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Occurrence and diversity of *Phytophthora* species in declining broadleaf forests in western Ukraine

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Abstract

In western Ukraine, forest decline and dieback of several broadleaved tree species have become increasingly evident during recent years, and surveys in some areas have shown symptoms indicative of *Phytophthora* infections. In this study, we aimed to determine the occurrence and diversity of *Phytophthora* species associated with several broadleaved tree species (*Alnus glutinosa*, *Betula pendula*, *Castanea sativa*, *Fagus sylvatica* and *Quercus robur*) from forest stands where dieback has been observed. Rhizosphere soil samples were collected from 14 forest stands during 2017 and 2018 and tested for the presence of *Phytophthora* species using morphological and molecular methods. Seven *Phytophthora* species (*P. bilorbang*, *P. cactorum*, *P. gallica*, *P. gonapodyides*, *P. lacustris*, *P. plurivora and P. polonica*), and two other clade six taxa were detected from the various forest types, several of which are probable agents responsible for decline. Four of the *Phytophthora* species (*P. bilorbang*, *P. gallica*, *P. plurivora and P. polonica*) have previously never been reported from broadleaf forests in Ukraine.

KEYWORDS

Alnus glutinosa, baiting and isolation, *Betula pendula*, *Castanea sativa*, forest decline, *Quercus robur*

1 | INTRODUCTION

Across the globe, *Phytophthora* pathogens cause widespread devastation affecting numerous plant species in both agricultural and natural or seminatural ecosystems (Erwin & Ribeiro, 1996). In recent years, forest decline and dieback phenomena in forests have been reported to be correlated with damage caused by *Phytophthora* species (Classification: Straminipila, oomycetes) (Jung et al., 2018). For example, in European forests, several *Phytophthora* species are associated with the decline of different broadleaved trees such as alder (*Alnus* spp.) (Brasier et al., 1995; Černý & Strnadová, 2010; Jung & Blaschke, 2004), oak (*Quercus* spp.) (Balci & Halmschlager, 2003a; Jung et al., 2000, 2013) and European beech (*Fagus sylvatica* L.) (Jung, 2009; Jung et al., 2005; Jung et al., 2013).

Forests in Ukraine have enormous economic and environmental importance with more than 9.6 million ha of forested land constituting approximately 16% of the total land area (Tkach, 2012). Forest decline has been increasingly observed in Ukraine in recent years. Between 2009 and 2018, the area of declining forests increased 2.3 times from 176,000 ha to 413,000 ha (Bondar, 2019). Of that total forest areas, approximately 222,000 ha are mainly Scots pine (*Pinus sylvestris* L.) forests, 27 000 ha are Norway spruce (*Picea abies* [L.] H. Karst) forests, 100,000 ha are pedunculate oak (*Quercus robur* Sol.). and 64,000 ha are associated with other broadleaf tree species (Bondar, 2019).

Silver birch (*Betula pendula* Roth.) is a major component of native woodlands throughout the Polisia region and plays an important role as a pioneer species, especially on post-agricultural lands

TABLE 1 Host species, location and site characteristics from which soil rhizosphere samples were collected in western Ukraine

Host species	Sampling date	No. of soil samples	Site and Location	Site coordinates	Stand species composition[a]	Forest age (years)	Stand origin	Noted forest health problems
Betula pendula	22.09.2017	13	Chortoryj Forestry, Volyn region	49°08′41,3′′N 23°51′08,9′′E	B(70) P(30)	50	Natural regeneration (B); plantation (P)	Up to 10% of area showed birch decline (low tree vitality, crown dieback, lesions with exudates on trunks).
Betula pendula	23.09.2017	3	Kukly Forestry, Volyn region	51°34′39,8′′N 23°54′42,3′′E	B(60) A(20) O(10) P(10)	40–50	Natural regeneration, coppice (B, A); plantation (O, P)	Up to 20% of area showed birch decline (low tree vitality, crown dieback, lesions with exudates on trunks).
Alnus glutinosa	23.09.2017	2	Kukly Forestry, Volyn region	49°46′00,4′′N 24°05′31,1′′E	A(100)	70	Natural regeneration, coppice (A)	Up to 30% of alder trees showed decline symptoms including bleeding lesions on the trunk; 10% of trees had died
Alnus glutinosa *Salix caprea*	20.10.2017	5 3	Nyvytsi Forestry, Lviv region	49°46′00,4′′N 24°05′31,1′′E	A(100) + B, W	50–60	Natural regeneration and coppice (A)	Up to 30% of alder trees showed decline symptoms (crown dieback), up to 15% of alder trees had died.
Betula pendula	07.06.2018	8	Sarny Forestry, Rivne region	42°30′85,0′′N 42°54′98,4′′E	B(80) A(20)	40–50	Natural regeneration and coppice (B, A)	Separate birch trees showed crown dieback with exudates on the trunk; up to 5% of trees were dead.
Betula pendula	07.06.2018	6	Strashiv Forestry, Rivne region	42°30′85,0′′N 42°54′98,4′′E	B(100) + A	40	Natural regeneration (B)	Individual trees exhibited crown dieback with bleeding lesions on the trunk.
Betula pendula *Alnus glutinosa*	08.06.2018	2 3	Klesiv Forestry, Rivne region	42°30′85,0′′N 42°54′98,4′′E	B(100) + A	60–70	Natural regeneration and coppice (B, A)	Up to 60% of trees showed crown dieback; 10% of trees were dead.
Quercus robur *Carpinus betulus*	18.06.2018	2 3	Beregujfalu Forestry, Transcarpathia region	42°30′85,0′′N 42°54′98,4′′E	O(50) H(50)	O 60–70 H 40–50	Plantation (O); natural regeneration (H)	Decline symptoms noted. Hornbeam were also damaged by jewel beetles *Agrilus* spp. and trunk rot.
Quercus robur	18.06.2018	2	Beregujfalu Forestry, Transcarpathia region	42°30′85,0′′N 42°54′98,4′′E	O(80) H(20)	40	Plantation (O), natural regeneration (H)	Up to 10% of oak trees showed symptoms of crown dieback with exudation on the trunks.
Quercus robur	19.06.2018	2	Borzhava Forestry, Transcarpathia region	42°30′85,0′′N 42°54′98,4′′E	O(80), Ash(20) + H	150–200	Natural regeneration	Most trees appeared healthy; only isolated individual trees showed some exudates at the base of trunks.
Castanea sativa	19.06.2018	3	Lavkivske Forestry, Transcarpathia region	42°30′85,0′′N 42°54′98,4′′E	C(100)	40–50	Plantation	Trees were also affected by chestnut blight *Cryphonectria parasitica* and partly damaged by a previous low-level fires.
Alnus glutinosa *Quercus robur*	10.06.2018	2 3	Komarno Forestry, Lviv region	42°9′96,1′′N 45°19′18,3′′E	O(70), A(20) H(10) + As	O 120 A 50–70	Natural regeneration,	Oak trees exhibited symptoms of crown dieback.

(Continues)

TABLE 1 (Continued)

Host species	Sampling date	No. of soil samples	Site and Location	Site coordinates	Stand species composition[a]	Forest age (years)	Stand origin	Noted forest health problems
Fagus sylvatica *Quercus robur*	14.06.2018	9 3	Vikno Forestry, Ternopil region	42°30'85,0''N 42°54'98,4''E	Be(80) O(20)	180	Natural regeneration	Individual trees showed symptoms of crown dieback. Up to 3% trees showed evidence of other secondary decay on trunk.
Quercus robur	15.06.2018	3	Sataniv Forestry, Khmelnytskyj region	42°30'85,0''N 42°54'98,4''E	O(70) H(30) + Be	70	Plantation (O), natural regeneration (H, B)	Individual trees showed high crown transparency, crown dieback, and exudates on trunks.
Total		77						

Abbreviations: A, *Alnus glutinosa*; As, *Populus tremula*; Ash, *Fraxinus excelsior*; B, *Betula pendula*; Be, *Fagus sylvatica*; C, *Castanea sativa*; H, *Carpinus betulus*; O, *Quercus robur*; W, *Salix caprea*.

[a]Percentage correspondences of the main tree species in stand species composition.

where Scots pine are also planted. Birch decline was noted starting in the early 1990s in Zhytomyr and Chernihiv regions (Polissya) and after 2006 in Rivne and Volyn regions (Goychuk et al., 2018). Earlier studies from Ukraine, Russia and Belarus often attributed necrotic lesions on the outer bark of symptomatic birch to bacterial wet wood (*Enterobacter nimipressuralis* Carter, synonym *Erwinia multivora* Sch. Parf.) due to the foul smell of exudates distinct from *Phytophthora* and wet patches on the trunk (Gvozdyak et al., 2011; Gvozdyak & Yakovleva, 1979; Koshelyaeva, 2016, 2017; Shvets, 2015, 2016). In addition, birch bark beetles and the tremex wasp (*Tremex fuscicornis* Fabr.) were noted as vectors of the bacteria (Koshelyaeva, 2016, 2017; Shvets, 2017; Skrylnik et al., 2019). Moreover, episodes of drough and high temperatures during the growing season can cause more pronounced dieback of birch, which, according to Goychuk et al. (2018), can contribute to the spread of the bacterial disease.

Common alder (*Alnus glutinosa* [L.] Gaertn.) play a key role in riparian habitats due in part to its ability to provide stability and protection for banks adjacent to aquatic ecosystems. In Ukraine, common alder is mostly found in the wetlands in Polisia along both sides of the Prypiat River, especially in its western reaches (Volyn and Rivne regions), where it occupies large expanses along river valleys. In these woodlands, alder grows alongside birch, goat willow (*Salix caprea* L.) and Scots pine. Since 2010, alder dieback has been increasingly observed (Ustskiy & Bugayev, 2014). According to Ustskiy et al. (2015), the observed alder decline may be attributable to changes in the hydrological regime, Armillaria root rot and climatic or other anthropogenic factors.

Since 1995, an oak decline phenomenon has been observed throughout Ukraine (Ustskiy & Meshkova, 1998). Earlier reports have noted biotic factors (insect defoliators, cambium feeders such as *Agrilus* spp., root and stem rot diseases), climatic factors and the impact of previous unsatisfactory forest management activities as main contributing factors for the observed oak decline in Ukraine and surrounding region (Sazonov & Zviagintsev, 2010; Selochnik, 2008; Ustskiy & Meshkova, 1998). Similarly, European beech decline has been observed in western Ukraine during the last 15–20 years and attributed to biotic agents like *Nectria* spp. and other stem and root rot pathogens, as well as abiotic factors (Shyshkanynets & Mazepa, 2013; Slobodjan, 2004, 2005). Plantations of sweet chestnuts (*Castanea sativa* Mill.) were established in the 1950s in the Transcarpathia region (Gerbut & Turis, 2007). During this time, it was already known about chestnut health problems in the Caucasus where chestnut blight caused by *Cryphonectria parasitica* (Murrill) M.E.Barr and ink disease caused by *Blepharospora cambivora* Petri and *Melanconis modonia* Tul. were considered the main causal agents of the decline (Shcherbin-Parfenenko, 1950). These days, *Phytophthora cinnamomi* Rands. is reported as the main causal agent of ink disease in chestnut forests in Caucasus (Gninenko et al., 2017). In Ukraine, reports of a significant deterioration of the health condition of sweet chestnut plantations in Transcarpathia came from employees of forestry enterprises where it was cited chestnut blight as the main factor causing chestnut decline.

Historically, *Phytophthora* has not been associated with the declining broadleaf tree species in Ukraine. However, in 2010, surveys conducted in the forest enterprises in three regions of western

Ukraine documented forest decline and other symptoms on beech, alder, birch and oak (e.g. dieback and increased crown transparency, and bleeding stem lesions) indicative of *Phytophthora* infections (Kramarets et al., 2011). The aim of this work was to determine the occurrence of *Phytophthora* species in declining broadleaf forests in western Ukraine.

2 | MATERIALS AND METHODS

2.1 | Field sites and soil sampling

Field sampling was carried out in 14 broadleaf forest stands located in six regions in the western part of Ukraine: Lviv, Ternopil, Volyn, Rivne, Chmelnytskyj and Transcarpathia (Table 1). Stands were selected on the basis of earlier reports where general tree decline or suspected *Phytophthora*-infected trees were noted, or both (Kramarets et al., 2011; personal communication with foresters in the respective regions) (Figure 1a and b). The species composition and density varied depending on the site, but target hosts included: silver birch, common black alder, goat willow, pedunculate oak, common hornbeam (*Carpinus betulus* L.), beech and sweet chestnut (*C. sativa*). Soil samples were collected in September and October 2017, and in June 2018. At each site, soil samples were collected from symptomatic trees (Figure 1c and e) by removing the organic litter layer from two opposite directions of the tree at a distance between 50 and 150 cm from the base of the tree and from a depth of 10–15 cm (Drenth & Sendall, 2001; Jung et al., 2016). Two soil samples (each weighing approximately 1–1.5 kg) were collected in a plastic bag from around each sampled tree and thoroughly mixed to yield a single soil sample. Approximately 400 g of the mixed soil sample was transferred to a separate bag and shipped to the laboratory at the Swedish University of Agricultural Sciences in Alnarp, Sweden for subsequent baiting and analysis. In total, 77 symptomatic trees were sampled, each yielding a soil sample. Of the 77 rhizosphere soil samples collected, 26 were from Volyn and Lviv regions and collected during the autumn from predominantly mixed birch and pine forests and from floodplain alder stands, and 51 were collected from Rivne, Transcarpathia, Lviv, Ternopil and Khmelnytskyj regions comprising a variety of mixed broadleaved forest types (Table 1).

2.2 | Baiting and isolation of *Phytophthora* spp.

In the laboratory, soil samples were flooded with 1.5 L of distilled water in 30 cm × 30 cm × 20 cm plastic containers so that the distance between the surface of the soil and the water line was 3–4 cm. After several hours, any litter and debris floating at the surface were carefully removed with paper tissue, and then the surface of the water was covered by fresh young leaflets as baits and incubated at room temperature (approximately 21°C). Different baiting hosts were used depending on the season and availability of hosts from the nearby arboretum or growing in the greenhouse. Fresh young leaflets of pedunculate oak, northern red oak (*Q. rubra* L.), beech, horse chestnut (*Aesculus hippocastanum* L.), *Rhododendron* spp. and Saucer magnolia (*Magnolia* × *soulangeana* Soul.-Bod.) were used. When characteristic necrotic spots or lesions appeared on baits after approximately 3–4 days, leaves were rinsed in deionized water, dried on tissue paper, cut aseptically in approximately 3 × 3 mm pieces and plated onto 9 cm Petri dishes with *Phytophthora* selective PAR(PH) V8 agar (consisting of 10 μg/L rifampicin, 1 ml/L dimethyl sulfoxide [DMSO], 250 μg/L sodium ampicillin, 5 μg/L pimaricin, 100 μg/L pentachloronitrobenzene [PCNB], 50 μg/L hymexazol, and 10 μg/L benomyl). Petri dishes were examined daily under an OLYMPUS BX45 microscope (magnification 15–20×) to look for developing *Phytophthora* hyphae. When growth was observed, small agar plugs of mycelium at the colony margin were transferred onto fresh V8 or carrot media (17 g/L) to obtain pure cultures. Isolates were grouped according to morphological traits, and then representative samples of each morphotype were selected to identify species through DNA sequencing of the ITS1-5-8S-ITS2 region.

2.3 | Molecular analyses

To prepare for DNA extraction, plugs of selected isolates grown on V8-media for 7 days were transferred to 50 ml falcon tubes, filled with 35 ml of liquid V8 media. After 5 days of incubation at room temperature, residues of V8-agar were removed and the mycelia were filtrated using filter paper with particle retention of 11 μm (Munktell filters AB, Falun, Sweden). Mycelia were transferred to 2 ml eppendorf tubes with four glass beads and lyophilized for 2–3 days. Freeze-dried samples were then homogenized to fine powder using a Rescht MM400 ball mill (Retsch, Haan, Germany). DNA extraction was done using the Omega E.Z.N.A. Bio-Tek Fungal DNA Mini Kit D3390-02 (Norcross, GA, USA), following the manufacturer's instructions. The concentration of the DNA extracts was measured using NanoDrop® ND1000 (Wilmington, USA). The region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) of the ribosomal DNA was amplified by PCR using the primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) and ITS6 (5′-GAAGGTGAAGTCGTAACAAGG-3′) (Cooke et al., 2000). PCR was carried out in 20 μl reaction volumes containing: 1 μl (5 ng/ul) DNA template, 1.6 μl ITS4 (0.4 μM) and 1.6 μl ITS6 (0.4 μM) primers, 2 μl dNTP mix (Solis BioDyne), 0.08 μl Taq DNA polymerase, 2 μl 10x PCR Buffer, 1.2 μl 25 mM $MgCl_2$ and 10.5 μl sterile water (Cooke et al., 2000; Pérez-Sierra et al., 2010). All PCR reactions were carried out in an Eppendorf Mastercycler DNA Engine Thermal Cycler PCR (Hayward) under the following conditions: initial denaturation for 3 min at 94°C, followed by 35 cycles of denaturation 1 min at 94°C, annealing for 1 min at 55°C and extension for 1 min at 72°C. A final elongation step was carried out for 10 min at 72°C. PCR products were visualized on 1.5% agarose (Saveen Werner, Sweden) together with 1 μl Gel Red (Biotium) per 10 ml of 1X TAE buffer under UV light (UVP, BioDoc-It Imaging system) with a mounted camera (Computar).

The amplified PCR products were purified and cleaned using ExoSAP-IT High-Throughput PCR Product Cleanup (Affymetrix), and

the concentration of PCR products was measured with a Qubit® 3.0 Fluorometer (Thermo Fisher Scientific) using the dsDNA HS Assay Kit (Life technologies, Carlsbad, USA) following the manufacturer's instructions. The final product was divided into two replicates for each isolate. The resulting PCR products were sequenced in both directions by automated Sanger sequencing at the National Genomics Infrastructure (NGI) at Science for Life Laboratory (Scilifelab, Solna). The obtained sequences were checked manually, aligned and edited using BioEdit sequence Alignment Editor software (version 7.0.5.3 for Windows) and compared with known reference sequences in GenBank (National Center for Biotechnology Information, NCBI) using the Basic Local Alignment Search Tool (BLAST). Putative taxonomic assignment was given to each sequence following pairwise alignment against the closest representatives and sequence similarity of 99% or above. The sequences obtained for all *Phytophthora* isolates were registered in GenBank (Accession no.'s MT420377–MT420411).

3 | RESULTS

A total of 1,577 baited necrotic pieces plated on media yielded 448 isolates that represented locations in four of the six regions (Volyn, Rivne, Lviv and Transcarpathia). Samples from Ternopil and Khmelnytskyj regions did not yield any *Phytophthora* isolates. Of these 448 isolates, 124 were selected for DNA sequencing. These isolates represented samples collected during both seasons in 2017 and 2018, different host species and locations, and the different baits used (Table 2). In general, baits from all host plants developed necrotic lesions that yielded several isolates for sequencing, but more than half (58%) of the isolates obtained were from pedunculate oak and *Rhododendron* spp. baits (27% and 31%, respectively). Thirty-five of the 124 isolates were identified as *Phytophthora* spp. including *P. bilorbang* Aghighi, *P. cactorum* (Lebert & Cohn) J. Schröt, *P. gallica* Jung & Nechwatal, *P. gonapodyides* (H.E. Petersen) Buisman, *P. lacustris* Brasier, Cacciola, Nechwatal, Jung & Bakonyi, *P. plurivora* T. Jung & T.I. Burgess, and *P. polonica* Belbahri L, Moralejo E & Lefort F. sp. nov. and two which could not be identified to a species level. The remaining 89 isolates belonged to species of *Pythium* including *P. irregulare* Buisman, *P. intermedium* de Bary, *P. litorale* (Nechw.) Abad, de Cock, Bala Robideau, A.M. Lodhi & Lévesque, *P. mamillatumP. nodosum* (B. Paul, D. Galland, T. Bhatn. & Dulieu), *P. violae* Chesters & Hickman, as well as *Phytopythium citrinum* (B. Paul) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque, *Phytopythium litorale* (Nechw.) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque, and *Elongisporangium anandrum* (Drechsler) Uzuhasi, Tojo & Kakish. .

Four *Phytophthora* species, namely *P. bilorbang*, *P. lacustris*, *P. plurivora* and *P. polonica*, were recovered from the rhizosphere soil of declining alder forests in three regions of western Ukraine (Table 3, Figure 2). From declining birch forests, three *Phytophthora* spp. (*P. gonapodyides*, *P. plurivora* and *P. gallica*) were recovered but only in the Rivne region. In the Transcarpathia region, *P. plurivora* was predominant in the declining oak forest and *P. cactorum* in the declining sweet chestnut plantations (Table 3, Figure 2). The most abundant

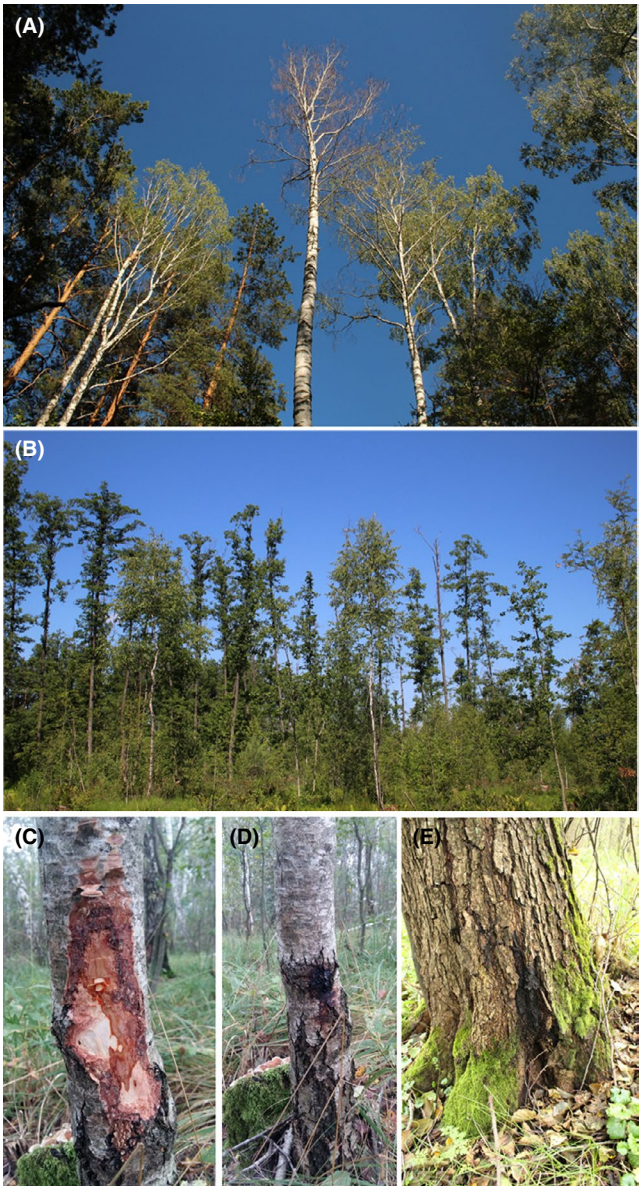

FIGURE 1 (a) *Betula pendula* growing in floodplain areas in the Volyn region exhibiting crown thinning and dieback; (b) *Alnus glutinosa* growing in floodplain areas in the Rivne region exhibiting crown dieback; (c and d) Bleeding lesions on stems of declining *Betula pendula* in the Volyn and Rivne regions; (e) Bleeding at the base of a *Quercus robur* tree in the Transcarpathia region, the tree was also showing branch and crown dieback

species from rhizosphere soil samples were *P. plurivora* and *P. polonica* representing 26% and 20% of the total number of soil samples, respectively. *Phytophthora bilorbang*, *P. polonica*, *P. gallica* and *P. plurivora* have never been recorded in forest soils in Ukraine.

4 | DISCUSSION

During the last decade, attention associated with the decline of several different forest types has increased locally in Ukraine with forest inventories often noting symptoms indicative of *Phytophthora*

infections on declining broadleaf trees (Kramarets et al., 2011). Since we found seven *Phytophthora* species (*P. bilorbang*, *P. cactorum*, *P. gallica*, *P. gonapodyides*, *P. lacustris*, *P. plurivora and P. polonica*) from the rhizosphere soil using traditional baiting and isolation techniques this suggests a possible role of these *Phytophthora* species in the decline of broadleaf forests in western Ukraine. Previously, *P. cactorum*, *P. gonapodyides* and *P. lacustris* have been found in rivers at the Polish-Ukrainian border (Matsiakh et al., 2012, 2016), but this is the first study looking at the occurrence and diversity of *Phytophthora* species sampled from soils in declining broadleaved forests. Four *Phytophthora* species (*P. bilorbang*, *P. gallica*, *P. plurivora* and *P. polonica*) have not been previously reported from broadleaf forests in Ukraine.

4.1 | Isolates from alder stands

The four *Phytophthora* species that were detected from three declining alder forests (Table 3) represent three clades. *Phytophthora bilorbang*, a species that has been commonly associated with the decline of European blackberry (*Rubus anglocandicans* A. Newton) in Western Australia (Aghighi et al., 2012, 2015), was found in both Volyn and Rivne regions (Figure 2). In Europe, *P. bilorbang* has been isolated from leaves of common alder (Scanu et al., 2014), and more recently the species was reported for the first time on *Olea europaea* L. in Italy (Santilli et al., 2020). Given these recent new reports, the distribution and host range for *P. bilorbang* in Europe needs further resolution.

Phytophthora plurivora was detected from one declining alder forest in Volyn region (Figure 2). The pathogen has previously been reported to cause lesions on the trunks of alder in Poland (Trzewik et al., 2015). Earlier, *P. plurivora* has been found to be present in the rhizosphere of soils beneath declining alder trees and causing aerial canker and collar rot on alder in Spain, Romania, Austria and Germany (Haque et al., 2014; Jung & Blaschke, 2004; Jung & Burgess, 2009). In a recent study from Turkey, *P. plurivora* was the most frequently isolated species of *Phytophthora* from rhizosphere soils from declining alder forests (Aday Kaya et al., 2018).

Phytophthora lacustris was isolated from soil in declining alder stands in Volyn region (Figure 2). Formerly classified as *Phytophthora* taxon Salixsoil due to it being first isolated from *Salix matsudana* Koidz. roots, *P. lacustris* has been reported widely in wetland and riparian habitats, including previously from rivers at the Polish-Ukrainian border (Matsiakh et al., 2012, 2016) and from rhizosphere soils in alder stands in Turkey (Aday Kaya et al., 2018). Though commonly recognized as a saprotroph which infects plant detritus, *P. lacustris* was recently shown also to cause significant damage to common alder and *Prunus persica* (L.) Batsch (Kanoun-Boulé et al., 2016; Nechwatal et al., 2013). Like other clade 6 species, *P. lacustris* is commonly aquatic and tolerant of high temperatures (Nechwatal et al., 2013), but may cause consequential damage under certain conditions (Jung et al., 2011; Kanoun-Boulé et al., 2016). The pathogenicity of *P. lacustris* on different host species does not appear to be fully recognized (Nechwatal et al., 2013; Nowak et al.&, 2015; Orłikowski et al., 2012).

Phytophthora polonica which was detected from three alder stands in Volyn, Lviv and Rivne regions (Figure 2) has been reported

TABLE 2 *Phytophthora* isolates obtained from baiting soil samples from the rhizosphere of several broadleaved tree species with leaves from different baiting hosts and those selected for DNA sequence identification

			No. of baited necrotic pieces plated on media/ No. isolates obtained/ sequenced isolates per baiting host*					
Region	**Locatity**	**Host species**	***Qro***	***Qru***	***Fs***	***Ah***	***Rh***	***Ms***
Volyn	Chortoryj	*Betula pendula*	147/-/-	8/-/-	71/3/2	125/3/1	-/-/-	-/-/-
Volyn	Kukly	*Betula pendula*	117/7/-	-/-/-	2/10/-	60/2/1	-/-/-	-/-/-
Volyn	Kukly	*Alnus glutinosa*	96/40/16	27/5/3	55/11/2	21/6/2	-/-/-	-/-/-
Lviv	Nyvytsi	*Alnus glutinosa*	109/38/25	25/9/8	32/12/11	57/2/2	-/-/-	-/-/-
Lviv	Komarno	*Alnus glutinosa*	-/-/-	-/-/-	-/-/-	-/-/-	45/22/2	20/11/-
Rivne	Strashiv	*Betula pendula*	21/8/1	-/-/-	-/-/-	112/6/2	77/31/10	26/17/4
Rivne	Klesiv	*Betula pendula*	-/-/-	-/-/-	-/-/-	29/21/3	30/19/4	19/19/-
Rivne	Klesiv	*Alnus glutinosa*	8/4/0	-/-/-	-/-/-	6/4/4	36/18/5	14/4/1
Transcarpathia	Borzhava	*Quercus robur*	20/18/2	-/-/-	-/-/-	50/45/1	91/35/5	14/7/2
Transcarpathia	Lavkivske	*Castanea sativa*	8/2/-	-/-/-	-/-/-	-/-/-	14/9/4	-/-/-
Ternopil	Vikno	*Fagus sylvatica*, *Quescus robur*	8/-/-	-/-/-	-/-/-	5/-/-	18/-/-	25/-/-
Khmelnytskyj	Sataniv	*Quescus robur*	15/-/-	-/-/-	-/-/-	-/-/-	-/-/-	15/-/-
		Total	549/117/45	60/14/11	160/36/15	364/89/16	311/134/30	133/58/7

Note: Baiting hosts*: *Qro*, *Quercus robur*, *Qru*, *Quercus rubra*, *Fs*, *Fagus sylvatica*, *Ah*, *Aesculus hippocastanum*, *Rh.*, *Rhododendron spp.*, *Ms*, *Magnolia soulangeana*

TABLE 3 *Phytophthora* species identified from rhizosphere soil samples in western Ukraine

Putative taxon	Clade	Host species	Locality	Baiting hosts	Closest GenBank Accession No	Similarity%	No of isolate sequences deposited to GenBank	GenBank accession number(s)
Soil samples collected in September-October 2017								
Phytophthora lacustris	6	*Alnus glutinosa*	Kukly Forestry, Volyn region	*Q. robur*	MN045219.1	99	1	MT420402
Phytophthora lacustris	6				MN045221.1	100	1	MT420403
Phytophthora plurivora	2				KT383059.2	99	1	MT420404
Phytophthora bilorbang	6			*Q. rubra*	MN589654.1	99	1	MT420401
Phytophthora polonica	9			*F. sylvatica*	KF234760.1	99	1	MT420405
Phytophthora polonica	9				KX618507.1	100	1	MT420406
Phytophthora polonica	9	*Alnus glutinosa*	Nyvytsi Forestry, Lviv region	*F. sylvatica*	KX618507.1	99	1	MT420411
Soil samples collected in June 2018								
Phytophthora sp.	-	*Betula pendula*	Strashiv, Rivne region	*Rhododendron* spp.	-	-	1	MT420392
Phytophthora gonapodyides	6				KT383041.2	99	1	MT420398
Phytophthora gonapodyides	6				KT383041.2	100	1	MT420400
Phytophthora gallica	10				MG865497.1	99	3	MT420393-95
Phytophthora sp.	-				-	-	1	MT420399
Phytophthora gallica	10			*M. soulangeana*	MF441618.1	99	1	MT420396
Phytophthora gallica	10			*A. hippocastanum*	MG865497.1	99	1	MT420397
Phytophthora plurivora	2	*Betula pendula*	Klesiv Forestry, Rivne region	*Rhododendron* spp.	MH037150.1	100	4	MT420379-82
Phytophthora bilorbang	6	*Alnus glutinosa*	Klesiv Forestry, Rivne region	*Rhododendron* spp.	MT328692.1	99	1	MT420377
Phytophthora bilorbang	6				MG696534.1	99	1	MT420378
Phytophthora polonica	9			*A. hippocastanum*	JX276065.1	100	1	MT420383
Phytophthora polonica	9				KY465650.1	100	1	MT420384
Phytophthora polonica	9				KF234760.1	99	1	MT420385
Phytophthora polonica	9				JX276064.1	99	1	MT420386
Phytophthora plurivora	2	*Quercus robur*	Borzhava Forestry, Transcarpathia region	*Q. robur*	MN589655.1	100	1	MT420407
Phytophthora plurivora	2				MN517992.1	100	1	MT420408
Phytophthora plurivora	2			*M. soulangeana*	MN517992.1	100	1	MT420409
Phytophthora plurivora	2			*A. hippocastanum*	MH037150.1	99	1	MT420410
Phytophthora cactorum	1	*Castanea sativa*	Lavkivske Forestry, Transcarpathia region	*Q. robur*	MG696466.1	99	1	MT420387
Phytophthora cactorum	1			*Rhododendron* spp.	MK534114.1	99	1	MT420388
Phytophthora cactorum	1				MG783385.1	100	1	MT420389
Phytophthora cactorum	1				MN398584.7	100	1	MT420390
Phytophthora cactorum	1				EU044728.2	100	1	MT420391

in declining alder stands in Poland together with *P. alni* (Belbahri et al., 2006). Belbahri et al. (2006) suggest *P. polonica* is a poor bark colonizer of alder, however, in Hungary, the species was isolated from the necrotic tissues of symptomatic roots and was associated with rapid tree mortality of wild cherry trees (*Prunus avium*) in a mixed deciduous forest (Sárándi-Kovács et al., 2016).

Interestingly, in this study, we did not detect *P. xalni* s.l. from any of the alder sites. Studies of alder decline in neighbouring Poland have reported several *Phytophthora* species including *P. xalni*, *P. xcambivora* and *P. plurivora* from diseased trunks and *P. lacustris* and *P. gonapodyides* from soils (Orlikowski et al., 2003; Trzewik et al., 2015). Serious alder decline and mortality in Hungary did not identify to *P. xalni*, but rather several other *Phytophthora* species including *P. gonapodyides*, *P. inundata*, *P. lacustris*, *P. megasperma*, *P. plurivora* and *P. taxon hungarica* (Szabó et al., 2013). Similarly, Aday Kaya et al. (2018) did not detect *P. ×alni* in rhizosphere soils from declining *Alnus* stands in Turkey.

4.2 | Isolates from birch stands

The three *Phytophthora* species (*P. gallica*, *P. gonapodyides and P. plurivora*) and two other taxa to which species level could not be discerned that were detected from declining birch forests in the Rivne region (Table 3, Figure 2) represent three clades. Studies of dieback on birch elsewhere in Europe have been associated with other damaging agents including the fungi *Annisogramma virgultorum* (Fr.) Theiss and Syd. and *Marssonina betulae* (Lib.) Magnus (De Silva et al., 2008; Green & MacAskill, 2007; Witzell & Karlsson, 2002). In Finland, necrotic lesions caused by *Phytophthora cactorum* have been reported in nurseries (Hantula et al., 1997; Lilja et al., 1996). Little information exists on *Phytophthora* species in birch forests, though Jung et al. (2007) reported the presence of *P. cactorum*, *P. citricola* s.l., *P. gonapodyides* and *P.* taxon 'raspberry' on *Betula* spp. in the western and southern parts of Germany and Switzerland.

Phytophthora gallica which was found in a declining birch stand in Rivne region in Ukraine has not been associated with birch elsewhere in Europe. However, *P. gallica* has been previously isolated from the rhizosphere soil of a declining oak stand in Northeast France (Jung & Nechwatal, 2008), and the species was detected in a water source in Poland (Oszako et al., 2017). In the former study, Jung and Nechwatal (2008) showed that *P. gallica* was moderately aggressive to common alder and beech, weakly aggressive to pedunculate oak and *Salix alba* .L, and non-pathogenic to common ash (*Fraxinus excelsior* L.).

Phytophthora gonapodyides which was detected from declining birch stands in Rivne region (Figure 2) is likely indigenous to

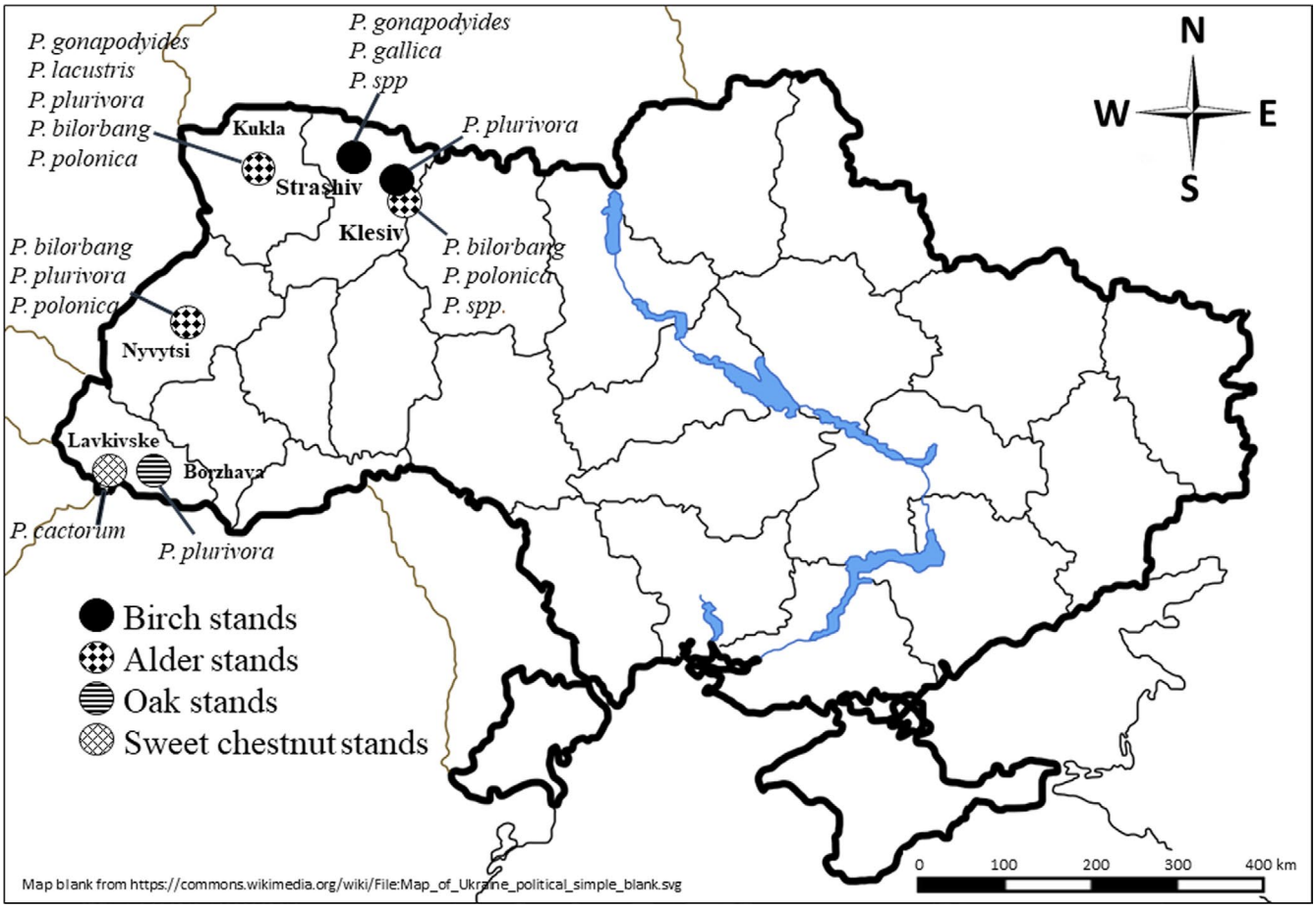

FIGURE 2 Map showing locations of declining broadleaved forest sites in western Ukraine and detected *Phytophthora* species

Europe (cf. Hansen, 2008; Hansen & Delatour, 1999) and is generally known as a weak parasite with saprophytic abilities, usually associated with aquatic environments such as rivers, riparian areas and wetlands (Brasier et al., 2003; Brasier et al., 1993; Jung & Blaschke, 2006). However, in some cases, *P. gonapodyides* can be an opportunistic pathogen capable of causing disease on trees (Brasier & Jung, 2003; Brown & Brasier, 2007; Cleary et al., 2016; Jung & Blaschke, 1996; Jung et al., 1996). *Phytophthora gonapodyides* has been detected from the soil in southwestern Spain where an intensive dieback of holm oak (*Quercus ilex* L.) was recorded (Corcobado et al., 2010).

4.3 | Isolates from oak stands

Phytophthora plurivora (clade two) which was the only species isolated from rhizosphere soil samples in a declining oak stand near the Borzhava river in Transcarpathia region (Figure 2), has been widely detected in European oak stands in Germany (Jung et al., 1996; Jung, Blaschke, & Osswald, 2000), France (Hansen & Delatour, 1999), Austria (Balcì & Halmschlager, 2003b), Czech Republic (Mrázková et al., 2013), Italy (Vettraino et al., 2002), Poland (Jankowiak et al., 2014) and Turkey (Balcì & Halmschlager, 2003b). *Phytophthora plurivora* has a very broad host range including several broadleaf hosts (Jung et al., 2016). *Phytophthora plurivora* is presumed to be native to the regions of South and East Asia, as it was found in Taiwan, Nepal and Yunnan (Jung et al., 2017; Jung et al., 2020). Since *P. plurivora* is one of the most common *Phytophthora* species found in European nurseries (Jung et al., 2016), it is probable that large reforestations and afforestation efforts using infested nursery plants have contributed to the species' widespread distribution in forests across Europe.

4.4 | Isolates from chestnut stands

Phytopthhora cactorum (clade one) was the one *Phytophthora* species isolated from the rhizosphere of the declining chestnut stand in Transcarpathia (Figure 2). Earlier, the species has been detected in Ukraine as responsible for causing 'damping-off' in oak and beech seedlings for many years (Tsilyurik & Shevchenko, 2008). Though, *P. x cambivora* and *P. cinnamomi* are the main species associated with ink disease on sweet chestnut in central and southeastern Europe (Jung et al., 2018; Vannini & Vettraino, 2001), *P. cactorum* is on a long list together with other *Phytophthora* species that have been found associated with declining chestnuts (Jung et al., 2018). The blight pathogen *Cryphonectria parasitica* is continuing to impact sweet chestnut plantations in western Ukraine, and forest enterprises are continuously removing diseased trees as a result. Synergistic effects from the presence of both *C. parasitica* and *P. cactorum* may well accelerate the decline of sweet chestut in the future.

5 | CONCLUSION

This study provides a first look at the occurrence and diversity of *Phytophthora* species associated with different declining broadleaved forest tree species in western Ukraine using traditional baiting and isolation techniques. It is noteable that we observed variable success isolating *Phytophthora* species with different host baits. *Rhododendron* spp. and oak appeared to be more efficient than other hosts accounting for more than half of the isolates obtained. Aghighi et al. (2015) found that baiting the soil with the youngest fully expanded leaves of oak (holm oak and cork oak (*Q. suber* L.)) permitted isolation of up to seven *Phytophthora* species from declining *Rubus anglocandicans* A. Newton. Differences in isolation success have been similarly reported in other studies (Jung et al., 2002; Vettraino et al., 2005). Different host species used as baits vary in their susceptibility to different *Phytophthora* species which may influence the efficiency of detection (Erwin & Ribeiro, 1996; Marks & Kassaby, 1974). In some sites which showed clear evidence of forest decline, we did not yield any *Phytophthora* by soil baiting. However, a negative result does not indicate the absence of *Phytophthora* species as several factors can influence the efficiency of detection by traditional techniques (Cooke et al., 2007). For example, O'Brien et al. (2009) and Jung et al. (2002) suggest that for some *Phytophthora* species the efficiency of detection can be wide-ranging depending on the season. In our study, a one-time sampling was conducted during two seasons. It is unclear whether season affected isolation success, though we noted that autumn sampling in birch stands yielded only *Mortierella macrocystis* and several *Pythium* spp. and no *Phytophthora* species were detected. Cultures of *Pythium* and *Phytopythium* species were often dominant in the isolation plates and may have overgrown *Phytophthora* spp. Thus, we do not exclude that the rhizosphere communities are in fact more diverse than what we could detect using traditional soil baiting techniques. Though several studies have reported the occurrence of *Pythium* and *Phytopythium* species in forest soils (e.g. Balci and Halmschlager, 2003a, 2003b; Jankowiak et al., 2015; Jung Blaschke & Oßwald, 2000), information about their role on tree health is rather limited, but the study by Jankowiak et al. (2015) suggests a possible association with the presence of *Phytopythium* and *Pythium* species in declining oak stands in southern Poland. Thus, further investigations are needed to better understand the role of *Phytopythium* and *Pythium* species in declining broadleaved forests and their possible potential of interaction with other *Phytophthora* species in causing root damage on trees.

We have identified seven *Phytophthora* species that have shown to have clear association with decline syndromes in either the same or other broadleaved tree species in other European countries, which suggests that at least some, are probable causal agents for the observed forest decline in western Ukraine. These findings highlight the potential risk for further damage caused by these organisms in the future, especially with changing climate

conditions that promotes pathogen infection and spread. Further work is needed to determine the pathogenicity of the obtained *Phytophthora* species to different economically and ecologically important broadleaved hosts and wider monitoring of *Phytophthora* species associations with forest decline in western Ukraine.

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PEER REVIEW

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