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Short Notes

Phytophthora hibernalis, *P. lacustris* and *P. multivora* associated with declining *Ligustrum lucidum* trees in an urban park in Portugal

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Summary. During a monitoring survey carried out in a park in Aveiro, Portugal, typical *Phytophthora* symptoms of root rot, stem bleeding cankers and extensive canopy dieback were observed on mature ornamental glossy privet trees (*Ligustrum lucidum*). A study carried out in spring 2022 aimed to isolate the causal agents, as there is was no available knowledge on potential root pathogens of this host. Thirty-two *Phytophthora* isolates were obtained from inner bark tissues and/or rhizosphere samples (soil and fine roots) collected from 27 declining glossy privet trees. Based on morpho-biometric data and phylogeny of concatenated ITS and *cox*1 sequences, *Phytophthora* isolates were identified as *P. hibernalis* (14 isolates), *P. multivora* (12) and *P. lacustris* (6). Pathogenicity tests confirmed the virulence of the three species on glossy privet. *Phytophthora lacustris* was the most aggressive species, while *P. hibernalis* was most abundant. These results give new insights into emerging *Phytophthora*-related tree diseases in urban areas, and highlight the importance of enhancing biosecurity measures against these invasive pathogens.

Keywords. Emerging disease, *Oomycetes*, urban green areas.

INTRODUCTION

Urban forests and green areas are important components of city life, contributing to a wide range of ecosystem services as well as environmental quality and social well-being (Kabisch *et al*., 2015). However, urban vegetation is particularly vulnerable to disturbances and imbalances arising from biotic, abiotic or anthropogenic factors (Stenhouse, 2005; Threlfall *et al*., 2016). Some external abiotic and anthropogenic stresses, such as air pollution, climate change, limited soil availability, pruning activities and other mechanical damage, can increase plant susceptibility to pathogens and pests

(Oldfield *et al*., 2013; Vettraino *et al*., 2025). Close proximity of ornamental plants with different origins can also facilitate host jumps of pathogens and emergence of new diseases, which can be further promoted by the introduction of infected nursery material (Laurence *et al*., 2024).

Phytophthora species are major pathogens threatening plant health (Scott *et al*., 2019). Research on *Phytophthora* has mainly focused on forest ecosystems, nurseries and agricultural systems (Bose *et al*., 2023; Bregant *et al*., 2023a), whereas urban areas have been relatively understudied for *Phytophthora* presence and impacts on trees. Recent studies have highlighted how urban vegetation is susceptible to root infections by *Phytophthora* (Khdiar *et al*., 2020; Antonelli *et al*., 2023, Laurence *et al*., 2024). Urban areas can be unintentionally exposed to *Phytophthora* pathogens through introduction of infected nursery plants, which may lead to widespread disease outbreaks (Laurence *et al*., 2024). In cities, close proximity of exotic and native species for ornamental purposes may also facilitate host jumps and new diseases. Urban green areas may also serve as reservoirs of *Phytophthora* diversity, and function as entry points for the spread of *Phytophthora* into managed and natural ecosystems. These pathogens are easily moved by human activities, and their abilities to infect plants under a variety of conditions poses challenges for management of urban green spaces (Hulbert *et al*., 2017).

In Portugal, the impact of *Phytophthora* species has been studied in natural ecosystems, forest plantations and nurseries, showing a wide diversity of these organisms, many of which are invasive and polyphagous (Diogo *et al*., 2022; Bregant *et al*., 2023b, 2025; Horta Jung *et al*., 2024). However, no study has investigated the occurrence of these pathogens in urban green spaces in Portugal.

Severe and unusual decline of mature glossy privet trees (*Ligustrum lucidum*) was discovered in a public park in the city of Aveiro (northern Portugal). Research carried out to isolate and characterize the pathogens involved is outlined in the present paper.

MATERIAL AND METHODS

A survey was carried out during winter and spring 2022 in a public park in Aveiro, Portugal (40°38’05.4”N, 8°39’11.0”W). The area includes a series of wetlands and lakes, with many ornamental plant species and a dominance of symptomatic glossy privet (*Ligustrum lucidum*). Disease incidence and mortality rate were estimated along two 25 m transects as described by Bregant *et al*., (2023b). Rhizosphere soil samples including soil and symptomatic fine roots were collected from 27 declining glossy privet trees. Among these, seven trees were randomly chosen for the collection of inner bark tissue samples, which were taken from margins of necrotic lesions at the lower part of trunks.

Phytophthora colonies were isolated using the zoospore trap method of Linderman and Zeitoun (1977), with small changes. Soil plus fine root samples (each of 300 g) were placed in conical flasks and 0.8 L of distilled water was added to each flask. After 24 h, fresh cork oak and *Pittosporum* leaves were placed on the clean water surface and used as baits to capture *Phytophthora* zoospores. The flasks were kept at 18–20°C in laboratory conditions for 5 d. Leaves showing necrotic lesions were then washed in sterile water, cut in small pieces (5 mm^2) and placed in Petri dishes containing the selective substrate PDA+ (Bregant *et al*., 2020). *Phytophthora* isolations were also carried out from inner bark samples taken at tree collars. For each of these samples, outer bark was removed, and ten small fragments were aseptically cut from the lesion margin with a disinfected scalpel and were placed into a Petri dish containing PDA+. All plates were kept at 20°C in the dark. and examined every 12 h. Hyphal tips from emerging mycelium were sub-cultured onto carrot agar (CA) (Erwin and Ribeiro, 1996), and kept at 20°C in the dark.

All isolates obtained were initially grouped into morphotypes, based on colony appearance after 7 d incubation, and morpho-biometric data of reproductive structures (oogonia and sporangia) were recorded using a Motic BA410E microscope. Representative isolates of isolated species were stored on CA slants at -80 °C under 15% glycerol, in the culture collection of Prof Linaldeddu, at the University of Padova, Italy and in the Department of Biology, University of Aveiro, Portugal.

Identities of all isolates were confirmed by molecular analyses. Genomic DNA was extracted from mycelium of 5-d-old pure cultures (Bregant *et al*., 2023a), and internal transcribed spacer regions (ITS) for all isolates were amplified and sequenced using the primers ITS1 and ITS4 (White *et al*., 1990) according to Linaldeddu *et al*. (2023). The primer-pairs FM83/FM84 (Martin and Tooley, 2003) were also used to amplify and sequence a portion of the mitochondrial cytochrome c oxidase subunit I (*cox*1) regions. Amplicons were purified using a EUROGOLD gel extraction kit (EuroClone S.p.A.), following the manufacturer’s instructions, and were sequenced by BMR Genomics (University of Padova). Forward and reverse DNA sequences were read and analysed with FinchTV 1.4.0 (Geospiza Inc.). Resulting consensus sequences were then compared though BLAST analysis with reference sequences (ex-type culture or representative strains) available in GenBank (Altschul *et al*., 1990). Identities of the isolates from glossy privet trees were confirmed when the DNA sequences showed 100% similarity with those of ex-type or representative cultures. Representative ITS and *cox*1 sequences were register at GenBank (Accession numbers: *P. hibernalis* isolate CBP19: PV570382, PV611059; *P. lacustris* isolate CBP161: PV570383, PV611061; *P. multivora* isolate CBP157: PV570384, PV611060).

Concatenated ITS and *cox*1 sequences of three representative *Phytophthora* isolates obtained from glossy privet were analyzed together 24 sequences of nine other *Phytophthora* species representative of the major clades 2, 6 and 8 available in GenBank. Sequence alignments and phylogenetic analyses were carried out as described by Bregant *et al.* (2023a).

Koch's postulates were assessed by inoculating one representative isolate of each *Phytophthora* species (*P. hibernalis* CBP19, *P. lacustris* CBP161, *P. multivora* CBP157) onto the stems of asymptomatic 1-year-old glossy privet seedlings. Inoculations were carried out using the protocol of Linaldeddu *et al*., (2023). Individual inoculation points were each wrapped with sterile damp cotton wool and covered with aluminium foil. Six seedlings were inoculated with each isolate, and six seedlings were each inoculated with a sterile plug of potato dextrose agar (PDA, Oxoid Ltd) as controls. The seedlings were then maintained in natural conditions at 22°C and watered every 2 d. After 15 d, the seedlings were inspected for external and internal disease symptoms, and the extent of necrotic lesions on the stems were assessed. Re-isolations were carried out by placing ten small inner bark samples taken from the margin of each necrotic lesion onto PDA+. Resulting colonies were transferred into PDA and CA plates, and resulting organisms were identified as described above.

Data of necrotic lesion size were checked for normality, then subjected to analysis of variance (one-way ANOVA). Statistically significant differences ($P \leq 0.05$) among mean values were evaluated using Fisher's least significant difference multiple range test in XLSTAT 2008 software (Addinsoft).

RESULTS AND DISCUSSION

The field survey conducted in the public green urban area of Aveiro showed severe decline symptoms and death of ornamental glossy privet trees, including root and collar rot related to soilborne *Phytophthora* infections. Loss of the fine root systems was reflected on the tree canopies by the leaf chloroses, stunted growth, progressive branch dieback, and secondary sudden death (Figure 1). Symptoms were most evident on mature plants, particularly those growing placed close to water. Average disease incidence was 88% of trees affected, with 25% of trees dead.

Direct and indirect (baiting) isolations from glossy privet samples yielded 32 *Phytophthora* isolates belonging to three species (Figure 2). Of these isolates, 27 were from rhizosphere samples and five were from inner bark tissues collected at collar. Based on biometric data of sporangia, colony appearance, and nuclear and mitochondrial sequence data, the 32 isolates were identified as *Phytophthora hibernalis* (14 isolates), *Phytophthora multivora* (12) and *Phytophthora lacustris* (6). *Phytophthora hibernalis* was obtained from 12 rhizosphere samples and two inner bark samples, *P. multivora* was identified from nine rhizosphere samples and three inner bark samples, and *P. lacustris* was identified from six rhizosphere samples. Five of the sampled plants were positive for *P. hibernalis* and *P. multivora*.

Phylogenies obtained using concatenated ITS and *cox*1 sequences placed the isolates in three terminal clades including the ex-type strains of *P. multivora, P. lacustris* and *P. hibernalis* confirming the identifications (Figure 3).

Fifteen days after inoculations the three *Phytophthora* species were pathogenic on glossy privet seedlings, causing dark brown inner bark lesions that spread up and down from the stem inoculation points (Figure 4). Mean lesion sizes differed ($P \leq 0.05$) according to species. The lesions caused after *P. lacustris* inoculations (mean length = 32 ± 5 mm) were larger than those induced by *P. multivora* (19 ± 2 mm) or *P. hibernalis* (14 ± 4 mm). Control seedlings showed oxidative browning at the inoculation sites. *Phytophthora hibernalis, P. lacustris* and *P. multivora* were re-isolated from all the respective inoculated seedlings. No fungi or oomycete colonies were obtained from control seedlings.

The results from this study are the first to demonstrate susceptibility of glossy privet to three *Phytophthora* species which have been associated with the decline of different riparian ecosystems in Europe (Bregant *et al*., 2024). Glossy privet was introduced into Europe for ornamental purposes in the 16th century from China (Fernandez *et al*., 2020). This tree is now naturalized and considered invasive in many European countries, including Portugal (Fernandez *et al*., 2020; Silva *et al*., 2023). Although many biotic threats have been reported for glossy privet, the impacts of *Phytophthora* were unknown (Shaw *et al*., 2018).

Phytophthora hibernalis, *P. lacustris* and *P. multivora* are reported here for the first time in an urban environment of Portugal, although the occurrence of these

Figure 1. Overview of *Phytophthora*-related symptoms observed on *Ligustrum lucidum* (glossy privet). **a** and **b**, photographs of the declining stand near water, examined in the present study. **c**, trees with chlorosis, and **d** and **e**, progressive canopy dieback. **f**, trunk base rot with exudates.

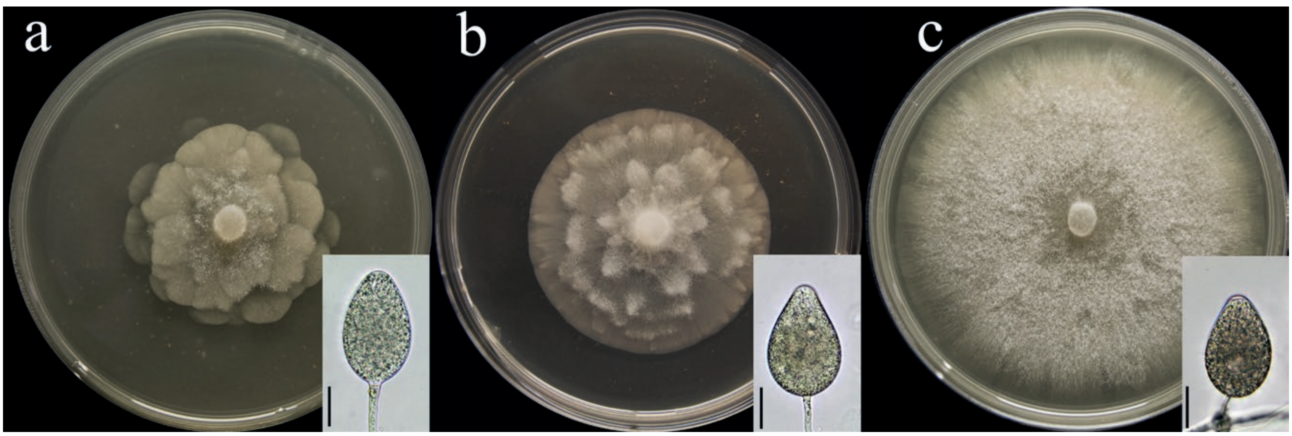

Figure 2. Colony morphologies of *Phytophthora hibernalis* (**a**), *P. lacustris* (**b**) and *P. multivora* (**c**), after 7 d on CA at 20°C in the dark. The insets accompanying the culture photographs show mature sporangia of the respective *Phytophthora* species. Scale bars = 20 µm.

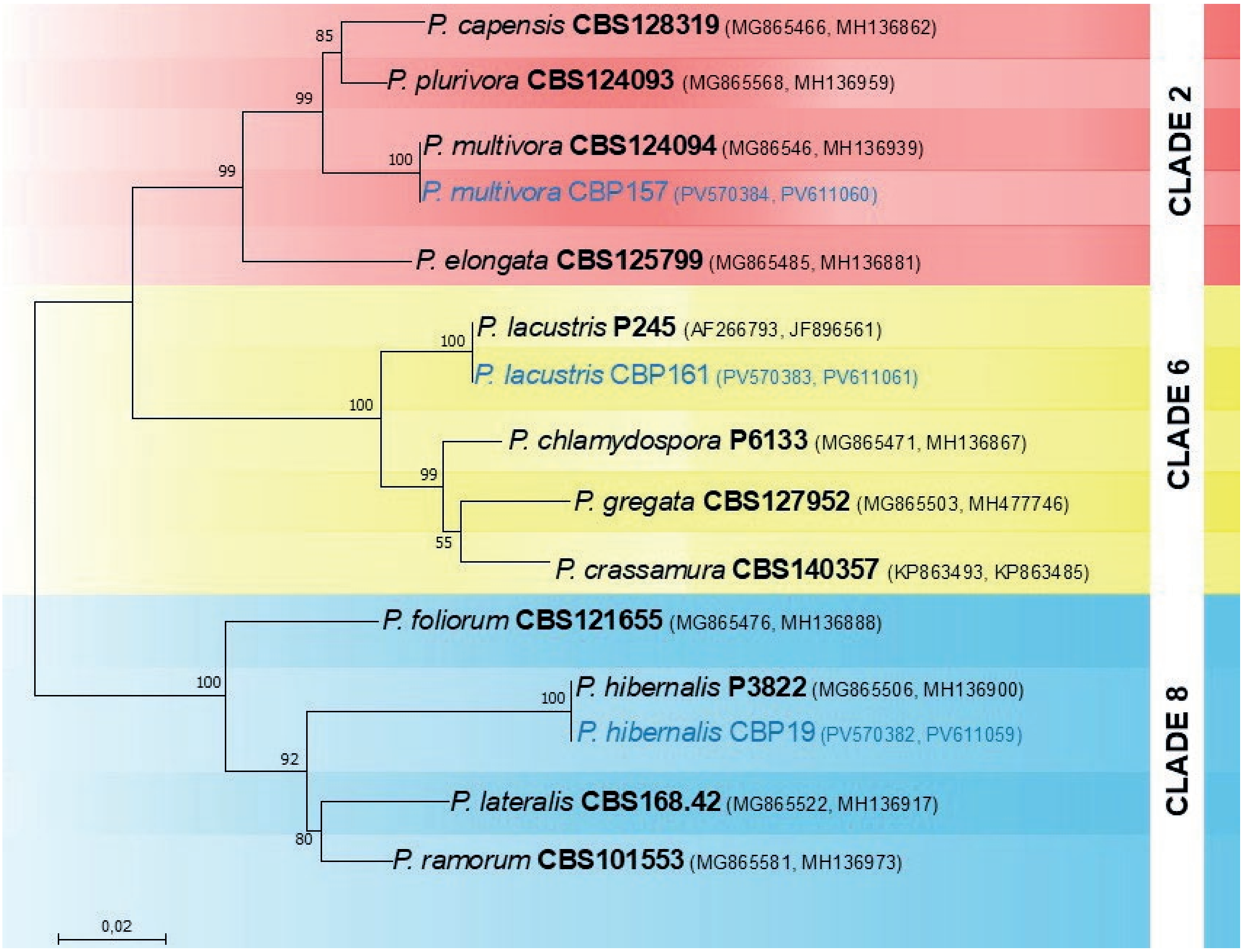

Figure 3. Maximum likelihood tree obtained from concatenated ITS and *cox*1 sequences of selected *Phytophthora* species belonging to the clades 2, 6 and 8. The data are based on the General Time Reversible model. A discrete Gamma distribution was used to model evolutionary rate differences among sites. The tree is drawn to scale, with branch lengths equivalent to numbers of substitutions per site. Bootstrap support values in percentages (1000 replicates) are given at the nodes. Ex-type cultures are in bold font, and isolates obtained in the present study in blue font.

organisms has been extensively documented in several Portuguese forests (Bregant *et al.*, 2025). All three species artificially inoculated on asymptomatic glossy privet seedlings colonized and necrotized inner bark tissues of glossy privet. *Phytophthora lacustris* was the most aggressive of these species, while *P. hibernalis* was the most widespread at the investigation site.

Phytophthora hibernalis is a pathogen with broad international distribution although a few records are available (Álvarez *et al.*, 2007; Schlenzig *et al.*, 2015). Originally, this organism has been associated with collar, root and fruit rots of *Citrus* spp., and recently also in *Eucalyptus globulus* plantations in Portugal near Aveiro (Carne, 1925; Nadel-Schiffmann, 1947; Graham and Feichtenberger, 2015; Bregant *et al.*, 2023c). *Phytophthora hibernalis* was the most frequently isolated species in the present study from the monitored site, suggesting an adaptability and potential epidemiological relevance in urban conditions.

Phytophthora lacustris is a widespread species in wet and aquatic environments across Europe (Nechwatal *et al.*, 2013). It has been commonly reported as an opportunistic pathogen, but can also be aggressive on some susceptible riparian trees such as *Alnus* spp. (Kanoun-Boulé *et al.*, 2016; Bregant *et al.*, 2023b; Rial-Martínez *et al.*, 2023). Although this species was isolated from rhizosphere soil, it was aggressive towards glossy privet, suggesting requirement for further investigation into the susceptibility of trees used in urban green areas and forests towards this water-borne *Phytophthora* species.

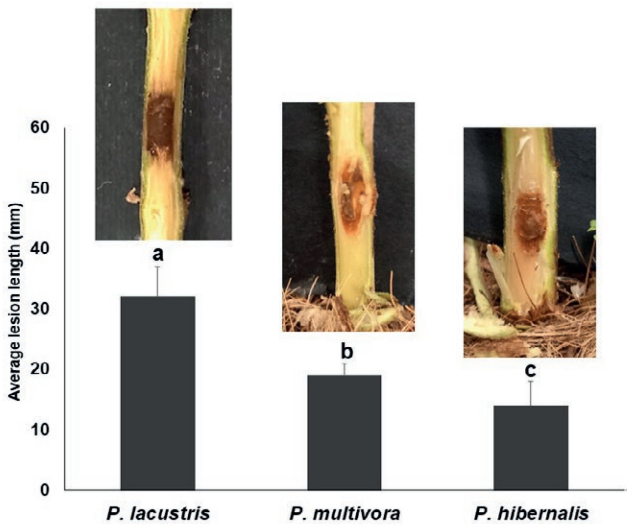

Figure 4. Mean lesion lengths (± standard deviations), and symptoms on 1-year-old *Ligustrum lucidum* seedlings, 15 d after inoculation with *Phytophthora hibernalis* (CBP19) (left), *P. lacustris* (CBP161) (centre), or *P. multivora* (CBP157) (right). The letters accompanying the means indicate differences ($P \leq 0.05$) according to LSD multiple range tests.

Phytophthora multivora is a polyphagous and invasive organism with broad international distribution (Scott *et al*., 2009). In Australia, *P. multivora* has contributed to destruction of many natural habitats dominated by *Eucalyptus* and *Banksia* host species, and several other plants have been reported as hosts, including urban ornamental trees (Burgess *et al*., 2021). In addition to natural environments, *P. multivora* is also widespread in nurseries and urban systems in Australia, where it is the dominant species (Khdiar *et al*., 2020; Burgess *et al*., 2021). The present study is the first to report *P. multivora* in an urban environment of Europe. This species is an emerging organism in Europe and North America with a wide distribution in areas with Mediterranean climate (Tsykun *et al*., 2022; Sims and Garbelotto, 2021; Bregant *et al*., 2023b). In Portugal, *P. multivora* is widespread and is a growing threat to natural ecosystems, timber plantations, and urban green areas as demonstrated in the present study.

In conclusion, results outlined here are the first to demonstrate occurrence of three *Phytophthora* species in an urban area of Portugal. Although interest in plant health within urban environments is increasing, studies addressing the impacts of *Oomycetes* on trees and shrubs used for ornamental proposes in urban contexts have been few (Khdiar *et al*., 2020; Antonelli *et al*., 2024; Laurence *et al*., 2024). These environments may be important for introduction and dissemination of invasive pathogens, and be significant reservoirs of inoculum. The global trade in ornamental plants, together with effects of climate change, may facilitate the spread of invasive species such as *P. multivora* into natural habitats and agroecosystems, posing serious ecological and economic issues.

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