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Short Notes

Pathogenicity of *Botryosphaeriaceae* and *Phytophthora* species associated with *Paulownia* dieback, canker and root rot in Italy

Carlo BREGANT[1], Francesca CARLONI[2], Mattia BALESTRA[2], Benedetto T. LINALDEDDU[1], Sergio MUROLO[2,*]

[1] *Dipartimento Territorio e Sistemi Agro-Forestali, Università degli Studi di Padova, Viale dell'Università, 16, 35020 Legnaro, Italy*
[2] *Department of Agricultural, Food and Environmental Sciences, Marche Polytechnic University, Via Brecce Bianche, I-60131, Ancona, Italy*
*Corresponding author. E-mail: s.murolo@staff.univpm.it

Summary. In recent years, an unusual decline and mortality has been observed in *Paulownia* plantations throughout the Marche region (Central Italy). Given the economic importance of this emerging forest crop, a study was conducted to determine which pathogens are directly involved in this syndrome. Field surveys performed in two plantations revealed the widespread occurrence of severe disease symptoms such as leaf chlorosis, crown thinning, shoot and branch dieback, sunken cankers, epicormic shoots and root rot. Disease incidence was also assessed by aerial remote sensing (RS) technologies using drones. Symptomatic samples collected from both stem and root tissues yielded fungal and fungal-like colonies representing two distinct families: *Botryosphaeriaceae* and *Peronosporaceae*. Morphological and DNA sequence data revealed five distinct species, identified as *Macrophomina phaseolina* and *Botryosphaeria dothidea* (*Botryosphaeriaceae*), *Phytophthora pseudocryptogea, P. citrophthora* and *P. erythroseptica* (*Peronosporaceae*). Given that all species are reported here for the first time on Paulownia, Koch's postulates were satisfied inoculating the three *Phytophthora* species and two *Botryosphaeriaceae* at the collar of the stem of potted 1-year-old rooted cuttings in June 2023. Thirty days after inoculation, all plants showed the same symptoms as those observed in the field.

Keywords: *Phytophthora pseudocryptogea*, *P. citrophthora*, *P. erythroseptica*, *Macrophomina phaseolina*, *Botryosphaeria dothidea*, emerging diseases.

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ORCID:

CB: 0000-0003-1353-7993
FC: 0009-0007-1245-5423
MB: 0000-0002-3741-3621
BTL: 0000-0003-2428-9905
SM: 0000-0001-7269-1734

INTRODUCTION

Paulownia spp., autochthonous and deciduous tree species from China, were, in few decades, rapidly introduced in different environments around the world, including Australia, USA, Asia, Europe, and Central Africa (Muthuri *et al*., 2004; Jakubowski, 2022). Paulownia popularity is ascribed to: i) the capability to grow in poor soil and marginal lands, ii) quick growing through a cultivation system called short rotation coppice, which allows

more harvests during their production cycle in a shorter time than traditional forest species (Hauk *et al*., 2014; Vanbeveren *et al*., 2017), iii) high carbon sequestration (Basu *et al*., 2016), and iv) high value and flexibility of wood, and eco-sustainable and alternative energy sources (Testa *et al*., 2022). For these beneficial characteristics, paulownia was considered in the first years as a promising crop, adapted for any soil types, and any environmental conditions.

Concerns about the ecological impact of Paulownia introduction arose recently, when in some countries, *Paulownia tomentosa* has been declared dangerous, and it has been recognized as an invasive species in Austria (Botond and Botta-Dukát, 2004; Essl, 2007). The invasiveness of *P. tomentosa*, and to a lesser rate *P. elongata*, is due to the ability to propagate vegetatively by suckering and resprouting after cutting, as well as to an impressive reproductive potential (Jakubowski, 2022). Sterile clones have been selected to prevent these plants from becoming invasive in new areas where extensive commercial plantings for wood production are established.

Furthermore, several researches focused on paulownia cultivation demonstrating that biomass production is particularly high in optimal conditions, but it resulted directly affected by stationary conditions (i.e. drought, salinity, pH value, temperature, nutrient content) (Ivanova *et al*., 2016; Sage and Sultmanis, 2016; Wang *et al*., 2019; Wozniak *et al*., 2022).

A further impact is due to several pathogens, which can take advantage of stress conditions or can directly come from the nursery, where propagation for many clones is via *in vitro*, rarely by seeds, and more frequently by root cuttings (Stuepp *et al*., 2015; Pozoga *et al*., 2019; Temirov *et al*., 2021).

The most well-known phytosanitary problems on *Paulownia* spp. are witches' broom determined by phytoplasma (Gao *et al*., 2008), Phytophthora root and collar rot (Aloi *et al*., 2021), wood decay caused by *Trametes hirsuta* (Milenkovic *et al*., 2018) and root-knot nematodes (Skwiercz *et al*., 2019). Other diseases affecting *Paulownia* spp. are blight caused by *Alternaria,* Paulownia scab caused by *Elsinoe ampelina* and *Sphaceloma paulowniae*, leaf spot caused by *Phyllosticta* sp., leaf brown spot caused by *Cercospora* sp., and canker caused by *Valsa paulowniae* (Ray *et al*., 2005; Pleysier *et al*., 2006; Pasiecznick, 2019; Liu *et al*., 2022).

Given the growing expansion of decline and mortality events in several paulownia plantations in central Italy and the lack of information available about the aetiology, the goal of this present study was to isolate and identify the main pathogens associated with the disease, as well as to test their pathogenicity.

MATERIAL AND METHODS

Study site, field surveys and decline assessment

From spring 2022 to spring 2023, the phytosanitary status of two 6-year-old plantations of *Paulownia elongata* × *P. fortune* hybrid was monitored for the occurrence of disease symptoms. The two plantations of about 3 and 1 ha, respectively, are located in the province of Pesaro Urbino (central-eastern Italy) and are characterized by tree spacing of 4 × 6 m and clay-loam soil. The propagative material was composed of 1-year old cuttings with roots, provided from Germany, and planted in April 2016. The first technical cut was carried out in 2017, one year after planting. No chemical control was applied on the canopy, nor to disinfect the technical cut, nor for the soil management, for which mechanical processing was carried out three times per year. The plantations were not equipped with an autonomous irrigation system, but water was provided when needed according to the trend of climatic conditions.

In 2022, in each plantation, 10 linear transects, consisting of 25 trees/each, were established *at random* and for each tree, dendrometric data (tree diameter and height) and severity of disease symptoms on the canopy including leaf chlorosis, crown thinning, shoot and branch dieback, sunken cankers, epicormic shoots and root rot were assessed according to an empirical scale with four disease severity levels: 0 = healthy plant, 1 ≤ 30%, 2 = 30–50%, 3 ≥ 50%, 4 = dead plant.

In July 2023, the phytosanitary status of the two plantations was monitored and assessed by aerial remote sensing (RS) using drones (Unmanned Aerial Vehicles, UAV), equipped with digital, multispectral, fluorescence sensors that offer finer resolution of plant diseases and assist in plant disease detection at an earlier stage.

The flight was made using a DJI Mavic 3 Multispectral drone. A carefully planned flight path covered the entire study area with an additional 10-metre buffer to ensure complete coverage of all target plants for evaluation. The Real-time Kinematic (RTK) service was utilized, enabling precise positioning and navigation data without the need for Ground Control Points (GCPs). The UAV maintained a constant speed of 3.5 m/s, capturing an image every 3 seconds. The sensors automatically and simultaneously took 5 images: one for RGB representation and the others to record reflectance values in the GREEN, RED, near-infrared (NIR), and RedEdge regions. The RGB images have dimensions of 5280 × 3956 pixels with a bit depth of 24 bits and were acquired with an exposure time of 1/1000 sec. Conversely, the multispectral images are 2592 × 1944 pixels, 16-bit, with an exposure time of 1/640 sec. All

images were acquired at 96 dpi. The focal length for the RGB images was 12 mm, while for the individual band acquisitions, it was 4 mm. The georeferenced orthomosaic processing for each study area was conducted using Agisoft Metashape software. The first study area, covering 0.122 km^2, has a total error of 2.33 cm, with a ground resolution of 49.2 cm/pixel. The orthomosaic of the second study area, covering 0.081 km^2, has an error of 2.27 cm, with a ground resolution of 87.1 cm/pixel. The flight altitude for the first area was 70 metres, while for the second area, it was 80 metres.

Sample collection, pathogens isolation and characterization

During 2022, twenty-five symptomatic paulownia trees were selected and labelled and from each tree 500 g of rhizosphere samples and root tissues were collected around the collar. At the same time, bark samples were excised from stems showing sunken cankers and inner bark necrotic lesions. From three additional plants, showing aerial and extensive sunken cankers, two stems were cut and collected. The samples were analysed in the laboratory to determine the causal agents involved in the symptoms observed. In particular, 300 g of soil samples were placed in plastic containers with about 2 L of distilled water. A few hours later, when the soil particles became sediment, young leaves of *Q. ilex* were added on the water surface and left at 18–20°C for 3–5 days. The leaves showing dark-brown necrotic spots were placed in Petri dishes containing potato dextrose agar (PDA, Oxoid Ltd., Basingstoke, UK) amended with 100 mL L^{-1} of carrot juice, 0.013 g L^{-1} of pimaricin and 0.05 g L^{-1} of hymexazol (PDA+) (Linaldeddu *et al*., 2020). For root samples, the isolation was performed directly from the necrotic tissues, removing small inner bark fragments and placing them both in PDA + and PDA amended with ampicillin (150 mg L^{-1}) and streptomycin (150 mg L^{-1}).

Morphological identification of the colonies obtained in pure culture was performed according to the colony appearance on PDA or carrot agar (CA) after 7 days at 20°C in the dark, and biometric data of mycelium and reproductive structures (conidia and sporangia) visualized under light microscope. All isolates were stored in PDA tubes at 5°C in the collection of the Department of Agriculture, Food and Environmental Science (Marche Polytechnic University, Ancona, Italy) and sterile paraffin oil in the culture collection of the Dipartimento Territorio e Sistemi Agro-Forestali (Università degli Studi di Padova, Italy).

Molecular analysis and phylogeny

The identity of all the isolates was inferred by molecular tools. Genomic DNA from mycelium of pure culture was extracted according to Bregant *et al*. (2020) and the ITS region was amplified and sequenced for all isolates with the primers ITS1 and ITS4 (White *et al*., 1990) according to Linaldeddu *et al*. (2023). In addition, the primer-pairs TUBUF2/TUBUR1 (Kroon *et al*., 2004) were used to amplify and sequence a portions of the β-tubulin (Btub) region of a representative set of *Phytophthora* isolates; whereas for *Botryosphaeriaceae* isolates a portion of the translation elongation factor 1 alpha gene (*tef1*-α) was amplified and sequenced with primers EF446f and EF1035r (Inderbitzin *et al*., 2010).

Amplicons were purified, sequenced by BMR Genomics (Padova) and then edited with BioEdit software. The *consensus* sequences were compared with reference sequences (ex-type culture or representative strains) available in GenBank. The species was assigned when the nucleotide identity was 100% with sequences of ex-type culture. ITS and *tef1*-α sequences of two representative isolates of *Botryosphaeria dothidea* (accession numbers: OR551463, OR784637) and *Macrophomina phaseolina* (OR551464, OR784638) as well as ITS and Btub sequences of *Phytophthora citrophthora* isolate (OR551465, OR784639), *P. erythroseptica* (OR551466, OR784640) and *P. pseudocryptogea* (OR551467, OR784641) were deposited at GenBank.

In addition, a multigene phylogeny based on concatenated ITS and Btub sequences for *Phytophthora* spp. and ITS and *tef1-α* sequences for *Botryosphaeriaceae* was performed. Sequences were aligned with ClustalX v. 1.83 (Thompson *et al*., 1997), using the parameters reported by Bregant *et al*. (2020). Phylogenetic reconstructions were performed with MEGA-X 10.1.8, including all gaps in the analyses. The best model of DNA sequence evolution was determined automatically by the software (Kumar *et al*., 2018). Maximum likelihood (ML) analysis was performed with a neighbourjoining (NJ) starting tree generated by the software.

Pathogenicity tests

Given that all species isolated were not reported on paulownia, Koch's postulates were satisfied. Three *Phytophthora* species and two fungal species belonging to *Botryosphaeriaceae* were inoculated at the collar of potted 1-year-old rooted cuttings in June 2023, when they were highly vigorous and 60 cm tall according to Linaldeddu *et al*. (2023). The plants inoculated with a representative isolate of each species (ten plants per patho-

gen) were maintained in controlled conditions at around 22°C for 30 days. At the end of the experimental period, the presence of internal (necrotic lesion) and external (wilting and exudates) disease symptoms as well as the impact on the root systems was recorded. The size of the necrotic lesions was estimated by removing the outer bark. Finally, re-isolation of the pathogens was performed taking five pieces of symptomatic inner bark tissue and transferring them onto PDA+ (for *Phytophthora*) and PDA (for *Botryosphaeriaceae*).

Results of the pathogenicity test were checked for normality, then subjected to analysis of variance (ANOVA). Significant differences among mean values were determined by Fisher's Least Significant Difference (LSD) test ($P = 0.05$) using XLSTAT 2008 software (Addinsoft, Paris, France).

RESULTS AND DISCUSSION

Field surveys conducted in both plantations over a two-year period, showed a high percentage of symptomatic plants. The first symptoms of vegetation suffering, characterized by yellowing and small sized leaves,

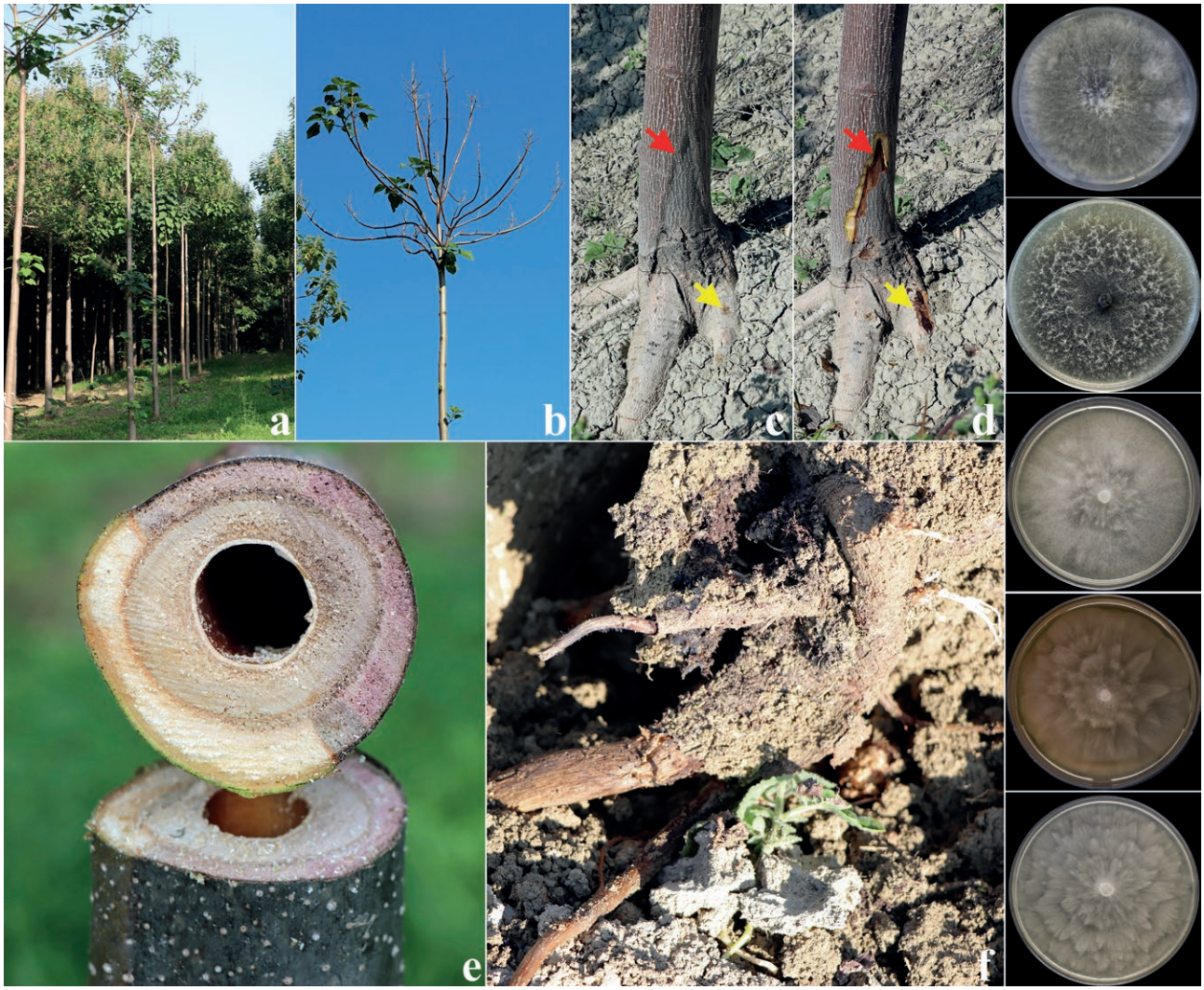

Figure 1. Overview of symptoms detected on the paulownia plants monitored in the study: extensive canopy dieback (A and B); tree showing a sunken canker at the collar caused by *Macrophomina phaseolina* (red arrow) and a *Phytophthora* bleeding canker on the main root caused by *Phytophthora pseudocryptogea* (yellow arrow) (C), particular of the internal (inner bark) necrotic lesion on the same tree (D), sunken canker in cross section (E), typical *Phytophthora* root rot symptoms (F). From top to bottom, colony morphology of *Botryosphaeria dothidea*, *Macrophomina phaseolina, Phytophthora citrophthora*, *Phytophthora erythroseptica* and *Phytophthora pseudocryptogea* after 7 days growth at 20 °C on PDA (*Botryosphaeriaceae*) and CA (*Phytophthora*) in the dark.

Table 1. Number of paulownia trees, cultivated in Site 1 and 2, showing different degrees of symptom severity in July 2022 and 2023.

Symptom severity	Site 1		Site 2	
	2022	2023	2022	2023
0	1668	1620	484	13
1	17	45	256	357
2	45	62	52	243
3	60	92	83	135
4	25	56	125	252
Total	1875		1000	

as well as percentage of canopy desiccation around 30%, were recorded in spring (Figure 1). The phytosanitary situation drastically declined during July, when canopy dieback was very frequent, with a percentage of desiccation around 30–50%, and dead plants.

In Site 1, the disease assessment performed during July 2022 and 2023 allowed an increment of symptomatic plants from 147 to 255 to be detected, as well as of dead plants. Most of the symptomatic plants showed an advanced status of canopy dieback (severity class 3) (Table 1).

In site 2, the phytosanitary status was completely deteriorated. More than 50% of plants were symptomatic in 2022 and in 2023 only 13 plants were without symptoms. In Site 2 in 2023 about 600 plants were evaluated with symptom severity in class 1 and 2, characterized by canopy dieback corresponding to <30% and between 30 and 50%, and 252 dead plants, double the number with respect to 2022 (Table 1). Among the 250 trees monitored, 173 showed both *Phytophthora* (bleeding) and *Botryosphaeriaceae* (sunken) cankers, 9 only *Phytophthora* bleeding cankers and 4 only *Botryosphaeriaceae* cankers, whereas 60 trees were dead (Figure 1).

By monitoring and disease assessing using aerial remote sensing (RS) in 2023, in Site 1 it was clear that the disease focus was not strictly related to dead plants, but there are areas, located near them, in which the pale green canopy captured during the flight, well correlated with the data collected in the field (Figure 2A). In Site 2, the aerial picture confirmed the dramatic phytosanitary *status*, extending to whole plantation (Figure 2B).

From 25 samples collected in the two paulownia plantations, we were able to isolate 22 *Phytophthora* colonies, of which 3 were obtained directly from necrotic canker tissues, and 19 indirectly from rhizosphere and root samples using the baiting technique. The morphological identification corroborated by molecular data, based on the sequences of the ITS and Btub regions, allowed three

Figure 2. Disease assessing by aerial remote sensing (RS) using drones in 2023: orthomosaic images of Site 1 (A) and Site 2 (B), collected with the DJI Mavic 3M.

different *Phytophthora* species to be defined, namely *P. citrophthora* (8 isolates), *P. erythroseptica* (3 isolates), *P. pseudocryptogea* (11 isolates) (Figure 1 and S1).

From 12 samples collected from stems showing aerial sunken canker, we were able to isolate 11 fungal colonies belonging to *Botryosphaeriaceae*. In particular, three isolates were identified as *Botryosphaeria dothidea* and 8 as *Macrophomina phaseolina* (Figure 1 and S2,3).

The five species used in the artificial inoculation showed to be pathogenic on paulownia. The average lesion size differed significantly according to the species, e.g., the lesions caused by *B. dothidea* (7.50 ± 0.46a cm; mean ± standard deviation) and *M. phaseolina* (7.45 ± 0.32a) were significantly bigger than those caused by *P. pseudocryptogea* (6.20 ± 0.18b), *P. erythroseptica* (4.51 ± 0.23c) and *P. citrophthora* (4.30 ± 0.29c).

The three *Phytophthora* species caused a range of both not specific symptoms such as yellowing, progressive dehydration and desiccation of leaves and specific symptoms such as inner bark necrosis on stem and root rot (Figure 3 e-j). The most severe symptoms were induced by *P. pseudocryptogea*, the necrotic lesion caused by this species expanded from the inoculation site to the root system. Less severe symptoms of decline were recorded for plants artificially inoculated with *P. erythroseptica*. The necrotic lesions caused by *P. citrophthora* were confined to inner bark tissues.

Macrophomina phaseolina and *B. dothidea* showed to be very aggressive on paulownia. The necrosis developed very quickly and progressively girdled the stem

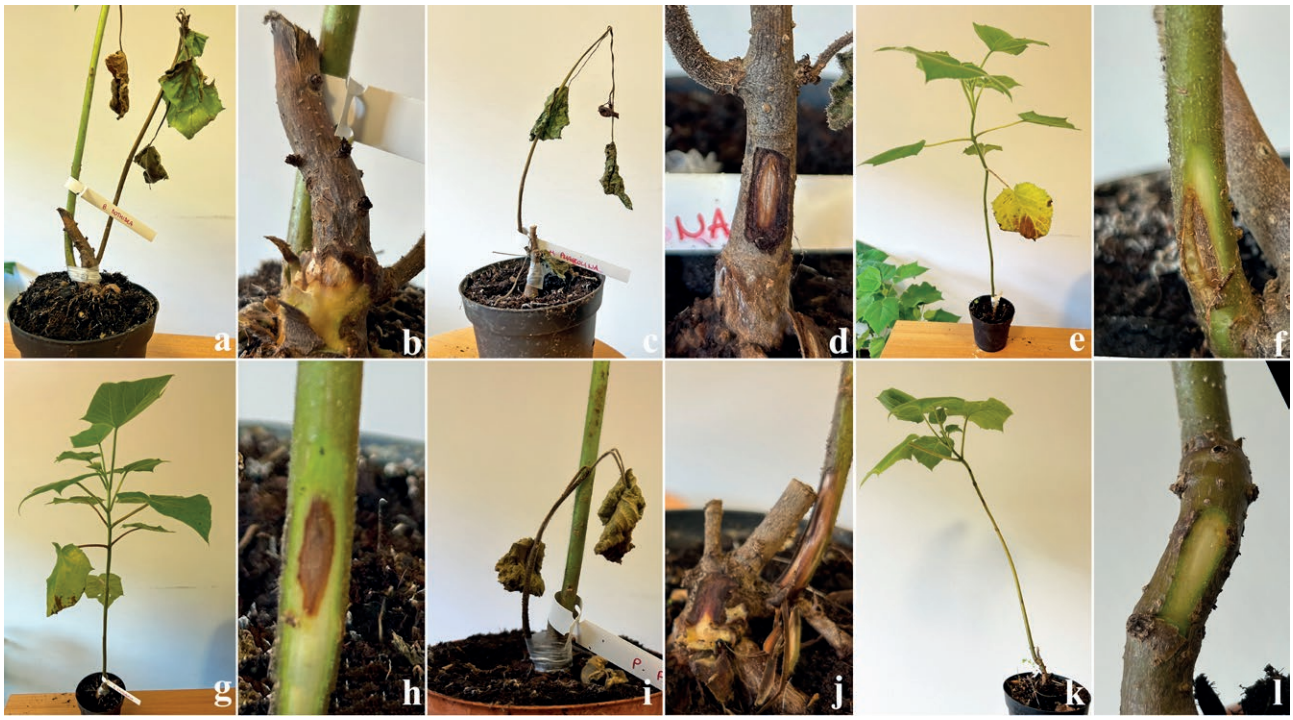

Figure 3. Artificial inoculation of *Botryosphaeria dothidea* (a,b), *Macrophomina phaseolina* (c,d), *Phytophthora citrophthora* (e,f), *P. erythroseptica* (g,h) and *P. pseudocryptogea* (i,j) on 1-year-old Paulownia plants in accordance with Koch's postulates. Control seedling (k,l).

causing wilting symptoms, with dead leaves remaining attached to the plant (Figure 3 a-d).

The symptoms induced by *Phytophthora* species, *M. phaseolina* and *B. dothidea* were identical to those observed in the two plantations of paulownia, except for exudates that have not been recorded on the young plants, artificially inoculated.

All five pathogens were successfully re-isolated from the margin on necrotic inner bark lesions of all seedlings, thus fulfilling Koch's postulates. All species are reported here for the first time as paulownia pathogens worldwide.

In conclusion, the findings obtained in this study allowed us to define the aetiology of the decline affecting paulownia trees in Italy, contributing to expand knowledge on the hosts range of some aggressive pathogens belonging to the genera *Botryosphaeria*, *Macrophomina* and *Phytophthora*. The co-occurrence of *Phytophthora* and *Botryosphaeriaceae* species was recently detected in several emerging diseases affecting forest trees and agriculture crops in Italy (Benigno *et al*., 2023; Linaldeddu *et al*., 2023). This complex aetiology indicates that multitrophic interactions are common in forest plantations and represent an important and concrete aspect of tree-pathogen relationships, providing a more realistic picture of the dynamics contributing to tree decline and mortality.

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