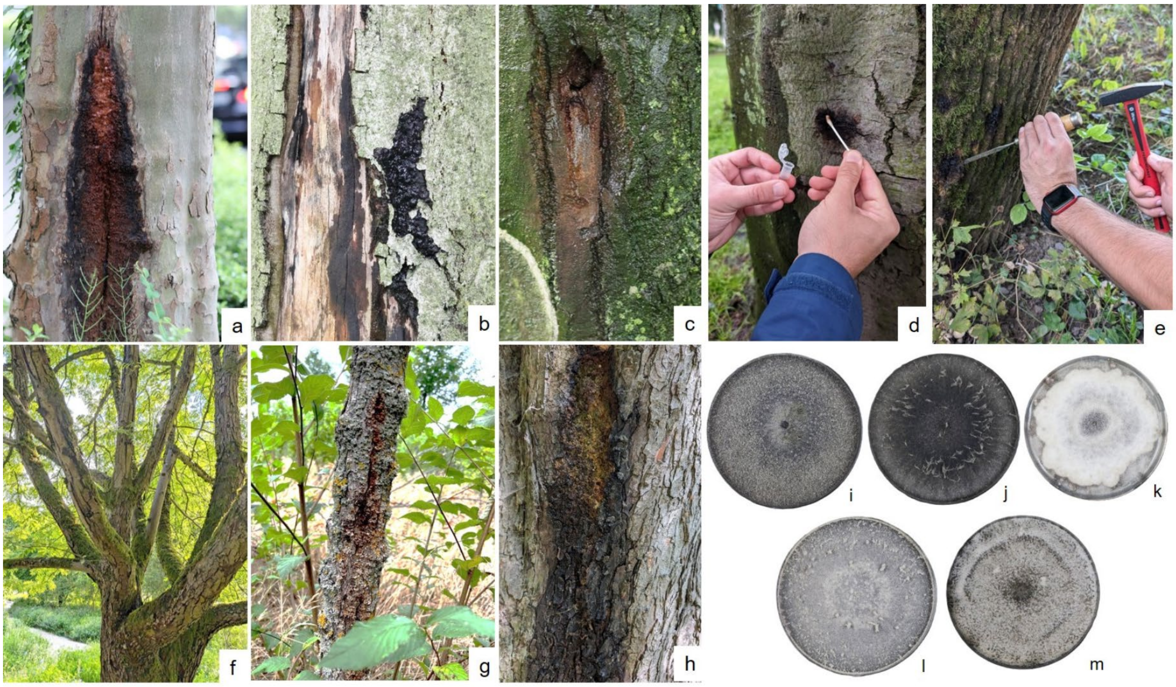

Fig. 1 Canker and oozing symptoms (**a**: *P.* × *hispanica,* **b**: *F. sylvatica,* **c**: *C. betulus,* **f**: *S. domestica,* **g**: *Ulmus* sp., **h**: *A. hippocastanum),* fungal cultures (**i**: *D. mutila,* **j**: *D. seriata,* **k**: *D. molarum,* **l**: *D. sapinea,* **m**: *D. juglandis)* and sampling methods (**d**: Cotton swab, **e:** Hammer and chisel)

samples were stored at low temperature for subsequent processing.

(2) *Wood chip samples*: In addition to the tissue swabs, wood chips were collected from symptomatic bark and phloem tissues using a sterilized hammer and chisel (Fig. 1e). These wood chips were placed in sterile bags and transported to the lab for further investigation. A portion of these wood chips was also directly placed onto MEA Petri dishes in the field to facilitate immediate microbial analysis.

Both sampling methods were engaged to obtain comprehensive data on both the microbial community associated with the surface of the cankers (swab samples) and the microorganisms potentially embedded within the wood tissues (wood chip samples). The combination of these two types of samples was chosen to cover the microbial diversity present at different tissue depths and regions of the infected trees.

Laboratory analysis

For wood sampling, three wood chips were collected from each tree species. While one chip was already plated in the lab, two were processed further in the lab. Each wood chip was cut into three smaller pieces and plated individually onto MEA Petri dishes. For liquid cultures, the buffer containing microorganisms was diluted to concentrations of 100%, 50%, and 25% and poured onto three separate MEA plates for each dilution. Surface sterilization of the wood chips was performed using two different methods: (1) washing samples with 70% ethanol and sodium chloride, and (2) flame sterilization. All samples were cultivated on 2% MEA, except in cases where fungi and bacteria appeared inseparable. In those instances, MEA plates supplemented with antibiotics (Streptomycin, Tetracyclin, and Penicillin) were used to inhibit bacterial growth. Additionally, MEA-Thiabendazole media was applied to prevent excessive mold growth, such as *Mucor* spp., when wood chips were plated directly in the field.

In total, 88% of the *Diplodia* isolates were obtained from wood chips, while 12% were isolated from the buffer collected in Eppendorf tubes, demonstrating that even though fungal isolation from wood chips was more effective, the buffer collection method also proved efficient. The following *Diplodia* isolates were obtained from the respective host species: *Aesculus hippocastanum*: 5 isolates, *Carpinus betulus*: 4 isolates, *Crataegus persimilis*: 3 isolate, *Fagus sylvatica*: 12 isolates, *Platanus* × *hispanica*: 37 isolates, *Sorbus domestica*: 6 isolates and *Ulmus* sp.: 6 isolates.

Once fungal cultures were isolated, they were grouped based on their morphological characteristics, following the method described by Blumenstein et al. (2021). The initial morphological grouping suggested the presence of distinct fungal species, which was later confirmed through molecular analyses.

DNA extraction was carried out using a modified protocol based on Keriö et al. (2020). PCR was performed using 7.5 µl of DNA, 2.5 µl of primer pairs—such as the Internal Transcribed Spacer universal primers (ITS-u1/u4), Large Subunit primers (LSU/LR6), Elongation Factor alpha primers (EF1a 526/1567R), and Beta-tubulin primers (T1/T22)—as well as species-specific primers for *Diplodia sapinea* (DiSapi-F/Diplo-R) and *Diplodia seriata* (DS3.8S3 F/DS3.8 R6) (Table 1). The reaction mixture also contained 12.5 µl of FastGene Optima PCR HotStart ReadyMix Master Mix. PCR amplification was confirmed through gel electrophoresis, and amplified DNA products were purified using magnetic beads according to the HighPrep PCR clean-up protocol. The cleaned PCR products were subsequently sent for sequencing to Microsynth Seqlab in Göttingen, Germany. Sequences were analyzed and cross-referenced with the NCBI database for accurate species identification.

Pathogenicity test

A pathogenicity test was conducted to assess the pathogenic potential of the isolated fungi. Seven healthy tree species were inoculated with fungal cultures derived from stem cankers of corresponding tree species. For each fungal culture, three trees from the same species were inoculated. Each tree was inoculated three times with the same fungal culture, ensuring adequate space between the inoculations to prevent overlap of necrotic areas. The inoculation involved creating small, precise cuts on the tree surface using a sterilized scalpel, ensuring that the outer bark remained intact. The outer bark was then pressed over the agar plug containing the fungal culture, securing it in place, and the wound was sealed with parafilm to prevent contamination and maintain contact between the inoculum and the tree tissue. During all procedures, gloves were worn, and the scalpels were sterilized by dipping in 95% alcohol and flaming before each new wound and fungal application. One additional tree per species was used as a control, inoculated with the same method as described above but with pure agar plugs only.

Following inoculation, the host plants were monitored for a month, with daily watering and no additional stressors. After about 30 days, all trees, including those from the control group, were harvested. The inoculated stems were carefully peeled with scalpels to examine necrotic areas on the phloem tissues. Fungi were reisolated from these necrotic areas to fulfill Koch's postulates, thus confirming the pathogenicity of the fungal isolates. The symptoms observed in the inoculated trees were recorded and compared with those in the control group.

Results

Field observations

We characterized the necrotic areas from freshly pruned or unpruned branches as a reddish oozing, observable on both lower and higher branches. The tissues of the stem cankers had a slimy-texture (Fig. 1 a-c) that could be above or below DBH. Further to this study, we made the observation that the oozing from the corresponding trees can be visible from spring to autumn. Field observations revealed that the spatial distribution of canker development showed no consistent or predictable pattern. In some instances, cankers were observed on individual trees that were spatially isolated from conspecifics or closely related species.

Conversely, other cases involved multiple trees affected within a localized area, such as a valley, where the disease spread irregularly—impacting either a single tree or several nearby individuals. Cankers were typically confined to one side of the tree; however, in certain cases, they were dispersed across multiple locations on the same tree, manifesting at various points along the stem and branches, indicating a non-uniform progression of the infection.

Laboratory findings

The identification of the fungal isolates was confirmed through sequence comparisons with the NCBI database. Several primers were used, and species-specific primers were applied where applicable to confirm the taxonomy of the fungal isolates. In addition to molecular methods, two fungi were morphology-matched to previously identified species, further supporting the identification (Table 2). A total of five *Diplodia* species—*D. malorum* Fuckel (Fig. 1k), *D. sapinea* (Fig. 1l), *D. mutila* (Fr.) Fr. (Fig. 1i), *D. juglandis* (Fr.) Fr. (Fig. 1m), and *D. seriata* De Not. (Fig. 1j)—were distinguished.

The fungal isolates were obtained from seven host tree species (Table 2). *D. juglandis* has been isolated from *A. hippocastanum* and *P.* × *hispanica*. *D. malorum* was isolated from *S. domestica*. *D. mutila* was detected on *C. betulus*, *F. sylvatica*, *P.* × *hispanica*, and *C. persimilis*, with frequent occurrences on *F. sylvatica* and *P.* × *hispanica*. *D. sapinea* was found on *Ulmus* spp., while *D. seriata* was recorded on multiple hosts, including *A. hippocastanum*, *P.* × *hispanica* (with multiple occurrences), *Ulmus* spp., *C. persimilis*, and *C. betulus* (Table 2).

Morphologically, the isolates showed the following features based on mycelial growth and photographs taken before DNA extraction, with all cultures plated on MEA

Table 2 List of the specifications for the fungal species and their identification based on several primers

			Assession number at NCBI		Specimen voucher		Identification with species-specific primer		
			Primers						
Fungal species	Host species	City of origin	ITSu1/ ITSu4[1]	LSU[2]/ LR6[3]	Efa1 526[4]/ Efa1 1567R[5]	Beta-tubulin T1/T22[6]	DiSapi-F/ Diplo-R[7]	DS3.8S3-F/ DS3.8R6-R[8]	Identity confirmed based on morphology
Diplodia juglandis	*Aesculus hippocastanum*	Frankfurt							+
D. juglandis	*Platanus x hispanica*	Frankfurt	PQ467092		UF-PT 789				
Diplodia malarum	*Sorbus domestica*	Frankfurt	PQ467090			UF-PT 767			
Diplodia mutila	*Carpinus betulus*	Düsseldorf	PQ467091	PQ469166	UF-PT 788				
D. mutila	*Fagus sylvatica*	Düsseldorf							+
D. mutila	*Platanus x hispanica*	Freiburg	PQ467084	PQ469164	UF-PT 326				
D. mutila	*Fagus sylvatica*	Stuttgart	PV363540	PV363558					
D. mutila	*Crataegus persimilis*	Stuttgart							+
Diplodia sapinea	*Ulmus* sp.	Potsdam	PQ467086	PQ469165			+		
Diplodia seriata	*Aesculus hippocastanum*	Freiburg	PQ467089		UF-PT 678			+	
D. seriata	*Platanus x hispanica*	Frankfurt	PQ467093		UF-PT 790			+	
D. seriata	*Platanus x hispanica*	Freiburg	PQ467088		UF-PT 625			+	
D. seriata	*Platanus x hispanica*	Göttingen	PQ467087		UF-PT 570			+	
D. seriata	*Ulmus* sp.	Darmstadt	PQ467085		UF-PT 506			+	
D. seriata	*Crataegus persimilis*	Stuttgart						+	
D. seriata	*Carpinus betulus*	Stuttgart						+	

media. The reisolated cultures were also plated on MEA, as different media types might affect the morphology:

- *D. malorum* (Fig. 1k) exhibits a white, dense mycelium with a cottony texture.
- *D. sapinea* (Fig. 1l) appears with aerial mycelium where the hyphae form tower-like structures, though less pronounced compared to *D. seriata* (Fig. 1j), and a whitish-gray mycelial pigmentation.
- *D. mutila* (Fig. 1i) has a fluffy, extensive mycelial growth on the surface, with a darker, gray color compared to *D. sapinea* (Fig. 1l).
- *D. juglandis* (Fig. 1m) has slow growing white mycelium with a silvery appearance.
- *D. seriata* (Fig. 1j) shows a dense aerial mycelium with well-distinctive tower-like hyphal structures and a darker grayish-brown hue compared to the other species.

Pathogenicity test

Based on the pathogenicity test, the results showed that all seven tree species exhibited varying degrees of susceptibility to the fungal isolates. Necrotic areas were observed on the phloem tissues of the inoculated stems, with the severity of symptoms differing among tree species. The five fungal cultures, isolated from their corresponding tree species and inoculated back onto the same hosts (Table 2), caused necrotic tissue in 88.46% of the cases, fulfilling Koch's postulates and confirming their pathogenic potential. This indicates that the vast majority of inoculations were successful, with only a small number failing, which underscores the effectiveness of the approach used in this study. In contrast, the control groups, which were treated with only agar plugs, showed no signs of necrosis or disease symptoms, further validating the pathogenicity of the fungal isolates.

The cross-species inoculations were conducted to assess whether these five fungal species, despite not being originally isolated from certain tree species, could still infect them. This was done to evaluate the potential future spread of these fungi within an urban environment where all tree species coexist. To ensure consistency, the inoculations were performed again on the same seven tree species used in the study. The results demonstrated that several fungal cultures could successfully infect multiple tree species, highlighting the possibility of a broader host range and potential disease outbreaks.

D. mutila was tested on *A. platanoides*, *A. hippocastanum*, and *Ulmus* spp., all of which developed necrotic symptoms, confirming its pathogenicity across these hosts. *D. malorum* was evaluated on *C. betulus*, *A. hippocastanum*, *Ulmus* spp., *F. sylvatica*, and *P.* × *hispanica*; necrotic symptoms were observed on all except *Platanus*, which did not exhibit any pathogenic activity. *D. seriata* was tested on *S. domestica*, *A. platanoides*, *F. sylvatica*, and *C. betulus*, with pathogenic effects recorded on all species. Similarly, *D. sapinea* was inoculated on *P.* × *hispanica*, *A. hippocastanum*, *C. betulus*, and *F. sylvatica*, all of which showed necrotic activity.

Discussion

This study presents the first recorded instances of *D. seriata*, *D. sapinea*, *D. mutila*, *D. malorum* and *D. juglandis* on Elm hybrids, London plane, European common horse chestnut, European beech, European hornbeam, plumleaf hawthorn and service trees according to our research and the CABI and EPPO databases. The identification process confirms the presence of these fungi in new hosts and validates previous studies about its ability to jump hosts (Zlatković et al. 2017; Blumenstein et al. 2022), thereby expanding our understanding of its ecological range (Crous et al. 2006). Previously, in Europe *Diplodia* spp. had been documented mostly on conifers, such as pines (e.g., Bußkamp 2018; Brodde et al. 2019)), or fruit trees, such as apples and walnuts (Zugschwerdt et al. 2023). Finding *Diplodia* spp. on deciduous urban trees suggests a broader host range than previously thought, indicating its adaptability and potential to affect a wider variety of tree species.

While identifying *Diplodia* species on new hosts, the inoculation experiments confirmed Koch's postulates, demonstrating that these fungi are pathogenic on multiple tree species. The high success rate of these inoculations, particularly across a wide range of hosts, is alarming and suggests that these pathogens could cause a significant risk to urban trees in the future. Further studies are needed to assess the long-term ecological impact of *Diplodia* on various urban tree species. Beyond the sampling areas in this project, similar bleeding or slimy canker tissues have additionally been observed and reported on the same tree species in Hamburg, Berlin, Dresden, Nürnberg. This suggests that *Diplodia* spp. on urban trees might be widespread across Germany and countries with similar climate conditions and tree species composition. Such widespread distribution highlights the importance of monitoring and managing this pathogen to protect urban tree health and maintain the ecological and recreational benefits that urban trees provide.

Conclusion

Understanding the dynamics of these pathogens is crucial for enhancing our knowledge of tree-pathogen interactions and informing urban tree management strategies to mitigate potential disease outbreaks and broader ecological consequences. This finding highlights the importance of ongoing surveillance of fungal biodiversity and its potential implications for tree health. Urban trees are vital to city environments, offering ecological, aesthetic, and recreational benefits. Introduced a new pathogen like *Diplodia* spp. raises concerns about the health and sustainability of these urban forests. Detailed monitoring and proactive management practices are essential to mitigate the risks posed by this pathogen and preserve the benefits of urban trees.

Acknowledgements We are especially grateful to Sabine Diener for her great support in the laboratory. Further we like to express our gratitude to the Eva-Mayr-Stihl Foundation for financing our project and supporting the field of tree pathology. We also like to thank our Faculty of Environment and Natural Resources of the University of Freiburg.

Funding Open Access funding enabled and organized by Projekt DEAL. This study was supported by the Eva-Mayr-Stihl Foundation and the Faculty of Environment and Natural Resources of the University of Freiburg.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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