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Phytophthora Root and Collar Rot of *Paulownia*, a New Disease for Europe

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Abstract: *Paulownia* species are fast growing trees native to China, which are being grown in managed plantings in several European countries for the production of wood and biomasses. In 2018, wilting, stunting, leaf yellowing, and collapse, as a consequence of root and crown rot, were observed in around 40% of trees of a 2-year-old planting of *Paulownia elongata* × *P. fortunei* in Calabria (Southern Italy). Two species of *Phytophthora* were consistently recovered from roots, basal stem bark, and rhizosphere soil of symptomatic trees and were identified as *Ph. nicotianae* and *Ph. palmivora* on the basis of both morphological characteristics and phylogenetic analysis of rDNA ITS sequences. Koch's postulates were fulfilled by reproducing the symptoms on potted paulownia saplings transplanted into infested soil or stem-inoculated by wounding. Both *Phytophthora* species were pathogenic and caused root rot and stem cankers. Even though *P. palmivora* was the only species recovered from roots of naturally infected plants, in pathogenicity tests through infested soil *P. nicotianae* was more virulent. This is the first report of Phytophthora root and crown rot of a *Paulownia* species in Europe. Strategies to prevent this emerging disease include the use of healthy nursery plants, choice of well-drained soils for new plantations, and proper irrigation management.

Keywords: princess tree; tree of life; *Phytophthora nicotianae*; *Phytophthora palmivora*; *Phytophthora heterospora*; DNA sequencing; phylogenetic analysis; *Paulownia elongata* × *P. fortunei*; biomass and timber; nursery plants; pathogenicity

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1. Introduction

The genus *Paulownia*, formerly in the family Scrophulariaceae and now regarded as a member of the mono-generic and mono-phyletic family Paulowniaceae according to the Fourth Angiosperm Phylogeny Group (APG IV) classification system, encompasses deciduous hardwood tree species, very probably native to south-eastern China [1,2]. *Paulownia* species are among the most popular cultivated trees in China. The species of greatest commercial importance worldwide are *P. tomentosa*, *P. elongata*, *P. fortunei*, *P. catalpifolia*, and *P. kawakamii*. *Paulownia tomentosa*, which has a long life and is cold-tolerant, is the most widespread species. It behaves as a pioneer species and has been largely used to reclaim disturbed natural, urban, and industrial sites. It was naturalized in Japan and Korea and was the first species of this genus to be known in Europe, where it has been grown as an ornamental tree for over 150 years. In some areas of the U.S.A., where *P. tomentosa* was

introduced in the 19th century, it has been naturalized and is considered invasive [3,4]. The invasiveness of *P. tomentosa* is due to the ability to propagate vegetatively by suckering and resprouting after cutting, as well as to an impressive reproductive potential. A single tree can produce around 20 million seeds, which are winged and can be dispersed by wind up to a distance of about 10 km. Moreover, this *Paulownia* species with its vigorous growth and the shadowing of its large leaves competes with native vegetation and prevents its regeneration [5]. Differently from the U.S.A., in European countries where it has been naturalized, including Great Britain, Germany, France, Switzerland, Austria, Italy, and Spain, *P. tomentosa* is not yet regarded as a nature conservation issue [6]. However, this is controversial and has to be re-examined also in the light of climate change which could favor the spread of *P. tomentosa* beyond its present distribution [7]. In any case, it would be advisable to cautiously spread this alien plant species and prevent it from escaping from ornamental or commercial plantations to more natural habitats as happened in the U.S.A. *Paulownia elongata* and *P. fortunei* are more thermophilic, very fast-growing tree species, suitable for biomass and wood production in warmer areas of southern Europe, such as Italy and Spain. They seem less invasive than *P. tomentosa* [5]. *Paulownia catalpifolia* is not so widely cultivated as other species as it is relatively slow-growing, but produces wood of better quality. *Paulownia kawakamii*, which is considered an endangered plant species in nature since it is almost extinct in the wild, is cultivated for its high quality wood. Paulownias are propagated by seeds, root cuttings, or micro-propagation and are known by several common names, such as princess tree, empress tree, foxglove tree, tree of life, and karri (in English) or kiri (in Japanese) tree, the last referring specifically to *P. tomentosa*. Many varieties, interspecific hybrids, and clonal selections with a better performance than the parental species are presently available and are being used in managed commercial plantings, mainly for timber production. Moreover, sterile clones have been selected to prevent these plants from becoming invasive in new areas where extensive commercial plantings for wood production are established. From an economic point of view, paulownias are multipurpose trees as they are utilized for obtaining large quantities of leafy biomass for fodder, fertilizer, and as mulch to control soil erosion, flowers for the production of honey (Figure 1), as well as short-rotation tree crops for the production of biomass and timber [3,8,9].

Paulownias, in general, are characterized by their fast growth, ability to re-sprout rapidly after cutting, as well as tolerance to drought and high soil acidity. Moreover, paulownias are efficient nitrogen fixers in temperate climates. Recently, in several European countries, including Spain, France, Italy, Rumenia, Bulgaria, Moldovia, Serbia, and Poland, there has been a renewed economic interest in paulownias as an agroforestry crop and there are private companies that publicize new patented clones, produce and sell nursery plants, and give advice to farmers on how to plant and grow them. It is estimated that during the last ten years around 1500 ha of paulownias have been planted in Italy but there are no official figures on this.

There is little information on infectious diseases of paulownias and most of those reported so far are caused by fungi and oomycetes [4,10–13].

In June 2018, a severe disease was noticed in a commercial, intensively managed paulownia planting for the production of timber in the Calabria region (Southern Italy). Symptoms were suggestive of Phytophthora root and crown rot, the common name of a disease affecting several herbaceous and woody plant species. There are diverse *Phytophthora* species causing this disease, which all provoke very similar symptoms [12,14]. The present study was aimed at identifying the etiology of this disease of paulownia.

Figure 1. (**A**,**B**) Blossoming commercial plantations of paulownia in Calabria (Southern Italy). (**C**) A commercial paulownia plantation in summer (Calabria).

2. Materials and Methods

2.1. Isolation and Morphological Identification of Isolates

In June 2018, a 2-year-old planting of *Paulownia elongata* × *P. fortunei* in the municipality of Montalto Uffugo, province of Cosenza, Calabria region (Southern Italy) (Geographic Coordinates, DATUM WGS 84: 39°39′58.0′′ N, 16°21′25.5′′ E, Cosenza, Calabria, Southern Italy) was visited by the research group for the first time. The plantation extended for about 1 ha with tree spacing of 4 × 6 m and clay-loam soil. Plants were watered with a drip irrigation system and water was from a well pumping from groundwater table. Potted 1-year-old rooted cuttings imported from Bulgaria had been used for the planting in late spring 2016. A second inspection was carried out a year later, in June 2019. In 2018, necrotic roots, stem bark, and rhizosphere soil were sampled from six distinct symptomatic *Paulownia elongata* × *P. fortunei* trees in a commercial orchard at Montalto Uffugo, in the province of Cosenza.

Bark pieces and root segments were washed thoroughly in tap water, disinfected in 1% NaClO for 2 min and immersed in 70% EtOH for 30 s, rinsed in sterile distilled water, dipped dry, and plated on selective PARPNH V8-agar medium which consisted of 100 mL V8 juice (Campbell Grocery Products Ltd., Ashford, UK), 15 g agar, 3 g $CaCO_3$, 200 mg ampicillin, 10 mg rifampicin, 25 mg pentachloronitrobenzene (PCNB), 50 mg nystatin, 50 mg hymexazol, and 1 L of deionized water [12]. After an incubation period of 24–48 h in the dark at 24 °C, pure cultures were obtained by transferring individual growing hyphae to V8-juice agar (V8A) [15].

Rhizosphere soil samples, including fine roots, were collected under the canopy of *Paulownia* trees after removing the upper organic soil layer. Soil sampling and isolation were performed in accordance with Riolo et al. [16]: four soil cores were collected from each tree, 50–150 cm away from the stem base, and were bulked together (about 2 L). Two subsamples of about 500 mL were used for baiting tests that were performed in a walk-in growth chamber with 12 h natural day light at 20 °C. Young leaves of carob-tree (*Ceratonia siliqua*) and oak (*Quercus* spp.) floated over flooded soil were used as baits. After 24–48 h

incubation, necrotic segments (2 mm side) from symptomatic leaves were plated in Petri dishes onto selective PARPNH agar medium. Petri dishes were incubated at 20 °C in the dark. Outgrowing *Phytophthora* hyphae were transferred onto V8 juice agar (V8A) under the stereomicroscope.

Morphological features and colony morphology of all isolates, including the morphology and dimensions of asexual reproductive structures, were determined on colonies grown on V8A at 24–26 °C in the dark in accordance with standard procedures [12]. Cardinal temperatures for radial growth were determined by growing the isolates on potato dextrose agar (PDA; Oxoid Ltd., Basingstoke, UK) in Petri dishes (9 cm diam.), and incubating the dishes at 5 °C, 10 °C, 15 °C, 20 °C, 25 °C, 27 °C, 30 °C, 32 °C, and 35 °C (all ± 0.5 °C), in the dark. In total, 17 isolates of *Phytophthora nicotianae* and 24 isolates of *Ph. palmivora*, with four replicates per isolate and temperature value, were tested. Sporangia production was stimulated with the method described by Santilli et al. [14]. Fragments of 2 mm were cut from the growing edge of 7-day-old cultures grown in Petri dishes (15 mm diam.) on V8A at 27 °C in the dark, were placed in a 5 cm diameter Petri dish and flooded with non-sterile soil extract water (200 g soil suspended in 1 L of de-ionized water for 24 h at room temperature and then filtered). After incubation at 27 °C in the dark for 24–48 h, dimensions and morphological features of 50 mature sporangia of each isolate were determined at ×400 magnification.

2.2. Molecular Identification of Isolates

All purified cultures were molecularly identified by the amplification and analysis of an Internal Transcribed Spacer (ITS) of ribosomal DNA (rDNA). Total DNA was extracted from 7-day-old cultures grown on V8-agar at 22 °C by using the PowerPlant® Pro DNA isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer's instructions. The PCR amplification was performed by using the primer pairs ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) [17] and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) [18] in a 25 µL reaction mix containing PCR Buffer (1X), dNTP mix (0.2 mM), MgCl2 (1.5 mM), forward and reverse primers (0.5 µM each), Taq DNA Polymerase (1 U), and 100 ng of DNA. The thermo-cycler conditions were as follows: 94 °C for 3 min; followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and then 72 °C for 10 min. Amplicons were detected in 1% agarose gel and sequenced in both directions by an external service (Macrogen, Amsterdam, The Netherlands). All the sequences were analyzed by using FinchTV v.1.4.0 [19]. Species identification was performed by blast searches in GenBank [20], *Phytophthora* database [21] and in a local database containing sequences of ex-type or key isolates from published studies. Isolates were assigned to a species when the respective consensus sequence was 100% identical to that of a reference isolate.

2.3. Pathogenicity Tests

The pathogenicity of the two *Phytophthora* species, recovered from roots and stem bark of *P. elongata* × *P. fortunei* trees in Calabria, was tested in two separate experiments, using the soil infestation method as described by La Spada et al. [22] and the method of stem inoculation by wounding as described by Aloi et al. [23], respectively. The isolates C2K3A (*Ph. nicotianae*) and C1K9E (*Ph. palmivora*), obtained from rhizosphere soil and roots of symptomatic *P. elongata* × *P. fortunei* trees, respectively, were used in the soil infestation test. Twelve 2-month-old saplings of *P. elongata* × *P. fortunei* were transplanted into free-draining pots containing a mixture of autoclaved universal potting soil (Terraricca ©, Cifo Srl, Giorgio di Piano, Bologna, Italy) and inoculum (20 cm^3 of inoculum per 1000 cm^3 of potting mixture). Inoculum consisted of a 21-day-old culture of the isolates grown in the dark at 25 °C in a 750 mL jar containing a sterilized medium made of a mixture of 50 mL of millet seeds and 50 mL V8-juice broth. Ten control plants were transplanted in free-draining pots containing non-infested potting mixture. After transplanting, all plants were maintained in saturated soil for 48 h and then transferred to a growth chamber at 23 °C, 80% relative humidity, and a photoperiod of 16 h of light and 8 h of dark. The trial

was concluded when inoculated plants showed severe symptoms of wilting, leaf yellowing, and defoliation (6 weeks post inoculation). At the end of the test, both *Phytophthora* species were re-isolated from necrotic roots and the identity was confirmed by sequencing the ITS. Symptoms were assessed visually. The severity of root rot was rated according to a scale of five root damage classes: 4 = healthy root system with dense fine root system and well developed taproot; 3 $\leq$ 25% fine root losses and well developed taproot; 2 = 26%–50% fine root losses, beginning taproot dieback and small necrotic lesions on woody roots and/or the collar; and 1 = 51%–75% fine root losses, advanced taproot dieback and large necrotic lesions on tap roots and/or the collar; 0 = 76–100% fine root losses, extensive taproot dieback and girdling necrotic lesions on taproot and/or the collar. The roots were then dried for 72 h at 65 °C and the dry weights of main roots (diam 2–10 mm) and fine roots (diam < 2 mm) were recorded for each plant [24]. Data were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons test by using R software. Differences at $p \leq 0.05$ were considered significant.

The pathogenicity of a *Ph. nicotianae* isolate and a *Ph. palmivora* isolate (P4K1C7 and P3K4A9, respectively), both from stem bark, was tested by wound inoculating on the stems of 6-month-old saplings of *P. elongata* × *P. fortunei* grown in plastic pots (10 cm diameter, 1 L volume). Six saplings were inoculated with each isolate, and four were used as control. The basal stem, near the collar, was disinfected with 70% ethanol, and a disk of bark (5 mm diameter) was removed with a flamed cork borer and replaced with an agar-mycelium plug taken from the margin of an actively growing colony on V8A as described by Aloi et al. [23]. The wound was covered by putting the excised bark disk back on the wound and then sealing it tightly with Parafilm®. The stem of each sapling was inoculated 10 cm above soil level with a single hole per stem. A sterile V8A plug was inserted into the wound of control saplings as described above. All inoculated seedlings were watered regularly for 30 days and kept in a growth chamber at 23 °C, 80% relative humidity, and a photoperiod of 16 h of light and 8 h of dark. The trial was concluded when inoculated plants showed severe symptoms of stem necrosis (4 weeks post inoculation). The length of necrotic lesion originating from each inoculation point was measured after removing the bark and both *Phytophthora* species were re-isolated from necrotic lesions using the selective PARPNH V8-agar medium and sequenced.

3. Results

3.1. Symptoms of the Disease

The first disease symptoms, including leaf chlorosis, wilting and stunting, were noticed by the farmer on just a few scattered plants in 2016, a few weeks after planting. When we made the first access, in June 2018, about 40% of the trees showed more or less severe above-ground symptoms including wilting, stunting, leaf yellowing, defoliation, basal stem necrosis, and final death of the entire plant (Figure 2). Occasionally, a viscous, dark brown, sticky exudate oozed from the basal part of non-lignified stems (Figure 3). Above-ground symptoms were always associated with severe root rot and often with basal stem necrosis (crown rot). By 2019, the overall number of symptomatic and dead trees had not increased, but symptoms had progressed and worsened in surviving trees compared to those observed in 2018. Conversely, no symptoms were observed in young saplings of *P. elongata* × *P. fortunei* from a local nursery that had been transplanted in 2018 to replace dead trees.

Figure 2. (**A**–**C**) Symptoms of wilting, stunting, leaf yellowing, defoliation, and final death of the entire plant in a young plantation of paulownia (*Paulownia elongata* × *P. fortunei*) in Calabria. (**D**,**E**) Leaf chlorosis, wilting, and stunting in 4-year-old trees (June 2019). (**E**) In the foreground, asymptomatic sapling from a different nursery transplanted in 2018 to replace a dead tree.

3.2. Morphological Characterisitcs of Phytophthora Isolates

Direct isolation from rotten roots and basal stem bark of six symptomatic trees selected randomly in the plantation of Montalto Uffugo consistently yielded two *Phytophthora* species with distinct colony morphologies on solid culture media (Figure 4).

Figure 3. (**A**) Basal stem necrosis with dark brown, sticky exudate on *Paulownia elongata* × *P. fortunei* trees. (**A**–**C**). Symptoms of severe crown rot on young trees (June 2018). (**D**,**E**). Symptoms of severe crown rot on 3-year-old trees (June 2019).

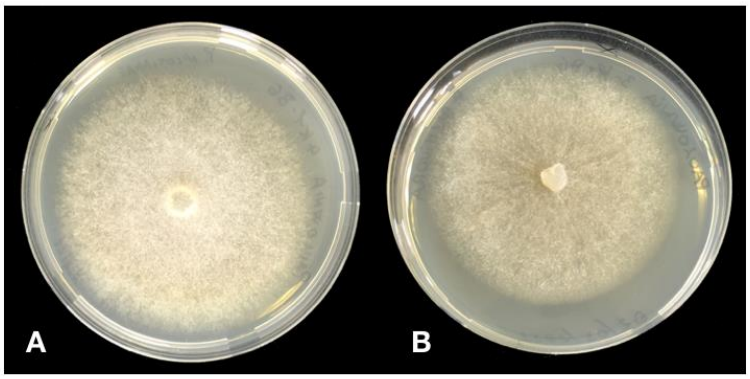

Figure 4. Morphology of 7-day-old colonies of *Ph. nicotianae* (**A**) and *Ph. palmivora* (**B**) grown on V8A at 25 °C in the dark.

The first species, identified subsequently as *Ph. nicotianae*, was recovered exclusively from basal stem bark, while the second species, identified subsequently as *Ph. palmivora*, was recovered from both roots and in lower proportion from basal stem bark. Both species were recovered from all six symptomatic plants. The proportions of isolates of each species recovered by direct isolation from stem bark were 70% and 30%, respectively. Moreover, both species were recovered by leaf-baiting from rhizosphere soil of symptomatic plants in proportions of 53% and 47%, respectively. Overall, 41 single-hypha isolates (17 and 24 isolates of each species, respectively) were selected randomly to be characterized (Table 1). On V8 agar (V8A) *Ph. nicotianae* formed fluffy colonies, with dense and cottony aerial mycelium. Colonies on PDA were typically arachnoid. They grew between 10 °C and 32 °C, with an optimum of 27 °C. Colony morphology of *Ph. palmivora* on V8A was stellate with aerial mycelium and coralloid hyphae. Colonies on PDA were uniform and slightly woolly, with stoloniferous hyphae. Minimum and maximum temperatures for growth were 10 °C and 35 °C, respectively, while optimum temperature was 27 °C. Colonies grew more slowly than those of *Ph. nicotianae*. On both solid V8A and V8A plugs flooded with non-sterile soil extract, *Ph. nicotianae* produced persistent, ovoid to spherical, papillate sporangia (dimensions 45.0 × 34.0 to 33.1 × 23.5 µm and mean length/breadth ratio 1.4:1), while *Ph. palmivora* produced ovoid-ellipsoid, caducous, papillate sporangia (dimensions 21.5 × 33.2 to 19.2 × 29.4 µm) with a short (mean length < 5 µm) pedicel. Isolates of both species were self-sterile and did not produce antheridia or oogonia in single cultures.

3.3. Molecular Identification

Amplification and sequencing of the Internal Transcribed Spacer (ITS) regions of ribosomal DNA (rDNA) of isolates of the two species recovered from paulownia plants revealed 99%–100% identity with the sequence of *Phytophthora nicotianae* isolates CBS 31062, CNRnico43RC, IMI 397474 and IMI 398989 (GenBank Accession Number HQ643302, KT148945, GU723474 and HQ596557, respectively [25–28]) and *P. palmivora* isolates CNR-pal44RC, CNRpal72RC, IMI 398987, IMI 398988, CBS 27433, IMI 503890 (MD5), IMI 503891 (MD6) and CBS 305.62 (GenBank Accession Number KT148931, KT148921, HQ596556, HQ596558, and HQ643308, KF823978, KF823979, MG865559, respectively [25–30]). In the tree obtained by the phylogenetic analysis of the combined data set of sequences from ITS region of isolates recovered from paulownia and sequences of *Phytophthora* species used as references, all isolates from roots, stem bark, and rhizosphere soil of paulownia trees clustered (bootstrap values of 1000 replicate) with the references isolates of these species (Figure 5). In particular, the isolates of *Ph. Palmivora* from paulownia grouped separately from those of *Ph. heterospora* [31], a very recently described sister species of *Ph. palmivora sensu stricto* segregated from the *Ph. palmivora* complex (Figure 5). The ITS sequences of isolates from roots, bark and rhizosphere soil of paulownia were deposited in GenBank (the respective GenBank accession numbers are given in Table 1).

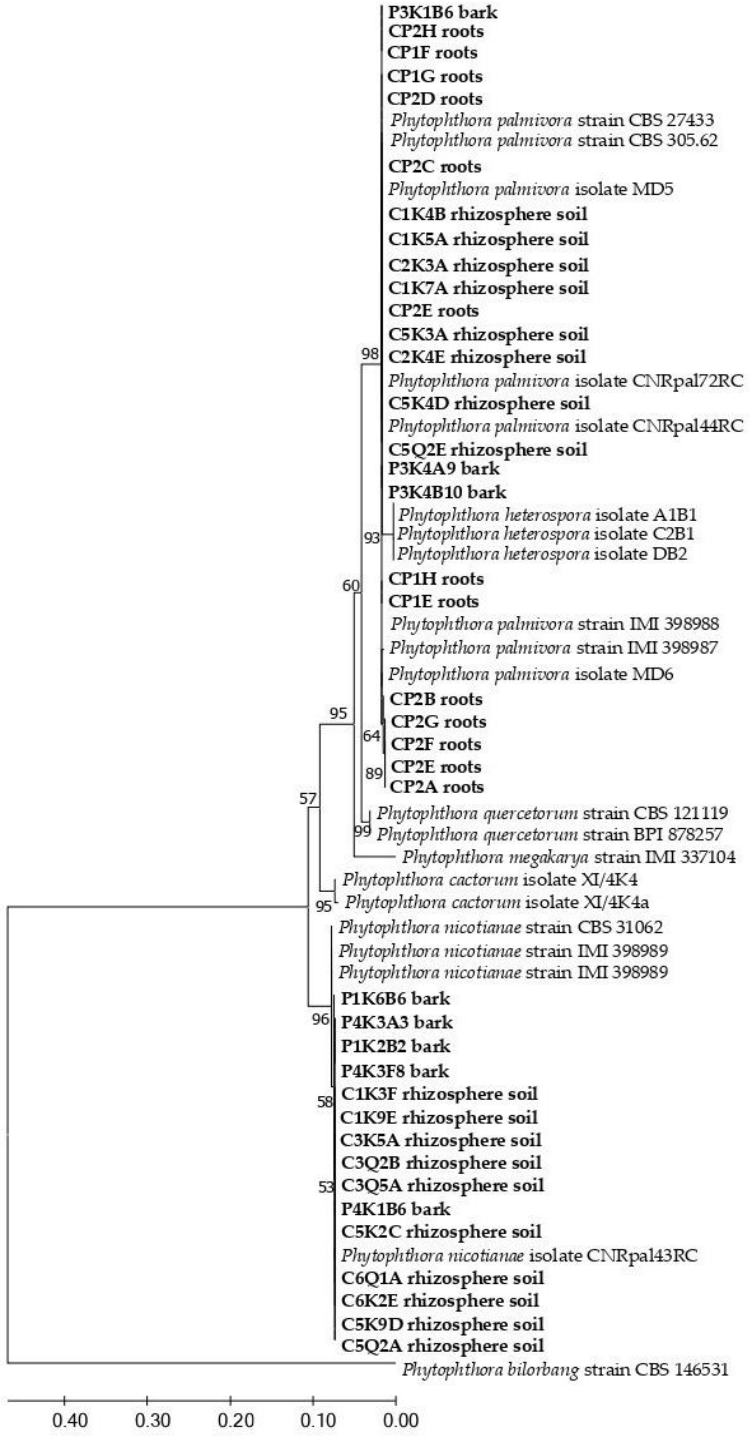

Figure 5. Phylogenetic tree for the ITS loci obtained by the maximum likelihood method, based on the Tamura–Nei model. Relationships between the 41 *Phytophthora* spp. Isolates from paulownia (in bold), the reference isolates of *Ph. nicotianae* and *Ph. palmivora* and other *Phytophthora* species within the ITS Clade 1 and Clade 4, including *Ph. heterospora* very recently separated from *Ph. palmivora*. *Ph. bilorbang* (CBS 146531) was used as outgroup taxon. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (below the branches).

Table 1. Isolates of *Phytophthora nicotiane* and *Ph. palmivora* sourced from different tissue of *Paulownia elongata* × *P. fortunei* trees in Calabria, characterized in this study.

Isolate Code	*Phytophthora* Taxa	Source	Accession Number
			ITS
P4K1C7	*Ph. nicotianae*	Bark	OK463579
P4K3A3	*Ph. nicotianae*	Bark	OK463580
P1K2B2	*Ph. nicotianae*	Bark	OK463581
P4K1B6	*Ph. nicotianae*	Bark	OK463582
P1K6B6	*Ph. nicotianae*	Bark	OK463583
P4K3F8	*Ph. nicotianae*	Bark	OK463584
P1K4A3	*Ph. nicotianae*	Bark	OK463585
P3K4A9	*Ph. palmivora*	Bark	OK463586
P3K1B6	*Ph. palmivora*	Bark	OK463587
P3K4B10	*Ph. palmivora*	Bark	OK463588
C1K9E	*Ph. nicotianae*	Rhizosphere soil	OK463589
C1K3F	*Ph. nicotianae*	Rhizosphere soil	OK463590
C3K5A	*Ph. nicotianae*	Rhizosphere soil	OK463591
C3Q5A	*Ph. nicotianae*	Rhizosphere soil	OK463592
C3Q2B	*Ph. nicotianae*	Rhizosphere soil	OK463593
C5K2C	*Ph. nicotianae*	Rhizosphere soil	OK463594
C5K9D	*Ph. nicotianae*	Rhizosphere soil	OK463595
C5Q2A	*Ph. nicotianae*	Rhizosphere soil	OK463596
C6Q1A	*Ph. nicotianae*	Rhizosphere soil	OK463597
C6K2E	*Ph. nicotianae*	Rhizosphere soil	OK463598
CP1F	*Ph. palmivora*	Roots	OK463599
CP2H	*Ph. palmivora*	Roots	OK463600
CP2A	*Ph. palmivora*	Roots	OK463601
CP2G	*Ph. palmivora*	Roots	OK463602
CP1H	*Ph. palmivora*	Roots	OK463603
CP1G	*Ph. palmivora*	Roots	OK463604
CP2D	*Ph. palmivora*	Roots	OK463605
CP2F	*Ph. palmivora*	Roots	OK463606
CP2E	*Ph. palmivora*	Roots	OK463607
CP2B	*Ph. palmivora*	Roots	OK463608
CP1E	*Ph. palmivora*	Roots	OK463609
CP2C	*Ph. palmivora*	Roots	OK463610
C1K5A	*Ph. palmivora*	Rhizosphere soil	OK463611
C1K4B	*Ph. palmivora*	Rhizosphere soil	OK463612
C1K7A	*Ph. palmivora*	Rhizosphere soil	OK463613
C2K3A	*Ph. palmivora*	Rhizosphere soil	OK463614
C2K5B	*Ph. palmivora*	Rhizosphere soil	OK463615
C2K4E	*Ph. palmivora*	Rhizosphere soil	OK463616
C5K3A	*Ph. palmivora*	Rhizosphere soil	OK463617
C5K4D	*Ph. palmivora*	Rhizosphere soil	OK463618
C5Q2E	*Ph. palmivora*	Rhizosphere soil	OK463619

3.4. Pathogenicity Test

The isolates C2K3A of *Ph. nicotianae* and C1K9E of *Ph. palmivora* from *P. elongata* × *P. fortunei* proved to be pathogenic on saplings of the same plant species. All 12 seedlings of *P. elongata* × *P. fortunei* transplanted into pots filled with soil infested with the two *Phytophthora* species developed severe symptoms of root rot, leaf chlorosis, defoliation, wilt, and death within four weeks after the transplanting (Figure 6). Conversely, control plants remained asymptomatic.

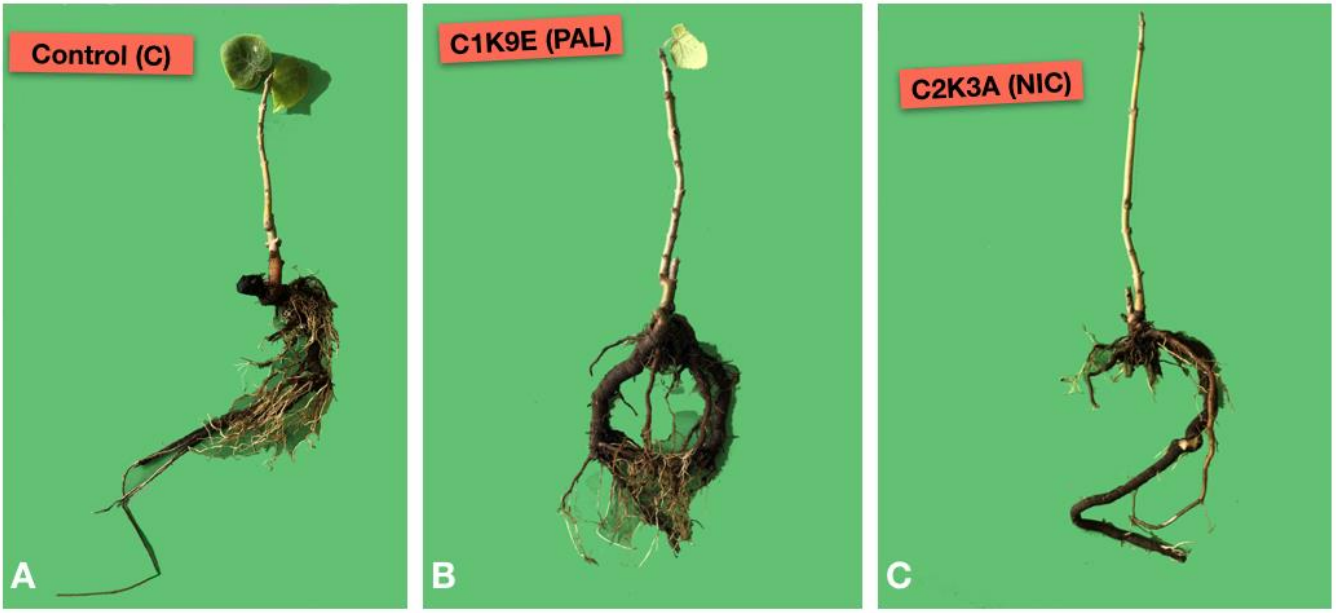

Figure 6. (**A**) No symptoms appeared in control *Paulownia elongata* × *P. fortunei* saplings 6 weeks after transplanting in uninfested soil; (**B**). Symptoms of root rot, leaf chlorosis, defoliation, wilt, and death on saplings 6 weeks after transplanting in soil infested with *Phytophthora palmivora* (C1K9E), and in (**C**). with *Ph. Nicotianae* (C2K3A).

The fine root/main root weight (frw/mrw) ratio in control plants and their root damage class were 1.2 and 3.8 ± 0.50, respectively (Figure 7). *Phytophthora nicotianae* was the most aggressive species causing 70% mortality of saplings 6 weeks after the transplanting, a frw/mrw ratio of 0.39 (67.5% reduction compared to the control), 74% reduction in fine root weight compared to control and a root damage class of 1.3 ± 0.46 (i.e., 67.5% fine root losses, severe decay of taproot and collar necrosis) (Figure 6). *Phytophthora palmivora* caused 30% mortality, a frw/mrw ratio of 0.74 (38.3% reduction compared to the control), and a root damage class of 2.0 ± 1.0 (Figure 6). Differences in root damage class and frw/mrw ratio compared to the control were statistically significant for both isolates C2K3A (*Ph. nicotianae*) and C1K9E (*Ph. palmivora*) ($p < 0.001$). *Phytophthora nicotianae* and *Ph. Palmivora* were re-isolated from roots of plants expressing symptoms, thus fulfilling Koch's postulates. The identity of isolates obtained from necrotic roots of symptomatic, artificially inoculated saplings was determined by the colony morphology, microscopic observations, and rDNA ITS sequencing. Isolates of *Ph. nicotianae* and *Ph. palmivora* from bark proved to be pathogenic on stems of *P. elongata* × *P. fortunei* saplings. All inoculated saplings showed dark brown inner bark lesions that spread up and down from the inoculation wound along the stem and extended to the outer layers of wood. Control plants remained asymptomatic (Figure 8). At the end of the experiment, mean lesion length from all seedlings was recorded. The lesions caused by both *Phytophthora* species were significantly larger than control plants, but did not differ significantly from each other (Figure 9). Both inoculated species were re-isolated from stem lesions.

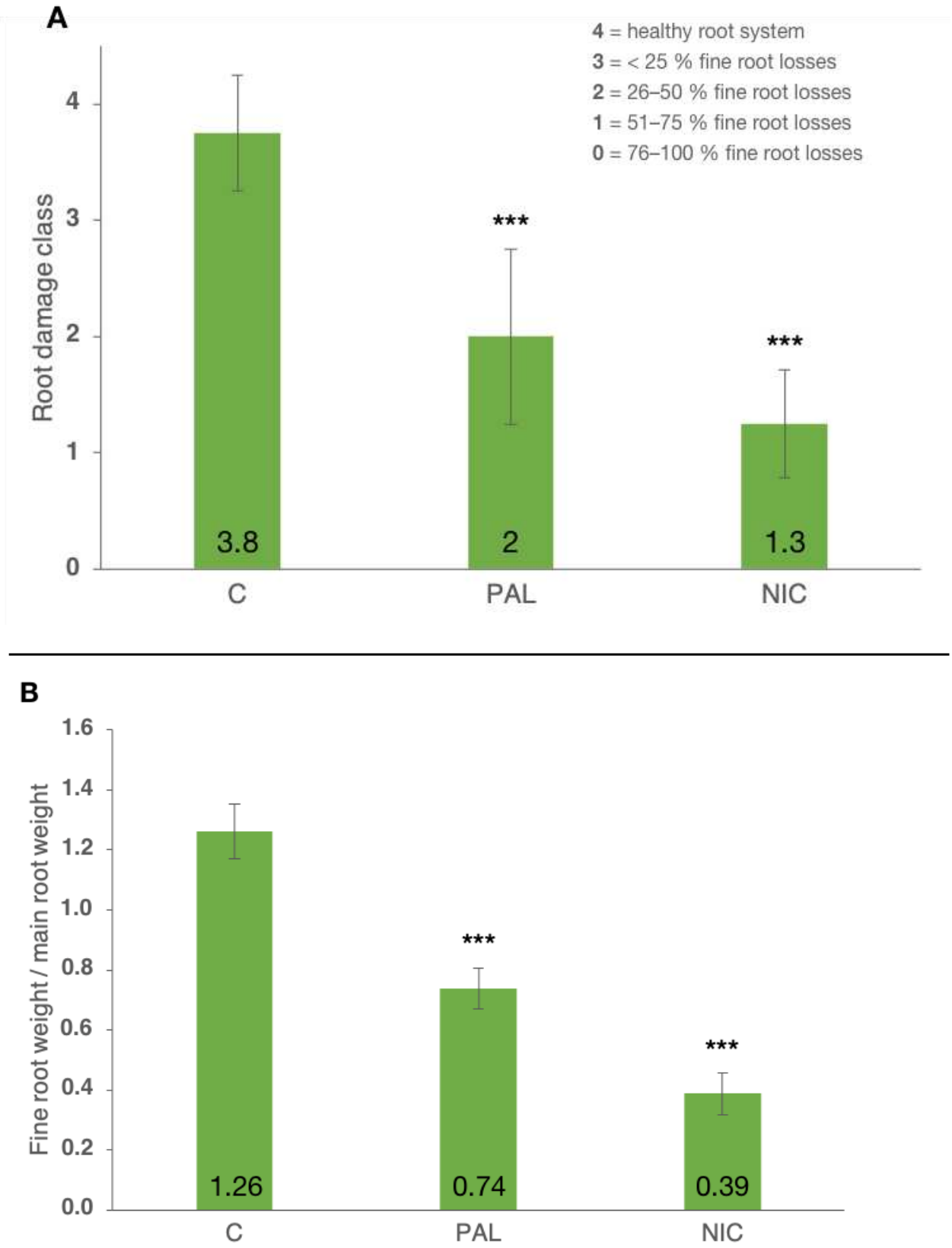

Figure 7. Mean root damage class (**A**) and mean fine root weight/main root weight (**B**) ratio of *Paulownia elongata* × *P. fortunei* saplings 6 weeks after transplanting in uninfested soil (Control: C) and in soil infested with *Phytophthora nicotianae* (NIC) and *Ph. palmivora* (PAL), singularly. Bars show standard deviations; asterisks represent statistical significances (*** = $p < 0.001$).

Figure 8. Internal stem necrosis from representative samples of *Paulownia elongata* × *P. fortunei* saplings non-inoculated (C) or inoculated with *Phytophthora palmivora* (PAL) and *Ph. nicotianae* (NIC) observed using a zoom stereomicroscope when inoculated plants showed severe symptoms of stem necrosis (4 weeks post inoculation).

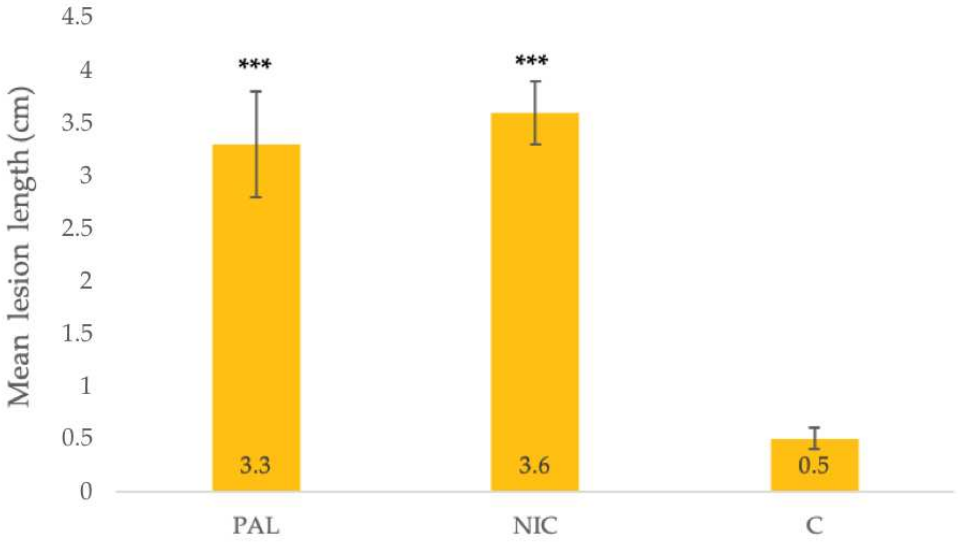

Figure 9. Mean lesion length (±SD) of *Paulownia elongata* × *P. fortunei* saplings 4 weeks after wound inoculation on the stem with *Ph. nicotianae* (NIC), *Ph. palmivora* (PAL) and control (with a sterile V8A plug). Bars show standard deviations; asterisks represent statistical significances (*** = $p < 0.05$).

4. Discussion

In this study, it was demonstrated that mixed infections of *P. nicotianae*, from *Phytophthora* clade 1, and *Ph. palmivora*, from *Phytophthora* clade 4, were responsible for the outbreak of Phytophthora crown and root rot of *P. elongata* × *P. fortunei* trees observed in a commercial plantation in Calabria. The etiological role of these two species was proved by completing Koch's postulates. Results of pathogenicity tests indicated that both species were able to induce stem cankers and root rot when inoculated singularly. Although both species were recovered from rhizosphere soil of symptomatic paulownia trees, *Ph. nicotianae* was isolated more frequently from stem bark than *Ph. palmivora* while the latter was the only species isolated directly from roots of trees with natural infections. This is in agreement with previous reports indicating *P. palmivora* is an aggressive pathogen

of roots of different woody host plants both alone and in combination with other pathogens or pests [25,32,33]. Mixed infections of different *Phytophthora* species on the same host plant are common [34–36]. In these cases, the prevalence as well as the incidence and isolation frequency of each species, and its distribution in different organs of the plant depends on several factors including, among others virulence and ecology of the species itself [36]. Mixed infections on the same host plant produce complex interactions resulting in synergism between pathogens or alternatively additive or neutral effects. Even though simultaneous infections by more than a single pathogen of the same genus or different genera in plants are frequent in natural conditions, the complex phenotypic and genetic responses of plants to multiple infections are still scarcely known. However, they are receiving increasing attention, and recent studies focused also on co-infections by species of *Phytophthora* [37,38].

Ph nicotianae and *Ph. palmivora* are polyphagous pathogens with a host range encompassing plant species of different families [39–44]. In Italy, *Ph. nicotianae* is widespread and is prevalently associated with agricultural crops. Its host range includes several economically important crops, such as citrus, tobacco, and vegetables. It is also the prevalent *Phtytophthora* species in nurseries of ornamentals [45,46]. Occasionally, it was also recovered from natural and forest ecosystems [16]. During the last twenty years, in the Mediterranean region the number of reports of *Ph. palmivora*, an exotic species probably native to the tropics, has increased noticeably. However, it is likely that many of these records refer to *Ph. heterospora*, a recently described sister species of *Ph. palmivora* [31]. Phylogenetic differences between these two species include 3–4 fixed polymorphisms of ITS. Accordingly, in the phylogenetic tree for the ITS loci obtained in this study the isolates from paulownia clustered separately from *P. heterospora* reference isolates and grouped together (100% similarity) with reference isolates of *Ph. palmivora sensu strictu*. Moreover, the isolates from paulownia were confidently identified as *Ph. palmivora* as they did not produce pseudo-conidia on solid medium, a unique distinctive trait of *Ph. heterospora*, and showed a maximum growth temperature of 35 °C, another key difference between *Ph. palmivora* and *Ph. heterospora* [31].

Although both *Ph. nicotianae* and *Ph. palmivora* are listed among the pathogens of paulownias in Asia, they were identified only on the basis of morphological characters and their impact on paulownia plantations has not been quantified [11]. *Ph. nicotianae* was also reported on paulownia in India [47], but as causal agent of leaf blight, a symptom not observed in Calabria. In a list of garden trees and shrubs susceptible to Phtytophthora root and crown rot published by the Royal Horticultural Society, paulownia is classified as a host plant affected only occasionally [48]. To our knowledge, this is the first documented report of Phtytophthora root and crown rot of paulownia in Europe and the first time ever that *Phytophthora* species associated with this disease have been identified using both morphological and molecular criteria. The severity of the outbreak of this emergent disease observed in Calabria indicates it is a potential threat for the expanding paulownia industry of some European countries and may have phytosanitary implications for the international trade of paulownia plantlets. In fact, *Ph. nicotianae* and *Ph. palmivora* as pathogens associated with paulownia are included in the list of quarantine or regulated pathogens by several countries, such as New Zealand and Sudan [49,50].

Although both *Ph. nicotianae* and *Ph. palmivora* are already established pathogens in Italy, it can be speculated that the outbreak of Phtytophthora root and crown rot of paulownia observed in Calabria originated from imported nursery plants. This hypothesis is based on circumstantial evidence, including the early appearance of first symptoms soon after planting, the uniform distribution of symptomatic trees throughout the entire plantation and the absence of symptoms on young plants supplied by a local nursery used to replace dead trees. The transport with infected nursery plants has proven to be the primary cause of the spread of *Phytophthora* species and their introduction into agricultural and forest ecosystems [31,51–56]. Likely, Phytophthora root and crown rot may be particularly harmful in nurseries and new plantings as young plants of paulownia

are more susceptible than mature trees to infections by *Phytophthora* species. Moreover, the intensive use of irrigation in nurseries and new plantings may favor the infections by these oomycetes. The use of healthy nursery plants is crucial for preventing this disease in commercial plantations. Paulownias are water-demanding trees and in Mediterranean climate watering is critical for the successful establishment of the planting and afterwards during hot dry summers to promote vigorous growth. In these intensively managed plantations another source of *Phytophthora* inoculum might be the irrigation water especially if it is recycled or comes from superficial reservoirs [30,57,58], which was not the case of the paulownia planting in Calabria. Soil waterlogging is conducive for Phytophthora root and crown rot. As a consequence, the choice of well-drained soil for establishing new plantings and careful management of irrigation are part of the strategy to prevent the disease. An additional aspect which is worth investigating is the susceptibility to this emerging disease of the new hybrid clones of paulownia selected mainly for their agronomic performance and the technological characteristics of the timber.

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