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Phytopythium litorale: A Novel Killer Pathogen of Plane (*Platanus orientalis*) Causing Canker Stain and Root and Collar Rot

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Abstract

Decline symptoms associated with lethal stem and branch canker stain along with root and collar rots were observed on 5- to 7-year-old roadside oriental plane trees (*Platanus orientalis*) in Diyarbakır, Turkey. Aboveground symptoms included leaf necrosis, leaf curling, extensive bluish or blackish staining of shoots, branches, stem bark, and wood surfaces, as well as stem cankers and exfoliation of branch bark scales. A general decline of the trees was distinctly visible from a distance. A *Phytophthora*/*Pythium*-like oomycete species with globose to ovoid, often papillate and internally proliferating sporangia was consistently isolated from the fine and coarse roots and stained branch parts and shoots. The pathogen was identified as *Phytopythium litorale* based on several morphological features. Partial DNA sequences of three loci, including nuclear rDNA internal transcribed spacer (ITS) and the large ribosomal subunit (LSU), and mitochondrial cytochrome c oxidase subunit II (*cox*II) confirmed the morphological identification. All *P. litorale* isolates were homothallic, developing gametangia, ornamented oogonia with elongate to lobate antheridia. Pathogenicity of *P. litorale* was tested by inoculation on excised shoots and by root inoculation on seedlings. *P. litorale* produced large lesions and blights on shoots in just 5 days and killed 100% of the seedlings in a month. This paper presents the first confirmed report of *P. litorale* as an important pathogen on a plant species causing branch and stem cankers, and root and collar rot, in and on *P. orientalis*, resulting in a rapid decline of trees and suggesting a threat to plane.

Keywords: *Platanus orientalis*, *Phytopythium litorale*, canker stain, oomycete, decline, root and collar rot

Oriental plane (*Platanus orientalis* L.; Platanaceae) is a widely planted, perennial, large, deciduous and ornamental shade tree known for its longevity and broad crown, occurring naturally throughout Turkey and many other countries. *P. orientalis*, the only *Platanus* species occurring naturally in Europe, is widely distributed in mountains, forests, and along streams and rivers in eastern Mediterranean regions, extending naturally from Southeastern Europe to Southwestern Asia in regions characterized by mild wet winters and warm dry summers (Besnard et al. 2002; Douda et al. 2016). In Turkey, some *P. orientalis* trees are recognized as veteran trees (natural monuments), growing up to 25 to 35 m in height with trunk diameters over 5 m and living for 2,000 years as a symbol of long and healthy life (Ozturk et al. 2017). The plane tree, known for its robustness, easy vegetative reproduction, rapid growth rate, attractive foliage, deep shade, and air purification ability, has also earned an important place as a prominent component of urban green space in parks, roadsides, and ornamental plantations in city centers. This tree species has provided environmental, aesthetic, cultural, and economic benefits beyond its natural range since ancient times.

A number of important diseases are known affecting plane trees, causing significant damage. The most destructive disease of planes is canker stain caused by *Ceratocystis platani* (J.M. Walter) Engelbrecht & Harrington. The pathogen was first reported in the United States as a widespread disease of plane trees in many states (Walter et al. 1952) and subsequently noted in Italy (Panconesi 1972) and other European countries, including France (Ferrari and Pichenot 1976), Spain (Riba 2011), Greece (Tsopelas and Angelopoulos 2004), Albania (Tsopelas et al. 2015), and the European part of Turkey (Lehtijärvi et al. 2018), killing many thousands of plane trees. The disease results in xylem staining, disruption of xylem flow, cankers, and finally death of the tree (Tsopelas et al. 2017). Another species causing cankers on plane trees is *Phytophthora cinnamomi* Rands, which was found to be the causal agent of stem canker and root rot of *Platanus* × *acerifolia* in a nursery in Rome (Pilotti et al. 2016). The same oomycete species was also recovered from plane trees affected by root rot (Frezzi 1977) or by resinous canker (Erwin and Ribeiro 1996) in Argentina and trunk rot in Australia (Greenhalgh and Challen 1979). Anthracnose caused by *Apiognomonia veneta* (Sacc. & Speg.) Höhn. (Ivanová et al. 2007), powdery mildew caused by *Erysiphe platani* (Howe) U. Braun & S. Takam. (de Oliveira et al. 2015; Heluta et al. 2013; Liang et al. 2008; Zhu and Pei 2017), wood decay caused by Agaricomycetes including *Ganoderma* spp. (Anselmi et al. 1994; Luchi et al. 2011; Moriondo and Santini 2002; Pilotti 2002; Pilotti et al. 2005), perennial canker, in which *Fusarium solani* (Mart.) Sacc. plays an important role (Pilotti 2002), dieback caused by fungi in the Botryosphaeriaceae (Pelleteret et al. 2017; Yu et al. 2018), and canker and dieback caused by *Diaporthe scabra* Nitschke (Grasso et al. 2012) are other examples of important diseases causing losses to *Platanus* spp. plantations in various countries.

During work on fungal diseases in Diyarbakır in May 2016, canker stain-like symptoms (canker and root rot) of unknown etiology were observed on roadside *P. orientalis* trees, similar to previous reports from the United States and Europe. *P. orientalis* was affected in increasing numbers with this severe disease, which eventually killed about 30% of the trees in the survey area in 2018. Isolations from necrotic tissues of roots and basal stem and branch cankers consistently yielded a *Phytophthora*/*Pythium*-like oomycete species exhibiting morphological features resembling those reported for *Phytopythium*

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species. The aim of this study, therefore, was to identify and characterize these isolates from plane trees using a polyphasic approach, comprising morphological and cultural observations, and sequencing of three loci located in nuclear DNA and mitochondrial DNA. In addition, pathogenicity tests were performed to fulfill Koch's postulates.

Materials and Methods

Observation of symptoms. Symptoms were observed on *Platanus* trees lining Mahabad Boulevard in the center of the Talaytepe neighborhood, in the center of Diyarbakır province, Turkey (37°55′20.9″N to 37°55′20.0″N, 40°08′58.4″E). All 5- to 7-year-old plane trees throughout this boulevard (500 trees) were inspected visually, and the appearance and progression of disease symptoms in and on trees were recorded. Soil surrounding root collars of the trees was removed to expose roots and lateral roots. Disease incidence was assessed by counting the number of symptomatic, asymptomatic, and dead trees.

Sampling and isolation protocols. Samples were collected from symptomatic tissues including twigs, branches, sapwood tissues at the stem base, and roots of affected trees. Basal stem and root tissues were washed in tap water and rinsed in sterile distilled water before cutting into 0.5- to 1-cm^2 pieces. These segments and symptomatic twig and branch segments of the same size were surface sterilized in 70% ethanol, blotted dry on sterile filter papers, transferred to potato dextrose agar (PDA; Merck, Darmstadt, Germany) or to grated apple corn meal agar (GACMA) (Türkölmez et al. 2015) amended with 5 mg of pimaricin, 250 mg of ampicillin, 10 mg of rifampicin, 100 mg of pentachloronitrobenzene, and 50 mg of hymexazol per liter (P_5ARPH) in 9-cm-diameter Petri dishes (four pieces per dish), and incubated at 26°C in the dark. Hyphal tips of resultant colonies with morphologies resembling *Phytophthora/Pythium* spp. were subcultured to fresh PDA, GACMA, and V8 agar (V8A) to obtain pure single-hyphal cultures and to determine the colony morphology. Five-millimeter plugs of 7-day-old mycelium grown on GACMA were transferred to autoclaved grass blades placed on V8A medium and incubated at room temperature for a few days until mycelial growth was observed around the grass blades. Colonized blades were transferred to fresh Petri dishes, and sterile distilled water was added (van der Plaats-Niterink 1981). Grass blade water cultures were incubated at 20°C for 5 to 10 days in the dark. Morphological features of the oomycete structures (sporangia, hyphal bodies, oogonia, antheridia, and oospores) on each medium were examined and recorded for five isolates (designated Dyrbkr01 to Dyrbkr05), performing at least 20 measurements for each isolate at 400× magnification under an Eclipse E200 light microscope (Nikon, Tokyo, Japan). All isolates were deposited in the culture collection of the Plant Pathology Laboratory at the GAP Agricultural Research Institute, Şanlıurfa, Turkey.

DNA extraction and PCR. The pure mycelial cultures of isolates Dyrbkr01 to Dyrbkr05 were incubated at 20°C for 7 to 10 days on V8A medium for DNA extraction. A small amount of mycelium was transferred to an Eppendorf tube, and DNA was extracted using the DNeasy Plant Mini kit (Qiagen, Germany). The concentration and quality of resultant DNA were estimated using a DS-11 FX+ spectrophotometer (DeNovix, Wilmington, DE) and diluted to 10 ng/μl with sterile ultra-pure water. The 50-μl reaction mixture for DNA amplification contained at final concentrations of 0.4 μM each primer, 1× DreamTaq PCR Master Mix (Thermo Fisher Scientific, Waltham, MA), and 1 to 2 μl of template DNA. Primer pairs of ITS6 and ITS4 were used to amplify the internal transcribed spacer (ITS) of rDNA (Cooke et al. 2000). The primer pairs NL1 and NL4 were used to amplify the large subunit (LSU) gene of rDNA (O'Donnell 1993), and FM58 and FM66 primer pairs were employed to produce the cytochrome oxidase II (*cox*II) gene of mitochondrial DNA (Martin and Tooley 2003). The amplification conditions were 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min for ITS and LSU loci and 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min for the *cox*II locus, with an initial denaturation step at 94°C for 3 min and a final extension step at 72°C for 10 min.

Sequence-based identification of isolates. After purification, amplicons were directly sequenced in both directions using the same primers in an automated sequencer using the Sanger method by a commercial company (Medsantek, Istanbul, Turkey). Sequences were aligned with Clustal W (Thompson et al. 1994), using the following parameters: pairwise alignment parameters (gap opening = 10, gap extension = 0.1) and multiple alignment parameters (gap opening = 10, gap extension = 0.2, transition weight = 0.5, delay divergent sequences = 30%) and edited manually using MEGA7 (Kumar et al. 2016) for high accuracy. The consensus sequences of each isolate were subjected to BLAST analysis (BLASTn) for species identification. Phylogenetic and molecular evolutionary analyses of sequence data of five isolates from this study and those of 28 *Phytophthora* spp. isolates used in Baten et al. (2014) and Baten et al. (2015) (Table 1) were performed using MEGA7 (Kumar et al. 2016) for maximum-likelihood (ML) analyses and PAUP version 4.0b10 (Swofford 2002) for maximum-parsimony (MP) analyses. MP analyses were conducted using the heuristic search option with 1,000 random taxa addition and tree bisection and reconnection as the branch-swapping algorithm. All characters were unordered and of equal weight, and gaps were treated as missing data. The robustness of the most parsimonious trees was evaluated from 1,000 bootstrap replications. The consistency index (CI), retention index (RI), and homoplasy index (HI) were also measured. ML analyses were performed on an MP starting tree automatically generated by the software. The evolutionary history was inferred by using the ML method based on the general time reversible model (Nei and Kumar 2000). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying the MP method. A discrete gamma distribution was used to model evolutionary rate differences among sites (six categories [+G, parameter = 0.2978]). Nearest neighbor interchange was used as the heuristic method for tree inference, and bootstrap resampling analysis for 1,000 replicates was conducted to estimate the confidence of tree topologies (Felsenstein 1985).

Pathogenicity tests. One representative isolate (Dyrbkr01) was tested for pathogenicity on 1- to 2-year-old excised twigs (approximately 1 cm diameter × 25 cm long) with foliage taken from healthy mature *P. orientalis* trees. The pathogenicity test was also made on 1-year-old potted *P. orientalis* seedlings. Following wiping with cotton wool soaked in 70% ethanol, 5-mm-diameter bark disks excised from shoots (one per shoot) and primary roots of each plant (two per plant) were replaced with 5-mm-diameter mycelial disks from the advancing margin of colonies grown on GACMA (mycelial side toward the vascular cambium). Controls were mock inoculated with sterile GACMA disks using the same procedure. Fifteen replicates of shoots and plants were used for each treatment and five for controls. Wounds on the excised shoots were immediately covered again using the excised bark tissue, wrapped in Parafilm, and finally covered with aluminum foil. One end of the inoculated twigs was covered with wax. The wax-free ends of the inoculated and control shoots were dipped individually in 100 to 150 ml of sterile distilled water in 250-ml Erlenmeyer flasks to reduce dehydration and incubated for 2 weeks at room temperature (21 to 27°C) under natural light on the bench. Wounds on the primary roots were not wrapped with Parafilm. Roots were covered with soil, and soil was kept saturated by daily watering. Seedlings were incubated for 1 month in a greenhouse, in which air temperatures ranged from 24 to 30°C from July to August. Individual twigs and plants were evaluated visually for the disease after 2 weeks. The outer bark was removed and the lesion lengths around inoculation points recorded for the inoculated twigs. Inoculated seedlings were scored twice at 2 weeks and 1 month after inoculation on a scale from 0 to 2, where 0 = no visible symptoms, 1 = wilt, and 2 = plant death.

Symptomatic tissues were excised from the margin of the canker and root rot lesions and plated onto P_5ARPH-GACMA medium to reisolate the pathogen in order to confirm Koch's postulates. The experiments were conducted twice at different times with a 3-month pause.

Results

Field observations and characteristics of the disease. During the 3 years of study, an increase in disease incidence and severity was noted in the surveys on plane trees on Mahabad Boulevard. Disease incidence increased from 20.6% (103 symptomatic trees) in 2016 to 31.2% (156 symptomatic trees) in 2017. In the first year, 58 (11.6%) trees died, whereas total mortality reached 98 (19.6%) in the second year of the survey. Dead trees were eventually removed from the boulevard. In 2018, approximately 40% of the trees exhibited characteristic symptoms of the disease, and almost 30% of 500 trees died in the boulevard.

Symptoms of leaf chlorosis and defoliation associated with branch and stem canker, root and basal stem rot, loss of canopy cover, and sudden collapse of the whole plant were observed on these plants (Fig. 1). Rapid and complete crown defoliation occurred within a few weeks of initial symptoms. The leaves of infected trees in the upper crown were chlorotic in appearance, undersized, and curled at the edges (Fig. 2a). Subsequently, the leaves turned brown and abscised. On the bark, the most characteristic symptom of the disease was the extensive bluish or blackish staining of shoots and branches (Fig. 2b), which also occurred on parts of the trunk that were free from external cankers (Fig. 3a). Cankers were apparent on young trees that had a relatively smooth bark, appearing as deep cracks on stems with no wound callus formation at the margins starting from the stem base and extending upward (Fig. 1). The stem regions and main branches distant from canker lesions, especially branch collars, were affected by extensive exfoliation of the rhytidome. On some old trees, if cankers were not severe, they could not be distinguished because of the thick and roughened outer bark of the trunks. The outer bark of these plants was cracked, and bluish or blackish staining or color changes were observed on the wound surface (Fig. 3a) or bark tissues (Fig. 3b). Collar and root rot symptoms were observed at the base of symptomatic trees. Under the bark and in wood tissue, reddish brown-black rot or necrosis occurred from the taproot and some large roots to the collar of the trunk (Fig. 3b to d), with severe reductions in lateral and fine roots (Fig. 3d). Cross-sections of the roots that were not completely rotten showed that primary xylem was forming a necrotic star-shaped pattern pointing toward the endodermis (Fig. 3e). Necrosis was also observed in the cortex along with the endodermis layer. During the 3-year observation period, severe basal wounds or cankers spread longitudinally along the main stem or lateral branches. Twigs and branches at the top of the tree died first. Eventually, larger branches and stems also died.

Isolation and morphological characterization of isolates. A total of 55 *Phytopythium*-like isolates were obtained from the fine and coarse roots (Fig. 4a) and stained branch parts and shoots (Fig. 4b) of 20 symptomatic plane trees. The oomycete had a conspicuous papilla (or with an outgrowth) and internally proliferating (nested and extended) zoosporangia. Zoospores were discharged in a vesicle at the tip; protoplasm moved through the tube to the vesicle and differentiated into zoospores. Cultures on GACMA and V8A had a radiate to petaloid or chrysanthemum-like pattern with sparse aerial mycelium toward the center of the colonies, submerged at the margins (Fig. 4a and b). On PDA, all isolates produced colonies with more aerial mycelium than on the GACMA and V8A, which had a distinct chrysanthemum pattern. The main hyphae were up to 6 μm wide. All isolates produced hyaline, subglobose, broadly ovoid or obpyriform sporangia in grass blade water cultures. Sporangia were 23.2 to 31.9 × 21.5 to 28.0 μm with a length/width ratio of 1.01 to 1.13. Encysted zoospores were 7.9 to 9.8 μm in diameter. Globose hyphal

Table 1. Accession numbers of the sequences of the internal transcribed spacer (ITS) and large ribosomal subunit (LSU) rDNA regions and mitochondrial cytochrome c oxidase subunit II (*cox*II) loci

Species	Isolates	GenBank accession number			References
		ITS	LSU	*cox*II	
Phytopythium litorale	Dyrbkr01	MN203107	MN197634	MN206728	This study
P. litorale	Dyrbkr02	MN203108	MN197635	MN206729	This study
P. litorale	Dyrbkr03	MN203109	MN197636	MN206730	This study
P. litorale	Dyrbkr04	MN203110	MN197637	MN206731	This study
P. litorale	Dyrbkr05	MN203111	MN197638	MN206732	This study
P. litorale	NBRC107451	AB690612	AB690583	AB690664	Baten et al. (2015)
P. litorale	GUCC1132	AB920536	AB920508	AB920501	Baten et al. (2014)
P. litorale	GUCC1072	AB920537	AB920507	AB920502	Baten et al. (2014)
P. aichiense	CBS137195	AB948197	AB948194	AB948192	Baten et al. (2015)
P. citrinum	CBS119171	AY197328	AB690597	AB690679	Baten et al. (2015)
P. delawarense	382B	AB725875	AB690591	AB690672	Baten et al. (2015)
P. carbonicum	CBS112544	AB725876	AB996605	AB690678	Baten et al. (2015)
P. montanum	CBS111349	AB725883	AB690586	AB690667	Baten et al. (2015)
P. boreale	CBS551.88	AB725879	AB690596	AB690677	Baten et al. (2014)
P. megacarpum	CBS112351	AB725881	AB690584	AB690665	Baten et al. (2014)
P. mercuriale	CBS122443	AB725882	AB690585	AB690666	Baten et al. (2015)
P. mercuriale	SZ14S6	AB920532	AB920509	AB920503	Baten et al. (2014)
P. iriomotense	GUCC0025	AB690622	AB690600	AB690682	Baten et al. (2015)
P. iriomotense	CBS137104	AB690629	AB690607	AB690689	Baten et al. (2015)
P. oedochilum	CBS252.70	AB690618	AB690592	AB690673	Baten et al. (2015)
P. oedochilum	CBS292.37	AB690619	AB690595	AB690676	Baten et al. (2015)
P. fagopyri	FP1	AB690621	AB690599	AB690681	Baten et al. (2014)
P. fagopyri	HonMa	AB690615	AB690588	AB690669	Baten et al. (2014)
P. fagopyri	CBS293.35	AB690617	AB690590	AB690671	Baten et al. (2014)
P. chamaehyphon	NBRC107441	AB948198	AB690603	AB690685	Baten et al. (2014)
P. chamaehyphon	NBRC107394	AB948199	AB690580	AB690661	Baten et al. (2014)
P. chamaehyphon	CBS259.30	AB690609	AB690593	AB690674	Baten et al. (2014)
P. helicoides	CBS286.31	AB725878	AB690594	AB690675	Baten et al. (2014)
P. helicoides	H5	AB690611	AB690582	AB690663	Baten et al. (2014)
P. helicoides	Roph3	AB690616	AB690589	AB690670	Baten et al. (2014)
P. cucurbitacearum	CBS748.96	AB725877	AB690598	AB690680	Baten et al. (2014)
P. vexans	NBRC107393	AB690630	AB690608	AB690690	Baten et al. (2015)
P. vexans	NBRC107442	AB690626	AB690604	AB690686	Baten et al. (2015)

swellings were spherical, abundant in GACMA, and approximately 20.0 to 35.6 μm in diameter (Fig. 4c). Moreover, all isolates produced large, ornamented oogonia, thick-walled plerotic oospores (Fig. 4d), and one to two elongate or lobate, paragynous, and diclinous antheridia that were laterally applied to the oogonium in single GACMA and V8A cultures after 1 month of incubation. Oogonia were 25 to 32 μm (average 29.1 μm) in diameter. Production of oospores was observed for the first time in this species during this

Fig. 1. Severe decline symptoms at an advanced stage of the disease: canker on trunk, bluish black discoloration of branches (white arrows), intense exfoliation on branch collar rhytidome, and defoliated crown due to *Phytopythium litorale* infection on a plane tree (*Platanus orientalis*) on the front side and a tree still retaining its browned leaves on the back side.

Fig. 2. a, Leaves of infected trees were initially chlorotic in appearance, undersized, and curled at the edges due to *Phytopythium litorale* on a plane tree. **b,** Close-up of bluish black discolored areas visible on plane branches (white arrows).

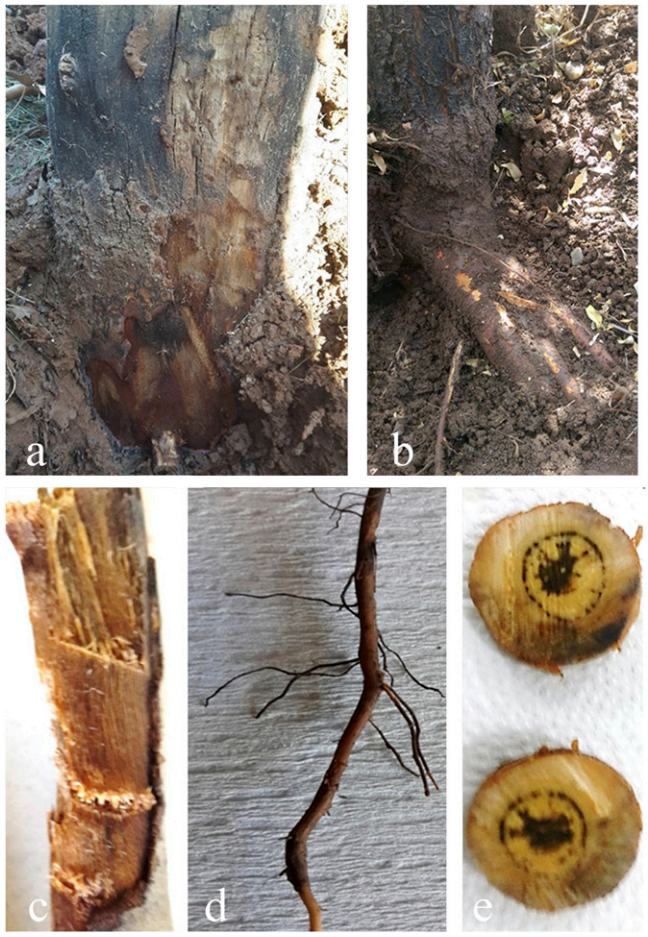

Fig. 3. a, Outer bark of a *Platanus orientalis* tree was cracked and eventually shed as the trunk and branches increased in diameter; bluish black discoloration and cracks on the surface of the corresponding wood and collar rot at the base. **b,** Bluish black discoloration on the surface of the thick and rough bark of a trunk and reddish brown discoloration on the surface of main roots. **c,** A dead, rotten root following *Phytopythium litorale* infection. **d,** Lesions and rots visible on the surface of roots with severe reductions in lateral and fine roots. **e,** Characteristic wood discoloration in cross-sections of root tissues that were not completely rotten.

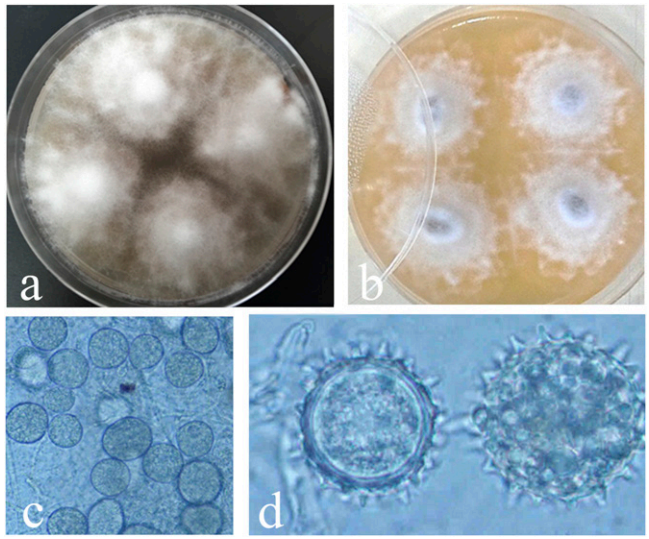

Fig. 4. a, *Phytopythium litorale* colonies from branch segments after 4 days of incubation. **b,** Colonies isolated from under bark tissues of basal stems. **c,** Terminal globose hyphal swellings of *P. litorale*. **d,** Ornamented surface of *P. litorale* oogonia with conical projections and large plerotic oospores with granulated cytoplasm.

investigation. These results established that homothallic (self-fertile) mating occurs in *P. litorale*.

Sequence-based identification of isolates. Sequences of the five isolates were deposited in GenBank, with the accession numbers MN203107 to MN203111 (ITS), MN197634 to MN197638 (LSU), and MN206728 to MN206732 (*cox*II), respectively. The accession numbers of sequences of 28 reference *Phytopythium* spp. isolates from the GenBank database are shown in Table 1. To produce the phylogenetic tree, we used a total of 99 sequences from 33 isolates. In the combined dataset of ITS, LSU, and *cox*II, the aligned data matrix of 33 isolates consisted of a total of 2,332 characters, of which 1,550 characters were constant (66.47%), 83 variable characters were parsimony-uninformative, and 699 characters were parsimony-informative for MP analyses. The MP analysis produced 925 equally parsimonious trees with a minimum possible length of 1,156 and a maximum possible length of 5,668 (consistency index (CI) = 0.715, retention index (RI) = 0.898, rescaled consistency index (RC) = 0.642, and homoplasy index (HI) = 0.285). The topology of the tree generated by ML analysis (Fig. 5) was similar to that of the MP tree. Isolates obtained in this study were clustered into same clade with reference *P. litorale* isolates (Fig. 5) and were differentiated from other *Phytopythium* species (*P. aichiense*, *P. boreale*, *P. carbonicum*, *P. chamaehyphon*, *P. citrinum*, *P. delawarense*, *P. fagopyri*, *P. helicoides*, *P. iriomotense*, *P. megacarpum*, *P. mercuriale*, *P. montanum*, and *P. oedochilum*).

Pathogenicity tests. *P. litorale* isolate Dyrbkr01 induced cankers, blight of leaves, petioles, and branches, and led to peeling of bark and darkened wood on plane shoots within 5 days of inoculation (Fig. 6). Two weeks after inoculation, *P. litorale* produced lesions averaging 7.5 (5.7 to 9.2) cm long and killed the seedlings, whereas no lesions occurred on control shoots. Similarly, with potted oriental plane seedlings, no disease occurred in the controls. However, the tested isolate of *P. litorale* caused dieback symptoms with blight on all foliar parts, dark brown to black root rot, extensive necrotic lesions around the taproot, and a reduction of the root system within 1 month of inoculation. The peeling of bark and darkening or discoloration of exposed wood were also observed on the surface of branches. The mean of disease severity for each isolate ranged from 1 to 2 with an average of 1.2 at 2 weeks after inoculation, increasing to 2.0 at 1 month after inoculation, when all seedlings had died. Koch's postulates were confirmed by reisolating the oomycete from inoculated shoots and roots. The reisolated culture was morphologically and molecularly confirmed as *P. litorale*. In repeated experiments, the symptoms observed were similar to those of the first experiments and those seen in field infections.

Discussion

In this research, a severe decline of oriental plane roadside trees was found in the Diyarbakır province. Based on our finding, an oomycete species, *P. litorale*, was consistently isolated from symptomatic tissues and considered responsible for occurrence of the

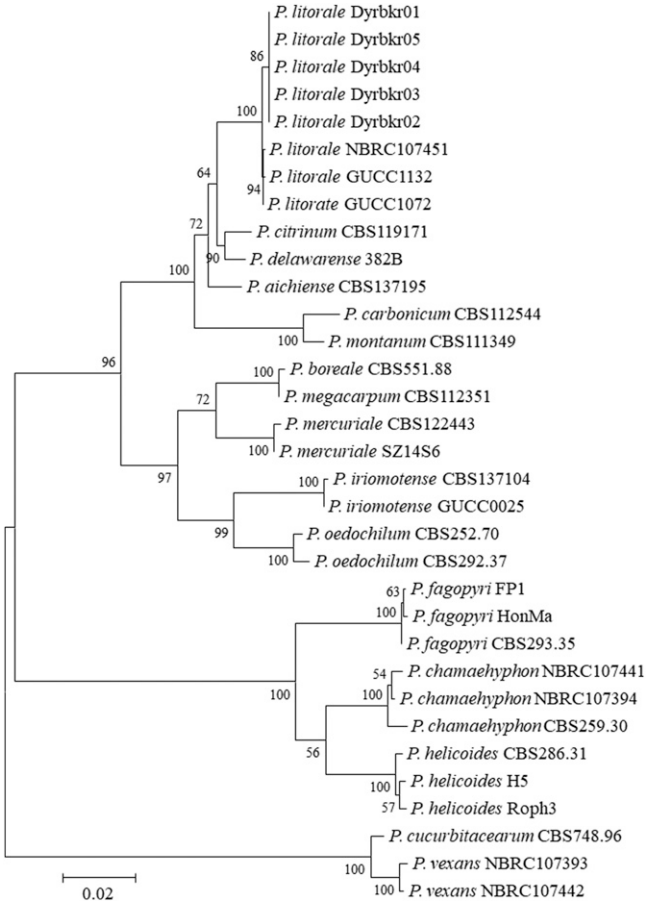

Fig. 5. Maximum likelihood tree of *Phytopythium* spp. isolates based on combined sequences of the internal transcribed spacer and large ribosomal subunit rDNA regions and mitochondrial cytochrome c oxidase subunit II loci. Numbers on the branches represent bootstrap values obtained from 1,000 bootstrap replications.

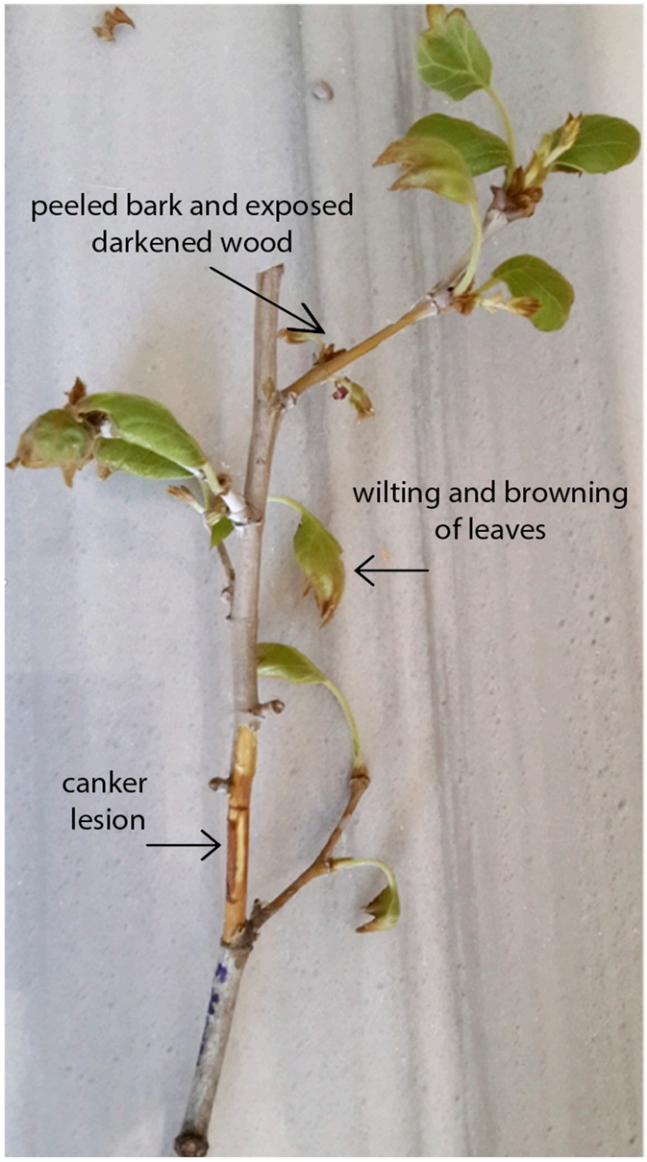

Fig. 6. Effect of inoculation on the young shoot: canker, progressive wilting and browning of leaves, and peeled bark and exposed darkened wood on branches of plane (*Platanus orientalis*) shoots 5 days after stem inoculation with *Phytopythium litorale*.

disease. Our pathogenicity tests revealed that *P. litorale* was a quite virulent species on *P. orientalis* plants. Under the conditions used for the pathogenicity tests, the disease symptoms developed rapidly on the excised shoots, resulting in the occurrence of large lesions and blights after 5 days. Furthermore, the symptoms on seedlings were also similar to those observed in the field, which confirmed the status of *P. litorale* as the pathogen of the plane species; all seedlings were dead in 1 month. During the course of the 3-year survey study throughout this boulevard, symptom severity and the disease incidence recorded on the basis of visual examination increased gradually. Interestingly, no other fungal or oomycete pathogenic species were found other than *P. litorale* in and on all studied trees. The isolation of symptomatic tissues in and on all sampled trees always yielded positive results for the pathogen's presence. This finding indicated that *P. litorale* was the cause of the disease throughout the surveyed boulevard. This study is also the first report worldwide concerning the occurrence of decline, root and collar rot, and stem and branch cankers of plane trees caused by *P. litorale*. From both pathogenicity and survey results, it can be concluded that the disease has an increasing trend, is able to kill many trees, and has the possibility to spread to other trees or plantations.

In this study, the species identification of pathogenic oomycete isolates obtained from plane trees as *P. litorale* (Nechw.) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque (Peronosporaceae, Peronosporales, Oomycota, Straminipila) was similar to descriptions cited by Nechwatal and Mendgen (2006), Parkunan and Ji (2013), and de Cock et al. (2015). Our morphological observations also confirmed the characteristics defining *Phytopythium* as a separate genus, with zoospores of *Phytopythium* species formed in a vesicle and sporangia with a papilla (Bala et al. 2010; de Cock et al. 2015; Gisi et al. 1979; Lévesque and de Cock 2004). *Phytopythium* has also internally proliferating sporangia and cylindrical or lobate antheridia (de Cock et al. 2015). *P. litorale* is placed in molecular clade 1 of *Phytopythium*, which shares several morphological characteristics with other species from that clade, such as the presence of papillate and internally proliferating sporangia and the presence of homothallic oospores and lobate antheridia (Baten et al. 2014; Jesus et al. 2016). However, in contrast to other species of *Phytopythium*, oogonia and oospores of *P. litorale* were ornamented, a trait not described for any other known species in the genus. In addition, none of the other 15 species (*P. aichiense*, *P. boreale*, *P. carbonicum*, *P. citrinum*, *P. delawarense*, *P. iriomotense*, *P. leanoi*, *P. mercuriale*, *P. megacarpum*, *P. mirpurense*, *P. montanum*, *P. oedochilum*, *P. ostracodes*, *P. sindhum*, and *P. sterilum*) in clade 1 of this genus (Baten et al. 2014; Bennett et al. 2017; Jesus et al. 2016) produce hyphal swellings in cultures. *P. litorale*, however, produced abundant hyphal swellings in cultures, as in the present work. This investigation is the first to show that this species produced oospores, under the conditions applied after prolonged incubation. The formation of oospores accounts for the ability of the pathogen to persist for long periods in soil without a living host. Molecular features also allowed the identification of *P. litorale*, coinciding with morphological features. The LSU, ITS, and *cox*II sequences of *P. litorale* isolates obtained from plane trees in this study showed high homology with those of *P. litorale* isolates in GenBank, clustering with them separately from other *Phytopythium* species, which is consistent with the results of Baten et al. (2014, 2015).

P. litorale was originally described from rhizosphere soil of reed stands (*Phragmites australis*) in Germany but was not a pathogen on reeds (Nechwatal and Mendgen 2006). The species was characterized by subglobose, papillate, and internally proliferating sporangia, globose hyphal swellings, the absence of oogonia in single cultures, and a high optimum growth temperature. It has been recorded several times in soil of pea (Alcala et al. 2016), soybean (Coffua et al. 2016; Radmer et al. 2017), and ash trees (Kranjec et al. 2017). *P. litorale* has also been reported in pond water used in vegetable irrigation (Parkunan and Ji 2013) in Georgia and Pennsylvania (Choudhary et al. 2016), in Tennessee streams (Hulvey et al. 2010; Shrestha et al. 2013), in waterways across Western Australia (Hüberli et al. 2013), and associated with root of English walnut (Guajardo et al. 2019). *P. litorale* isolated from irrigation water was pathogenic to squash in Georgia (Parkunan and Ji 2013) and was also pathogenic to soybean in Minnesota (Radmer et al. 2017). Therefore, this is the third host record of *P. litorale* as a pathogen (Parkunan and Ji 2013; Radmer et al. 2017).

Root and/or stem rot diseases caused by several other soil- and water-borne *Phytopythium* spp., including *P. cucurbitacearum*, *P. delawarense*, *P. chamaehyphon*, *P. helicoides*, *P. mercuriale*, and *P. vexans*, have been confirmed previously. For example, *P. helicoides* was found to be pathogenic to begonia in Virginia (Yang et al. 2013), to kiwifruit (Wang et al. 2015), citrus mandarin (Chen et al. 2016), and lotus (Yin et al. 2016) in China, and to pistachio rootstocks in California (Fichtner et al. 2016). *P. delawarense* was described and first recovered from diseased soybean seedlings in Ohio (Broders et al. 2009). Several species of *Phytopythium* such as *P. chamaehyphon*, *P. helicoides*, *P. litorale*, *P. mercuriale*, and *P. vexans* were pathogenic to soybeans in the United States and Ontario, Canada (Rojas et al. 2017). *P. vexans*, an important agricultural pathogen that causes damping-off and root rot of many economically important plants, exhibited pathogenicity on dry bean (Rossman et al. 2017), *Thuja occidentalis* and *Picea orientalis* seedlings (Lehtijärvi et al. 2017), and avocado (Rodríguez-Padrón et al. 2018), and it caused root and collar rot of kiwifruit in Turkey (Polat et al. 2017), patch canker on rubber trees (Zeng et al. 2005), stem rot on *Dendrobium* (Tao et al. 2011), and brown root rot on ramie plants (*Boehmeria nivea*) (Yu et al. 2016) in China.

P. litorale is causing a rapid decline, blights, cankers, root and collar rots, and mortality on *P. orientalis* trees in Turkey. This report is the first to describe *P. litorale* as a destructive canker stain agent on plane in its native habitat, holding potential for a devastating effect on *P. orientalis* health in specific geographical areas (i.e., the Mediterranean basin) or globally and posing new and urgent research challenges.

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