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Fishing for Estuarine Oomycetes

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Abstract: Oomycetes are water molds that are frequently isolated during a survey of waterways. Biodiversity of oomycetes in the estuary region of the Veleka River in Bulgaria was investigated in 2021. A total number of 32 isolates were derived using the baiting method. Species identification based on sequencing of the ITS region divided them into four different genera: *Phytophthora* (*P.*), *Phytopythium* (*Pp.*), *Pythium* (*Py.*) and *Elongisporangium* (*E.*). The most abundant species in the studied region was *P. lacustris* (sixteen isolates), followed by *P. honggalleglyana* (nine isolates). *P. bilorbang* and *P. inundata* were represented by only one isolate each and were recognized for the first time in Bulgaria. The genus *Phytopythium* was presented by two isolates that belong to different species, *Pp. litorale* and *Pp. citrinum*. In the obtained collection, the genera *Pythium* and *Elongisporangium* were represented by only one species each, *Py. angustatum* (one isolate) and *E. anandrum* (two isolates), respectively. Colony morphology of the eight collected oomycete species was characterized by cultivation of selected isolates on three different media. Potential host species of the isolated estuarine oomycetes were estimated by pathogenicity tests conducted with sixteen plants from ten diverse families. *P. lacustris* and *P. honggalleglyana* demonstrated a higher aggressiveness among *Phytophthora* isolates, whereas *P. bilorbang* and *P. inundata* showed less ability to infect the tested plant species. Similar pathogenicity and a potential host range for both *Phytopythium* species were observed. Less aggressive against analyzed plants in this study were *Py. angustatum* and *E. anandrum*.

Keywords: aquatic microorganisms; *Phytophthora*; *Pythium*; *Phytopythium*; *Elongisporangium*; baiting; ITS; diversity; pathogenicity

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1. Introduction

A number of oomycetes from the genera *Phytophthora*, *Pythium*, *Phytopythium* and *Elongisporangium* are important pathogens on agricultural crops and native plants, affecting both crop growing and natural ecosystems [1–3]. On the other hand, some oomycete species appear to be saprophytes in aquatic environments [4]. Oomycetes are water molds and are frequently isolated during a survey of waterways. Many of them are associated with wet soil conditions, and their spread is often favored by seasonal floods [5–8]. Numerous novel species and hybrids derived from riparian ecosystems in various areas of the world have been reported in recent years [8–11]. Among the most abundantly distributed in water habitats have been found to be *Phytophthora* species from clades 6 and 9 [12–14]. Some members of these groups are strongly associated with host plants from forests and riparian ecosystems [7,13–16]. However, knowledge on the diversity of oomycetes in estuarine and coastal environments is fragmentary. Out of about 2000 species of oomycetes reported, the highest diversity has been observed in terrestrial plant pathogens, and while some species have been isolated from water sources, only about 60 have been derived from marine environments [17,18]. Some of these species are associated with diseases on seaside and vegetated coastal ecosystems [19–21].

The Veleka is a river in the southeastern part of Bulgaria that flows into the Black Sea. The mouth of the river is a unique area with specific features because of its geographic characteristics. The estuary forms a partially enclosed coastal zone (sandbar) causing the formation of a transition area between a river and a marine environment. The fresh water mixes with the sea water at the lower and middle estuary of the river. Occasionally, the

mouth is blocked by the sand, which is especially pronounced during rough seas, and leads to flooding of the surrounding area. The region is characterized by transitional Mediterranean climatic conditions with a strong influence by the sea. The vegetation in the area is diverse, as there are forest and woody species like alder, bamboo, hornbeam, elm, ash and others along the banks. Dense emergent and sparse vegetation, as well as grassy meadow species, are widespread in the water and coastal wetland.

The isolation of oomycetes from diseased plants assists in their identification, as some pathogen species are strongly associated with specific host plants. However, when the species are derived from water sources, their pathogenicity and host range are unclear. A large amount of data is collected for some oomycetes, but for the species that are rarely isolated or have been identified recently, further investigations are needed. Frequently a limited number of susceptible plant species are known, and the importance of these oomycetes for the wide variety of potential hosts requires additional studies.

An investigation of oomycete diversity in the estuary region of the Veleka River is presented in this study. A total number of 32 isolates belonging to four different genera, *Phytophthora*, *Phytopythium*, *Pythium* and *Elongisporangium*, were collected and identified. Their ability to infect 16 different plants from 10 diverse families was evaluated. The most common oomycetes in the studied area and the most aggressive species among them were noted.

2. Materials and Methods

2.1. Sampling Area

The study covers the estuary region of the Veleka River (Figure 1).

Figure 1. Sampling area of the Veleka River: (**A**) map of the region and the studied sites; (**B**) photo of the river.

Different sites for investigation of oomycete diversity were selected:

- Next to a sandbar between the river and the sea—samples with numbers RVel2021/115 and RVel2021/120 (GPS coordinates: 42.066845, 27.971472). The river bottom in the region is sandy and the main vegetation is common reed (*Phragmites australis*).
- In the liman area of the river—samples with numbers RVel2021/114 and RVel2021/118 (GPS coordinates: 42.066310, 27.971529) and RVel2021/119 (GPS coordinates: 42.064896, 27.970419). In addition to the reed, there are grassy meadow species and dense riparian vegetation.
- In the forest area of the river—samples with numbers RVel2021/112 (GPS coordinates: 42.060125, 27.966534), RVel2021/113 and RVel2021/116 (GPS coordinates: 42.061555, 27.967234) and RVel2021/117 (GPS coordinates: 42.063026, 27.968246). There is forestry vegetation that includes alder, hornbeam, elm, ash, lianas and creeping plants.

At the time of collecting samples 119 and 120, the mouth of the river was blocked by the sand, and the water did not flow into the sea and spread over a large area of the surrounding region. This allowed zoospores of species that probably are not typical of the river, but rather of the wetlands around it, to be attracted by the baits and isolated.

2.2. Baiting and Isolation of Oomycetes

Baiting of oomycetes was performed in May, July and September of 2021. Zoospores of oomycetes were attracted to detached leaves of *Rhododendron*, oak and oleander that were placed into meshy baits in the river for a period of 3 days. Only young and healthy leaves were selected for the experiment. After collection of the baits, they were saved in a cooler and transferred to the laboratory.

The leaves from the baits were surface-sterilized, and pieces of developed necrosis with a part of healthy zone were cut out. They were cultivated on selective PARNHB media (carrot agar supplemented with 10 mg Pimaricin, 250 mg Ampicillin, 10 mg Rifampicin, 50 mg Nystatin, 50 mg Hymexazol and 15 mg Benomyl/L) at 20 °C for 5 days. A mycelium plug from each colony with different morphological type was transferred for cultivation on WA (water agar) for a period of 3 days. A hyphal tip from purified colonies was transferred onto a fresh CA medium (carrot agar; 16 g agar, 3 g $CaCO_3$, 100 mL carrot juice/L), and cultivation at 20 °C was performed.

2.3. Species Identification

DNA was isolated from mycelia of 10-day-old cultures using DNeasy Plant mini Kit (QIAGEN GmbH, Venlo, The Netherlands). PCR amplification of the ITS region was performed with primers ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The following PCR program was applied: 96 °C for 2 min, followed by 35 cycles of 96 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and final elongation at 72 °C for 10 min (Ristaino et al. 1998). The PCR was performed by PuReTaq™ Ready-To-Go™ PCR beads (GE Healthcare Life Sciences, Chicago, IL, USA), according to the manufacturer's instructions. The resulting PCR products were sequenced at GATC Biotech AG (Constance, Germany). Species identification of all isolates included in the study was performed by searching for the highest-homology ITS sequences within the NCBI database using BLAST.

2.4. Colony Morphology

One isolate from each species collected from the Veleka River was selected for characterization of the colony morphology, as follows: *P. lacustris* (RVel2021/113d), *P. bilorbang* (RVel2021/119a), *P. inundata* (RVel2021/119b), *P. honggalleglyana* (RVel2021/120c), *Pp. citrinum* (RVel2021/113a), *Pp. litorale* (RVel2021/113b), *Py. angustatum* (RVel2021/114c) and *E. anandrum* (RVel2021/115d). They were cultivated on three different media: CA, V8A (vegetable agar: 16 g agar, 3 g $CaCO_3$, 100 mL Campbell's V8 juice/L) and PDA (potato dextrose agar, Difco®) at 20 °C for a period of 10 days for *Phytophthora* isolates and 5 days for *Phytopythium*, *Pythium* and *Elongisporangium* isolates.

2.5. Testing for Potential Host Plants

Since all collected isolates were derived from the river, not from diseased plants, testing for potential hosts was performed. The isolates that were selected for characterization of the colony morphology were also used for these experiments. Plant species that are dominant in the ecosystem of the Veleka River or species that are among most common in the country were chosen for pathogenicity tests. A total number of sixteen different species from ten diverse families (Fabaceae, Fagaceae, Salicaceae, Rosaceae, Araliaceae, Vitaceae, Ranunculaceae, Asteraceae, Plantaginaceae and Poaceae) were selected. These are representatives of trees, including the Judas tree (*Cercis siliquastrum*), common oak (*Quercus robur*), European aspen (*Populus tremula*) and willow (*Salix babylonica*); bushes, such as dog rose (*Rosa canina*) and blackberry (*Rubus fruticosus*); as well as some perennial

and herbaceous plants like common ivy (*Hedera helix*), five-leaved ivy (*Parthenocissus quinquefolia*), old man's beard (*Clematis vitalba*), alfalfa (*Medicago sativa*), clover (*Trifolium repens*), common daisy (*Bellis perennis*), common yarrow (*Achillea millefolium*), ribwort plantain (*Plantago lanceolata*), broadleaf plantain (*Plantago major*) and green foxtail (*Setaria viridis*). The pathogenicity tests were performed with leaves and cuttings from the selected potential hosts. They were conducted twice with all tested isolated and plant species.

Detached leaves from healthy plants were selected, and the baiting technique was applied. The leaves were placed into plastic trays containing a mix of sterile distilled water and non-sterile spring water (1:1). Mycelial plugs (10 × 10 mm) of 7-day-old culture from each tested isolate were added into test variants, whereas control trays were prepared with the same water mixture, but without mycelial sample. All leaves were incubated at 22/20 °C (16 h day/8 h night) for a period of 5 to 14 days depending on the development of disease symptoms. Daily monitoring for appearance and expansion of necrotic lesions on tested leaves was performed.

Along with the pathogenicity tests with leaves, young cuttings (6–7 cm) of the selected plant species were arranged on wet paper in plastic trays. Mycelial plugs (3 × 3 mm) of 7-day-old culture from each tested oomycete isolate were placed on the middle part of the cuttings after slight surface injury. Plastic trays were covered with transparent film to maintain a high humidity and were incubated at 22/20 °C (16 h day/8 h night) for 2 weeks. A daily monitoring of the inoculated cuttings for appearance of disease symptoms was performed.

The severity of disease symptoms was evaluated according to the following scale: no symptoms—healthy leaf/cutting (-); small dot necroses—low susceptibility (+); necrotic area about 50% of the leaf/cutting—middle level of sensitivity (++); necrosis covers 90–100% of the leaf/cutting—totally affected (+++).

3. Results

3.1. Diversity of Oomycetes

A total number of 32 isolates were collected from the estuary region of the Veleka River (Table 1). The species identification of the isolates was determined by sequencing of the ITS region and is based on 99–100% homology with corresponding species in the NCBI database. The isolated oomycetes belong to four different genera: *Phytophthora, Phytopythium*, *Pythium* and *Elongisporangium*, represented by 27, two, one and two isolates, respectively.

The genus *Phytophthora* was represented by four species: *P. lacustris*, *P. honggalleglyana*, *P. bilorbang* and *P. inundata*. The most abundant species of oomycetes in the study was *P. lacustris* (sixteen isolates), followed by *P. honggalleglyana* (nine isolates), corresponding to 50% and 28% of the collected isolates, respectively. The two rarest species from the genus *Phytophthora*, *P. bilorbang* and *P. inundata*, which are represented by only one isolate each (3% of the isolates), were recognized for the first time in Bulgaria. The genus *Phytopythium* was represented by only two isolates that belong to different species: *Pp. litorale* and *Pp. citrinum* (Table 1). The first of them has been found in another river in the northern part of the country previously [22], whereas the second species has not been reported in Bulgaria up to now. The genera *Pythium* and *Elongisporangium* were represented by one species each, *Py. angustatum* (one isolate) and *E. anandrum* (two isolates), respectively (Table 1).

Table 1. List of *Phytophthora*, *Phytopythium*, *Pythium* and *Elongisporangium* isolates included in the study.

No. of Isolate	Species	GenBank No.	Type of Bait	Location	Baiting Period
RVel2021/112a	*P. lacustris*	PQ107011	rhododendron	42.060125, 27.966534	2 May 2021 (start) 5 May 2021 (end)
RVel2021/112c	*P. lacustris*	PQ107013	rhododendron		
RVel2021/112d	*P. lacustris*	PQ107014	oak		
RVel2021/112f	*P. lacustris*	PQ107016	oleander		
RVel2021/113a	*Pp. citrinum*	PQ110268	oak	42.061555, 27.967234	
RVel2021/113b	*Pp. litorale*	PQ107594	oak		
RVel2021/113c	*E. anandrum*	PQ107604	oak		
RVel2021/113d	*P. lacustris*	PQ107017	oleander		
RVel2021/113e	*P. lacustris*	PQ107019	oleander		
RVel2021/114a	*P. lacustris*	PQ107021	rhododendron	42.066310, 27.971529	
RVel2021/114b1	*P. lacustris*	PQ107022	rhododendron		
RVel2021/114b2	*P. lacustris*	PQ107024	rhododendron		
RVel2021/114c	*Py. angustatum*	PQ107654	oak		
RVel2021/114d	*P. lacustris*	PQ107026	oak		
RVel2021/114e	*P. lacustris*	PQ107025	oleander		
RVel2021/114f	*P. lacustris*	PQ107479	oleander		
RVel2021/115a	*P. lacustris*	PQ107487	rhododendron	42.066845, 27.971472	
RVel2021/115b	*P. lacustris*	PQ107488	rhododendron		
RVel2021/115c	*P. lacustris*	PQ107490	oak		
RVel2021/115d	*E. anandrum*	PQ107632	oak		
RVel2021/115e	*P. lacustris*	PQ107492	oleander		
RVel2021/116a	*P. honggalleglyana*	PQ107495	rhododendron	42.061555, 27.967234	16 July 2021 (start) 18 July 2021 (end)
RVel2021/116b	*P. honggalleglyana*	PQ107496	rhododendron		
RVel2021/117a	*P. honggalleglyana*	PQ107498	rhododendron	42.063026, 27.968246	
RVel2021/117b	*P. honggalleglyana*	PQ107497	rhododendron		
RVel2021/118a	*P. honggalleglyana*	PQ107499	rhododendron	42.066310, 27.971529	
RVel2021/118b	*P. honggalleglyana*	PQ107501	rhododendron		
RVel2021/119a	*P. bilorbang*	PQ107517	rhododendron	42.064896, 27.970419	9 September 2021 (start) 11 September 2021 (end)
RVel2021/119b	*P. inundata*	PQ107518	rhododendron		
RVel2021/120a	*P. honggalleglyana*	PQ107500	rhododendron	42.066845, 27.971472	
RVel2021/120b	*P. honggalleglyana*	PQ107504	rhododendron		
RVel2021/120c	*P. honggalleglyana*	PQ107514	rhododendron		

Incubation of the selected isolates from the eight oomycete species on three different media (CA, V8A and PDA) demonstrated diversity in the pattern of colony morphology, growth rate and preferences to nutritional compounds of some species. All *Phytophthora* isolates showed relatively fast growth on CA and V8A, whereas significant radial development on PDA was observed only for *P. inundata*, isolate RVel2021/119b (Figure 2). A slight increase in growth of *P. lacustris* (RVel2021/113d) and *P. honggalleglyana* (RVel2021/120c) colonies on potato dextrose agar was recorded, but *P. bilorbang*, isolate RVel2021/119a, was strongly restricted on this medium. Mycelial growth of *P. lacustris*, isolate RVel2021/113d, was characterized by chrysanthemum pattern and limited aerial mycelium on media CA and V8A (Figure 2A1–A3). *P. bilorbang*, isolate RVel2021/119a, formed submerged colonies, petaloid on CA and with a stellate morphology pattern on V8A (Figure 2B1–B3). *P. inundata*, isolate RVel2021/119b, showed petaloid colony morphology on CA and V8A, and a chrysanthemum pattern on PDA with aerial mycelium on all three media (Figure 2C1–C3). *P. honggalleglyana*, isolate RVel2021/120c, demonstrated a petaloid pattern of colony with aerial growth on all tested media, especially pronounced on CA (Figure 2D1–D3).

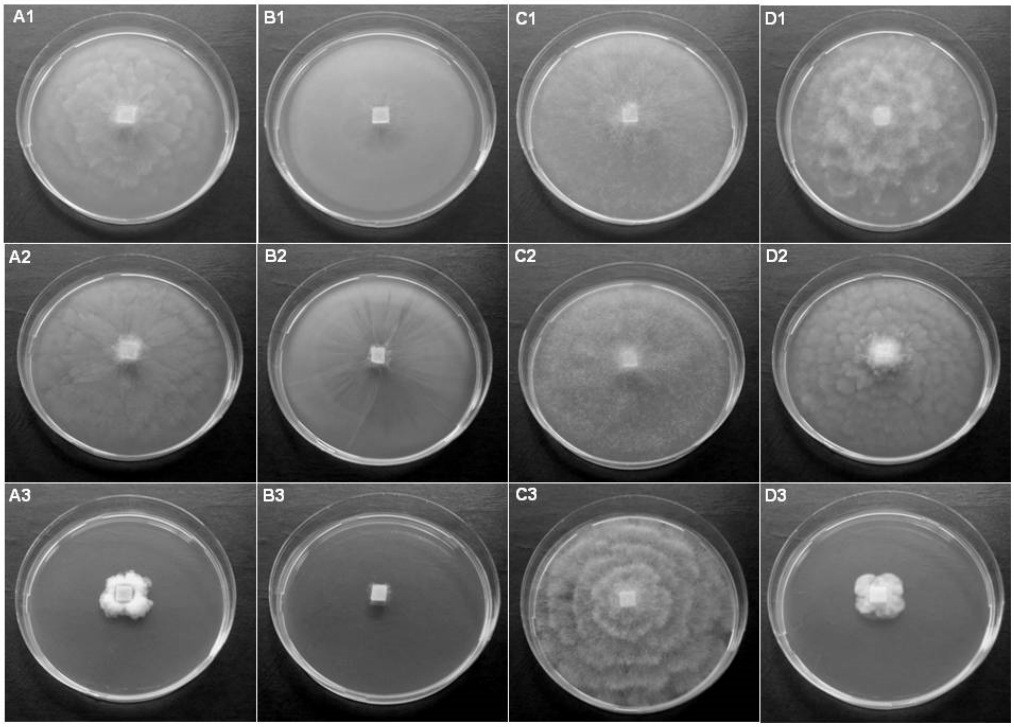

Figure 2. Colony morphology of *Phytophthora* isolates on CA (first row), V8A (second row) and PDA (third row) after 10 days cultivation at 20 °C. (**A1**–**A3**) *P. lacustris*, isolate RVel2021/113d, (**B1**–**B3**) *P. bilorbang*, isolate RVel2021/119a, (**C1**–**C3**) *P. inundata*, isolate RVel2021/119b, and (**D1**–**D3**) *P. honggalleglyana*, isolate RVel2021/120c.

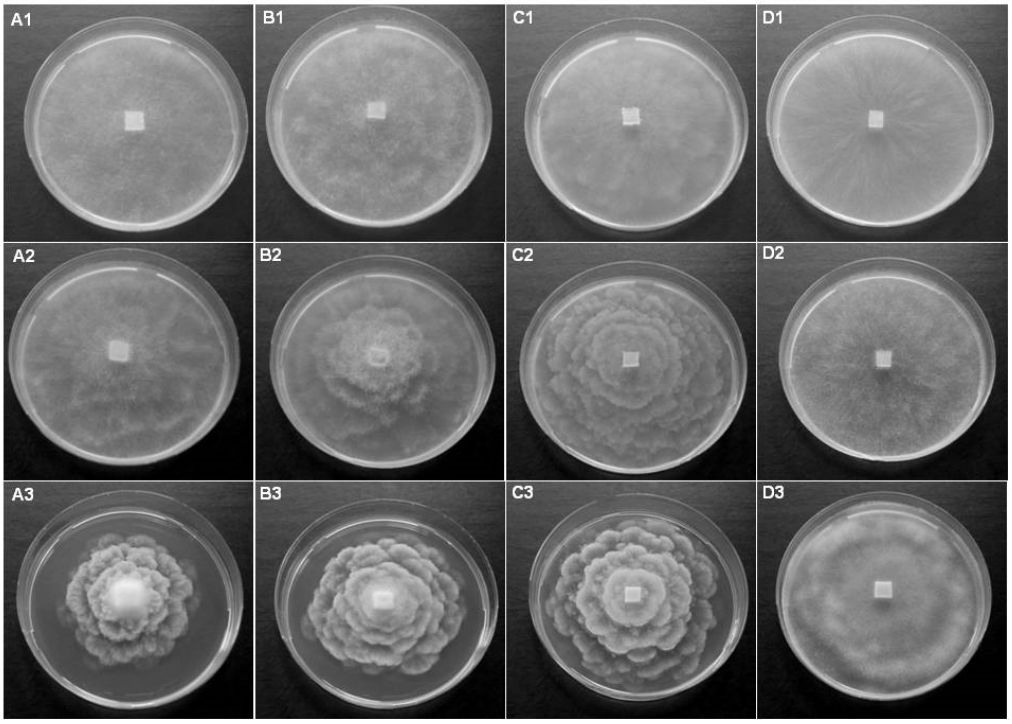

Figure 3. Colony morphology of *Phytopythium*, *Pythium* and *Elongisporangium* isolates on CA (first row), V8A (second row) and PDA (third row) after 5 days cultivation at 20 °C. (**A1**–**A3**) *Pp. citrinum*, isolate RVel2021/113a, (**B1**–**B3**) *Pp. litorale*, isolate RVel2021/113b, (**C1**–**C3**) *Py. angustatum*, isolate RVel2021/114c, and (**D1**–**D3**) *E. anandrum*, isolate RVel2021/115d.

An extremely rapid mycelial growth on all tested media of the studied isolates from the genera *Phytopythium*, *Pythium* and *Elongisporangium* was observed (Figure 3). Typical for all isolates was formation of aerial mycelium on all three media used. Similar morphology for both *Phytopythium* species was monitored. *Pp. citrinum*, isolate RVel2021/113a, and *Pp. litorale*, isolate RVel2021/113b, formed petaloid to rosaceous colonies on CA and V8A, and a rosette pattern of mycelial growth on PDA (Figure 3A1–A3,B1–B3). *Py. angustatum*, isolate RVel2021/114c, also showed petaloid to rosaceous colonies (Figure 3C1–C3). *E. anandrum*, isolate RVel2021/115d, demonstrated stellate mycelial growth on CA and V8A, and a rosette pattern on PDA with profuse aerial mycelium (Figure 3D1–D3).

3.2. Investigation of Potential Host Plants

In order to determine potential host species of isolates, a number of pathogenicity tests with detached leaves and cuttings from various plants were conducted (Table 2).

Table 2. Pathogenicity of the tested isolates *Phytophthora, Phytopythium*, *Pythium* and *Elongisporangium* on selected plant species.

Plant Species	***Phytophthora***								***Phytopythium***				***Pythium***		***Elongisporangium***	
	113d/*PL*		**119a/*PB***		**119b/*PI***		**120c/*PH***		**113a/*PpC***		**113b/*PpL***		**114c/*PyAg***		**115d/*EAn***	
Leaves/Cuttings	L	C	L	C	L	C	L	C	L	C	L	C	L	C	L	C
C. siliquastrum	+++	+++	+	++	-	+	+++	+++	+	+++	++	+++	-	+	-	+
Q. robur	+++	+++	+	+	+	+	+++	+++	++	+++	+++	++	+	+	+	+
P. tremula	+++	+++	++	++	-	-	+++	+++	+++	+++	+++	+++	-	-	+	+
S. babylonica	+++	-	-	+	-	-	+	-	+	-	-	+	-	-	-	-
R. canina	+++	+++	-	-	-	+	+++	+++	+	++	++	+++	-	-	+	-
R. fruticosus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H. helix	-	-	-	-	-	-	++	-	-	-	-	-	-	-	++	-
P. quinquefolia	+++	+	+	-	++	-	++	++	+++	++	+++	+	+	-	-	-
C. vitalba	++	+	-	-	+	-	++	-	++	-	++	-	-	-	-	-
M. sativa	-	-	-	-	-	-	+++	++	++	-	++	-	+	-	+	-
T. repens	+	na	+	na	++	na	+++	na	-	na	++	na	-	na	++	na
P. lanceolata	-	na	++	na	+++	na	+	na	++	na	-	na	++	na	-	na
P. major	-	na	-	na	-	na	+	na	-	na	-	na	-	na	-	na
B. perennis	++	na	++	na	+	na	++	na	-	na	++	na	++	na	-	na
A. millefolium	++	na	+	na	++	na	+	na	++	na	++	na	++	na	-	na
S. viridis	++	na	-	na	+	na	-	na	++	na	+	na	-	na	+	na

PL—P. lacustris; *PB—P. bilorbang*; *PI—P. inundata*; *PH—P. honggalleglyana*; *PpC—Pp. citrinum*; *PpL—Pp. litorale*; *PyAg—Py. angustatum*; *EAn—E. anandrum*. Disease symptoms severity scale: - no symptoms; + small dot necroses; ++ necrotic area about 50% of the leaf/cutting; +++ necrosis covers 90–100% of the leaf/cutting; na—not applicable. L—leaves; C—cuttings.

P. lacustris (RVel2021/113d) and *P. honggalleglyana* (RVel2021/120c) demonstrated higher aggressiveness among *Phytophthora* isolates, especially to the tree species, such as *C. siliquastrum*, *Q. robur* and *P. tremula*, as well as to some other plants such as *R. canina* and *P. quinquefolia.* Significant sensitivity of herbaceous plants *M. sativa* and *T. repens* to *P. honggalleglyana* was also observed. Moderate pathogenicity by *P. lacustris* against *S. babylonica*, *C. vitalba, B. perennis*, *A. millefolium* and *S. viridis* was detected. A similar effect to *H. helix*, *P. quinquefolia*, *C. vitalba* and *B. perennis* after inoculation with *P. honggalleglyana* was also observed. In contrast to both dominant *Phytophthora* species, *P. bilorbang* (RVel2021/119a) and *P. inundata* (RVel2021/119b) showed less ability to infect the tested plant species. More sensitive to *P. bilorbang* were various species including *P. tremula*, *P. lanceolata* and *B. perennis*, whereas *P. inundata* affected mainly the herbaceous plants *P. quinquefolia*, *T. repens*, *P. lanceolata* and *A. millefolium.* A total inability of the studied *Phytophthora* species to infect *R. fruticosus* was established. Relatively resistant to infection were also *H. helix* and *P. major*.

Both *Phytopythium* isolates (RVel2021/113a and RVel2021/113b) demonstrated comparable pathogenicity and potential host species. Similar to *Phytophthora* isolates, higher

aggressiveness against *C. siliquastrum*, *Q. robur*, *P. tremula, P. quinquefolia* and *R. canina* was monitored. Moderate ability to infect *C. vitalba, M. sativa* and *A. millefolium* by *Pp. citrinum* (RVel2021/113a) and *Pp. litorale* (RVel2021/113b) was observed. Herbaceous plants *R. fruticosus, H. helix* and *P. major* were resistant to the tested *Phytopythium* species. These results showed similar potential host plants for the pathogens from the genus *Phytophthora* and the genus *Phytopythium* among tested tree species, bushes and herbaceous plants.

Py. angustatum (RVel2021/114c) showed increased aggressiveness against *Q. robur* and the herbaceous plants *P. lanceolata*, *B. perennis* and *A. millefolium*. The species failed to infect most of the tested plants (nine out of 16). Similarly, eight plant species were not affected by *E. anandrum* (RVel2021/115d). The studied isolates demonstrated pathogenicity against *Q. robur*, *P. tremula*, *H. helix* and *T. repens*.

4. Discussion

A number of investigations on the diversity of the genus *Phytophthora* in Bulgaria were published in the last few years and some species, such as *P. pseudosyringae*, *P. gallica* and *P. polonica*, have been reported for the first time in the country [23–25], as well as a new hybrid species *P.* × *sansomeana* [11]. In contrast, information on the isolation and identification of the representatives from the genera *Phytopythium*, *Pythium* and *Elongisporangium* is limited [22,26–28]. Fishing for estuarine oomycetes in the firth region of the Veleka River resulted in accumulation of new data for species diversity in the country. Eight different species including *P. lacustris*, *P. bilorbang, P. inundata*, *P. honggalleglyana, Pp. litorale, Pp. citrinum, Py. angustatum* and *E. anandrum* were detected based on sequencing of the ITS region. Additional information for the collected oomycetes by characterization of colony morphology confirmed the species identification according to compliance with the main features described in the corresponding databases (IDphy, 1PPYTG, 1PYTHG).

In the present study, *P. lacustris* and *P. honggalleglyana* were determined as the most distributed species from the genus *Phytophthora* in the firth of the Veleka River. In contrast, two other members of the genus, *P. bilorbang* and *P. inundata*, were represented by only one isolate each and were identified for the first time in Bulgaria. The results also showed that clade 6 (*P. lacustris, P. bilorbang* and *P. inundata*) dominates in terms of species diversity and in number of *Phytophthora* isolates obtained (18 out of 27; 67%), whereas clade 9 is represented by nine isolates (33%) that belong to only one species (*P. honggalleglyana*). No species from the remaining nine *Phytophthora* clades were isolated at the time of this survey. These results confirm the statement that the *Phytophthora* species from clades 6 and 9 are among the most abundantly distributed in water ecosystems [12–14].

The species *P. lacustris* is widespread in Europe and has been reported as a weak to moderate pathogen to Alnus, Prunus and Salix [29]. According to a large-scale investigation of the potential hosts, the species was not able to infect a number of crop plants such as pumpkin (*Cucurbita* spp.), onion (*Allium cepa*), chili pepper (*Capsicum annuum*), alfalfa (*M. sativa*), corn (*Zea mays*), oats (*Avena sativa*) and barley (*Hordeum vulgare*) [15]. The results of our tests confirmed the moderate aggressiveness of *P. lacustris* to *S. babylonica*, as well as its pathogenicity to other forest species and a number of perennial and herbaceous plants. In addition, the resistance of *M. sativa* to the species reported previously [15] was also proved in this study.

The oomycete *P. honggalleglyana* is associated mainly with water ecosystems such as rivers, streams and irrigation sources, and has been reported in several European countries, including Austria, Spain and Italy [7,16,30,31]. The species has been isolated for the first time as a pathogen on ornamental plants [32]. Lately, *P. honggalleglyana* has been associated with alder decline across Europe, including Spain [16], Italy [31] and more recently in Bulgaria [25]. The current study presented a variety of potential host plants, including trees and perennial and herbaceous species. It is notable that all *P. honggalleglyana* isolates were obtained in the summer season, when the water temperature is the highest, while in the spring, this species was not detected in any of the collected samples. This is most likely due to the fact that species from clade 9, to which *P. honggalleglyana* belongs, tolerate

high temperatures and can grow even at 35–40 °C, in addition to their ability to develop quickly [32].

The species *P. bilorbang* has been recently designated as an exotic pathogen for European countries [33]. It has been isolated in Italy from the rhizosphere soil of *Platanus orientalis* by baiting [33] and has been also determined as a pathogen on trees from the Oleaceae family, including *Olea europaea* and *Phyllirea latifolia* [34,35]. Although *P. bilorbang* has been represented for the first time as a causal agent on declining *Rubus anglocandicans* in Australia [36], it appears that most hosts reported to date are woody plants. The results of the conducted pathogenicity tests showed that the isolated in Bulgaria *P. bilorbang* (RVel2021/119a) could affect tree species like *P. tremula* and *C. siliquastrum*, as well as herbaceous perennial plants such as *P. lanceolata* and *B. perennis*. The lack of sufficient information indicates that the identification of the natural hosts of this pathogen is an issue requiring further investigations.

The species *P. inundata* has been isolated from various plants and water environments in different countries in Europe (UK, France, Denmark, Spain, Italy, Turkey), as well as in some other continents [37–41]. Typical habitats of *P. inundata* are riparian ecosystems and vegetation areas after flooding. Similarly, in the present investigation, the species was derived after a large-scale spill of water in the area around the estuary of the Veleka River. *P. inundata* has been associated with diseases of woody plants and shrubs, including genera Aesculus, Olea, Salix, Prunus and Vitis [37]. It has been reported as a causal agent of olive tree decline, and root and collar rot of pomegranate in orchards in Turkey [40,41]. A former investigation of different potential hosts of *P. inundata* demonstrated that the species is capable of infecting roots of *Prunus amygdalus* and *Beta vulgaris* [39]. Additional pathogenicity tests conducted by the same authors with detached twigs of different tree species indicated several dormant hosts including *C. siliquastrum*, *Juglans regia*, *Magnolia grandiflora*, *Prunus domestica*, *Prunus domestica* and *R. canina*. The results of the present study showed that the isolated in Bulgaria *P. inundata* (RVel2021/119b) induced necrosis on cuttings of *C. siliquastrum* and *R. canina*, which confirms the potential of the species to infect these plants.

The species from the genus *Phytopythium* are mainly described as saprophytic organisms linked to water and soil habitats. However, some members of the group have been reported as dangerous plant pathogens. *Pp. litorale* has been derived from various types of water ecosystems such as rivers and streams in Austria, Slovakia and the Czech Republic [7] and mountain streams in Vietnam [8], as well as irrigation water tanks in the USA [42]. It has also been identified as a pathogenic species on diverse plant hosts, such as soya bean, almond and oriental plane trees *Platanus orientalis* [43–45]. Similar to previous reports, *Pp. litorale* (RVel2021/113b) derived from the Veleka River demonstrated aggressiveness to different forest species and perennial and herbaceous plants. Most sensitive were *C. siliquastrum*, *Q. robur*, *P. tremula*, *P. quinquefolia* and *R. canina*, which are therefore determined as potential host plants for the pathogen. Similar to *Pp. litorale*, *Pp. citrinum* has been isolated from various sources. The species has been identified in streams in the Czech Republic [7], nurseries in Sweden [46] and oak stands in Poland [47,48]. As presented in this study, *Pp. citrinum* (RVel2021/113a) showed moderate to high potential to infect a number of selected plants and was determined as a pathogen with a wide host range. The collected data suggest that both *Phytopythium* species are a potential threat for forest ecosystems, gardens and parks.

In this survey, less aggressive against the tested plants were *Py. angustatum* (RVel2021/114c) and *E. anandrum* (RVel2021/115d). *Py. angustatum* is associated with aquatic ecosystems and has been isolated from green algae, freshwater wetland soils invaded by European common reed (*P. australis*), a river in Ukraine and water courses in Poland [49–52]. Occurrence of *E. anandrum* in Europe is most often associated with oak stands, and it has been reported in Germany, Sweden, Austria, Turkey and Poland [48,53–57]. Although *E. anandrum* (RVel2021/115d) was isolated from the Veleka River by baiting with oak leaves, a low infestation of *Q. robur* was monitored. A moderate aggressiveness was established only

toward herbaceous plants *H. helix* and *T. repens*. In addition, *Py. angustatum* (RVel2021/114c) was weakly pathogenic to *Q. robur*, as well as most of the other tested plants. However, a moderate pathogenicity against *P. lanceolata*, *B. perennis* and *A. millefolium* was detected. These results indicate that *Py. angustatum* and *E. anandrum* are weak pathogens with various potential hosts.

5. Conclusions

Fishing for estuarine oomycetes in the firth region of the Veleka River in Bulgaria resulted in the isolation and identification of eight different species, including *P. lacustris*, *P. bilorbang*, *P. inundata*, *P. honggalleglyana*, *Pp. litorale*, *Pp. citrinum*, *Py. angustatum* and *E. anandrum*. A small collection of oomycetes from the genera *Phytophthora*, *Phytopythium*, *Pythium* and *Elongisporangium* derived from this specific habitat was established. Although a number of *Phytophthora* species have been reported for the first time in the country during the last few years, two other members of the genus (*P. bilorbang* and *P. inundata*) were identified, indicating that surveying various environments leads to the accumulation of new knowledge about oomycete diversity in Bulgaria.

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