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ORIGINAL PAPER

Occurrence of *Phytophthora* species in the rhizosphere of dying black alder *Alnus glutinosa* in central and northeastern Poland

Miłosz Tkaczyk✉, Hanna Szmidla, Katarzyna Sikora

Forest Protection Department, Forest Research Institute, Braci Leśnej 3, 05-090 Sękocin Stary, Poland

ABSTRACT

For many years, a progressive process in the decline of black alder has been observed in Poland. Symptoms of alder dieback include withering and death of branches, stunted growth, yellowing of leaves, and stagnation of the plant's development. The disease eventually leads to complete death of the alder. Previous observations and investigations indicate infections by pathogenic oomycetes of the genus *Phytophthora*. These organisms are responsible for damage to fine roots and necrosis of alder root collars. The objective of this study was to conduct an inventory on the occurrence of pathogens of the genus *Phytophthora* in the rhizosphere of dying alders. For this purpose, eight plots were selected in central and northeastern Poland from which soil was collected for analysis. Four of the plots were located in the Dobrocin Forest District (RDSF in Olsztyn), two in the Kutno Forest District (RDSF in Łódź) and two in the Radom Forest District (RDSF in Radom). Alder dieback was observed over a long period time on all plots together with thinning of crowns, discoloration of leaves and exudations at the base of stems. The research conducted, using the traditional method with young leaves of English oak *Quercus robur* and rhododendron *Rhododendron* sp. as plant traps, revealed the presence of four species of the genus *Phytophthora*: *P. plurivora*, *P. polonica*, *P. gallica* and *P. pseudocryptogea*. In addition, the presence of *Globisporangium megasporum* and *Phytopythium citrinum* was confirmed. All the aforementioned organisms have been reported to cause death of trees including black alder. In addition, two of these species *P. gallica* and *P. pseudocryptogea* were detected for the first time in the rhizosphere of dying alders in Poland. Information on the occurrence of these species is important because it increases knowledge about new pathogenic species that pose a direct threat to the persistence of alder stands in Poland.

KEY WORDS

pathways, *Phytophthora*, *Globisporangium*, *Phytopythium*, soilborne pathogens

Introduction

Black alder *Alnus glutinosa* (L.) Gaertn. is probably the most common tree species along riverbank habitats in Europe. It is a pioneer species characterized by large primary growth which can reach 90 cm (Kremer, 1995). Black alder plays an important role in aquatic ecosystems especially along the banks of rivers and lakes. Alder roots bind soil which help stabilize banks, reduce erosion,

✉e-mail: M.Tkaczyk@ibles.waw.pl

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and prevent flooding. In addition, black alder provides a habitat for many animal and plant species including birds, insects, fish and marsh plants. Alder is found mainly along watercourses and near lakes and ponds which makes it a very important addition to natural forests. The importance of this tree species make conducting studies on its health quite relevant. This research is important because a significant deterioration in the health of alder has been observed in Europe for over 30 years. The first reports of this phenomenon date back to the early 1990s in the United Kingdom (Brasier *et al*., 1995). Dieback was observed in these trees due to a disease that in the first stages was characterized by visible thinning and small discolored leaves, followed by dark spots on the stems near the root collar. After taking soil samples from the rhizosphere, a significant loss of fine roots was observed. Organisms of the genus *Phytophthora* were identified as having caused the damage to the root systems and root collar. In the following years, studies on the weakening of alders were conducted in other European countries including Germany (Jung and Blaschke, 2004), Austria (Cech *et al*., 1998), France (Streito *et al*., 2002), Czech Republic (Černy *et al*., 2003, 2008), Poland (Orlikowski *et al*., 2003) Hungary (Koltay *et al*., 2007), and Spain (Solla *et al*. 2010). Since then, numerous species of the genus *Phytophthora* have been described which are responsible for damage to alder root systems and lead to the death of entire trees. In Poland, these include *P.* × *alni* Brasier & S.A. Kirk, *P. cactorum* (Leb. & Cohn) Schröeter, *P.* × *cambivora* (Petri) Buisman, *P. plurivora* T. Jung & T. I. Burgess, *P. cinnamomi* Rands, *P. gonapodyides* Petersen, *P. megasperma* Engl., *P. pseudosyringae* T. Jung & Delatour, *P. syringae* Klebahn, *P. lacustris* Brasier, Cacciola, Nechwatal, Jung & Bakonyi, and *P. polonica* Belbahri L, Moralejo E & Lefort F. (Orlikowski *et al*., 2003; Orlikowski and Oszako, 2005; Oszako, 2005; Belbahri, *et al*., 2006; Trzewik, *et al*., 2015; Malewski *et al*. 2020). Despite the large number of species already described, new reports of *Phytophthora* occurrence in dying alder stands continue to be observed.

The aim of this study was to conduct an inventory of the species of the genus *Phytophthora* present in the rhizosphere of dying alders soils in central and northeastern Poland.

Materials and methods

Samples for analysis were collected in 2022 and 2023 in the Dobrocin Forest District (Regional Directorates of State Forests in Olsztyn), Kutno Forest District (RDSF in Łódź), and Radom Forest District (RDSF in Radom). Eight samples of rhizosphere soils of weakened alders were collected from all plots with one representative sample from each plot (Table 1). All plots were located in a depression with periodically stagnant water. Crown weakening was observed in the trees present there which was characterized by a reduction of leaves, thinning of the crown and death of branches. Dark exudates were observed in some trees (Fig. 1). Samples were taken from trees with visible cracks in the bark of the root collar at a distance of about one meter from the base of the trunk. After removing the top layer of soil, a 20×20×20 cm monolith was collected. The samples were then transported to the headquarters of the Forest Research Institute where they were analyzed using the baiting method (Jung *et al*., 1996). For this purpose, the collected soil was placed in sterile containers measuring 25×15×10 cm and then flooded with distilled water so that the water surface was about 4-5 cm above the soil surface. Then young leaves of English oak *Quercus robur* L. and rhododendron *Rhododendron* sp. were placed on the water surface. After three days, when dark spots were observed on the leaves, the leaves were removed from the water, dried and the affected tissues were cut into smaller pieces (about 5×5 mm). The leaf pieces were placed on V8-PARPNH selective medium [V8 agar – 16 g/l agar, 2 g/l $CaCO_3$, 100 ml/l vegetable juice with the addition of antibiotics 10 $\mu g \cdot ml^{-1}$ pimaricin, 200 $\mu g \cdot ml^{-1}$ ampicillin,

Table 1.
Study locations and basic data on the stands

Location	No.	Coordinates	Age	Average DBH	Symptoms observed
Dobrocin Forest District	1	53.936644 19.814748	53	27	crown transparency, yellowing of leaves
	2	53.935073 19.812553	73	29	crown transparency, yellowing of leaves
	3	53.919520 19.780470	53	28	crown transparency, yellowing of leaves, trunk base leaves
	4	53.919929 19.760751	55	26	crown transparency, broken branches
Kutno District Forest	5	52.197008 19.492987	16	6	yellowing of leaves, exudates at trunk base leaves
	6	52.193377 19.503570	88	37	crown transparency, yellowing of leaves
Radom Forest District	7	51.501377 21.169209	71	28	crown transparency, exudates at trunk base leaves
	8	51.487232 21.149476	10	4	crown transparency, exudates at trunk base leaves

Fig. 1.
Disease symptoms observed in the plots from which samples were taken for analysis

10 µg·ml^{-1} rifampicin, 25 µg·ml^{-1} pentachloronitrobenzene (PCNB), 50 µg·ml^{-1} nystatin, and 50 µg·ml^{-1} hymexazol] (Jung *et al*., 1996; Jung 2009). The prepared plates were incubated at room temperature (approximately 20°C) and observed daily under a ZEISS axiolab microscope at 20× magnification. When the first mycelia were observed, they were transplanted into new dishes containing V8 medium (20 g/l).

One-week-old isolates were sorted into morphotypes based on colony growth. The internal transcribed spacer (ITS) region of the nuclear rDNA of 5 isolates was amplified with universal ITS4 (White *et al*., 1990) and ITS 6 (Cooke *et al*., 2000) primers in direct PCR (diPCR) using Phire™ Plant Direct PCR Kits (Thermo Fisher Scientific Inc., Waltham, MA, USA). Mycelium from 7 day old colonies growing on V8 agar was scraped with a sterile tip and placed in 0.2 ml

Eppendorf tubes with 30 µl of Dilution Buffer (ThermoFisher Scientific Inc., Waltham, MA, USA). The 20 µl Phire PCR reaction mixture consisted of 0.5 µl of Dilution Buffer with young hypha (DNA template), 1 µl/0.5 µM of primers ITS4/ITS6, 10 µl 1 × Phire Plant PCR Buffer, and 0.4 µl Phire Hot Start II DNA Polymerase. The PCR conditions were as follows: 98°C for 5 min; 40 cycles of 98°C for 5 s, 55°C for 5 s, 72°C for 50 s, and 72°C for 7 min. The presence and size of PCR products were confirmed by analyzing 1 µl of product by electrophoresis in 1% TAE-agarose gel stained with GelRed™ NulceidAcid Dye (Biotium, Inc., Fremont, CA, USA). In prior sequencing, the PCR product was purified with the AntyInhibitor kit (A&A Biotechnology, Gdynia, Poland) following the manufacturer's insrtructions and sequenced with ABI 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Obtained sequences were checked and trimmed in FinchTV software (Geospiza) and compared to other sequences in the GenBank using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/). Isolates were assigned to a *Phytophthora* species when sequence matches were above a 99.7% cut-off in respect to those of extype isolates or key isolates. All ITS sequences obtained in this study were submitted to GenBank.

Results

A total of 193 necrotic spots were observed on leaves that served as plant traps and were subsequently applied to selective media. This resulted in 58 isolates representing locations in all three regions (RDSF Olsztyn, Łódź and Radom). The presence of pathogens was confirmed in five out of nine surfaces where observations were made. Of these 58 isolates, 10 were selected for DNA sequencing based on macroscopic and microscopic observations. These isolates were from samples collected during both the 2022 and 2023 seasons (Table 2). In general, baits from all host plants developed necrotic lesions, and the vast majority (78%) of isolates obtained were from English oak and the remaining 22% were from *Rhododendron* spp. Four out of ten isolates were identified as *Phytophthora* spp. including *P. gallica* Jung & Nechwatal, *P. plurivora*, *P. polonica* and *P. pseudocryptogea* Pethybr. & Laff. The remaining isolates belonged to the *Pythium* species including *Py. macrosporum* Vaartaja & Plaäts-Niterink and *Phytopythium citrinum* (B. Paul) Abad, de Cock.

Discussion

The problem of alder stand decline in Poland is not a new phenomenon (Oszako, 2005). Observations in these stands often reveal symptoms indicative of *Phytophthora* infections on declining deciduous trees (Orlikowski *et al*., 2003; Oszako *et al*., 2019). Since four *Phytophthora* species (*P. gallica*, *P. plurivora*, *P. pseudocryptogea*, and *P. polonica*) were found in the soil of the rhizosphere using conventional baiting and isolation techniques, this suggests the possible role of the identified *Phytophthora* species in the decline of alder forests in central and northeastern Poland. Two *Phytophthora* species *P. gallica* and *P. pseudocryptogea* identified in this study have not been previously detected in alder forests in Poland.

Phytophthora plurivora was detected in a dying alder forest in the RDSF in Łódź. This species is the most common of all *Phytophthora* species in dying oak stands in Poland (Tkaczyk *et al*., 2017; Solla *et al*., 2021). *P. plurivora* has already been found in the rhizosphere of soils under dying alders and has been observed to cause aerial cankers and collar rot on alders in Spain, Romania, Austria, Germany, Turkey, Poland, Ukraine and Slovakia (Jung and Blaschke, 2004; Jung and Burgess, 2009; Haque *et al*., 2014; Trzewik *et al*., 2015; Aday Kaya *et al*., 2018; Matsiakh *et al*., 2020; Tkaczyk *et al*., 2022).

Table 2.
Oomycetes species on black alder, origin of isolates, and number of isolates

Location	No	Oomycete species (number of isolates)	GenBank Accession Numbers of the representative isolates	GenBank reference	Identity [%]	*Phytophthora* Clades
Dobrocin Forest District	1	–				–
	2	*Phytophthora gallica* (3)	OR676922	OM281732	100	10
		Phytopythium citrinum (15)	OR676928	OP910308	100	–
	3	*Phytopythium citrinum* (5)	OR676929 OR676930	OP910308	100	–
	4	–				–
Kutno Forest District	5	*Phytophthora plurivora* (4)	OR676925 OR676926 OR676927	MT328705	100	2
	6	*Globisporangium macrosporum* (21)	OR676931	HQ643685	100	–
Radom Forest District	7	*Phytophthora polonica* (4)	OR676923	KF234760	100	9
		Phytophthora pseudocryptogea (6)	OR676924	MN833033	100	8
	8	–				–

Phytophthora polonica was first detected in disappearing alder stands in Poland where it co-occurred with *P.* × *alni* (Belbahri *et al*., 2006). The study showed that *P. polonica* is a weak colonizer of alder tissue. Nevertheless, as shown by studies of Sárándi-Kovács *et al*. (2016) in Hungary, this species was isolated from necrotic root tissue with disease symptoms and associated with the rapid death of cherry trees *Prunus avium* L. In alder stands, this species has been detected in Ukraine, Austria, and Portugal, among other countries (Matsiakh *et al*., 2020; Bregant *et al*., 2023; Corcobado *et al*., 2023).

Phytophthora gallica was first described in dying oak stands in Germany and France (Jung and Nechwatal, 2008). Since then, this species has been confirmed several times in dying stands including in Ukraine and Bulgaria (Matsiakh *et al*., 2020; Christova, 2022). In Bulgaria, this species along with other species of the genus *Phytophthora* was isolated from small watercourses from which it spread and infected poplars, black locust and hawthorns occurring in the area.

The last species that was isolated is *Phytophthora pseudocryptogea*. This species was recently distinguished from *Phytophthora cryptogea* (Safaiefarahani *et al*., 2015). Relatively little is known about its pathogenicity to forest trees. To date, it has been detected mainly on tobacco plants, spinach, sugar beet, wheat and oats (Blein *et al*., 1991; Larsson and Gerhardson, 1990). This species has also been described as the cause of the decline of wild olive trees in Italy (Deidda *et al*., 2023).

This paper is the first report on the occurrence of *P. gallica* and *P. pseudocryptogea* in dying alder stands in Poland. These organisms have been repeatedly identified as causal agents of black alder root rot (Jung *et al*., 2013; Sims *et al*., 2015; Feau *et al*., 2016; Jung *et al*., 2016). However, in order to confirm that these species are responsible for alder dieback in the areas studied, further research is needed to confirm Koch's hypothesis. The literature confirms situations where their presence cannot be clearly linked to the occurrence of dieback despite the isolation of pathogens of the genus *Phytophthora* from the soil of the rhizosphere of trees (Kurbetli *et al*., 2022).

Conclusions

In this study, results on the occurrence of *Phytophthora* in dying alder stands in Poland have been presented. The dieback of these stands is a serious problem due to their great economic importance. In this study, in addition to known species such as *P. plurivora*, the presence of *P. gallica* and *P. pseudocryptogea* in dying alder stands in Poland was confirmed for the first time. *P. gallica* was detected in stands in the northern part of the country, while *P. pseudocryptogea* occurred in central Poland. It is difficult to establish any correlations with respect to the distribution of these species in different parts of the country, especially since species of the genus *Phytophthora* can spread easily by soil or water even over long distances. However, information on the occurrence of these species is important because it adds new species to existing knowledge that pose a direct threat to the survival of alder stands in Poland.

Authors' contributions

All authors substantially conceived the ideas and contributed to conceptualization, resources, writing the original draft, reviewing, and editing of the text.

Conflicts of interest

Authors declare no personal circumstances or interests that may be perceived as inappropriately influencing the representation or interpretation of the reported research results.

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STRESZCZENIE

Występowanie rodzaju *Phytophthora* w glebie ryzosferowej zamierającej olszy czarnej *Alnus glutinosa* w centralnej oraz północno-wschodniej Polsce

Olsza czarna *Alnus glutinosa* odgrywa ważną rolę w ekosystemach wodnych, zwłaszcza wzdłuż brzegów rzek i jezior. Jej korzenie wiążą glebę, co pomaga w stabilizacji brzegów, redukcji erozji oraz zapobieganiu powodziom. Dodatkowo olsza czarna stanowi siedlisko dla wielu gatunków zwierząt i roślin, w tym ptaków, owadów, ryb i roślinności bagiennej. Od wielu lat na terenie Polski obserwuje się postępujący proces związany z zamieraniem olszy czarnej. Objawy zamierania obejmują zasychanie i obumieranie gałęzi, żółknięcie liści oraz zahamowanie wzrostu i rozwoju rośliny. Choroba prowadzi do całkowitego zamierania olszy. Prowadzone dotychczas obserwacje i badania wskazują na infekcje ze strony patogenicznych lęgniowców z rodzaju *Phytophthora*. Organizmy te są odpowiedzialne za uszkadzanie korzeni drobnych, ale również nekrozy na szyjach korzeniowych olszy. Jako głównego sprawcę tego zjawiska od wielu lat wskazuje się gatunek *Phytophthora* × *alni*. Celem przedstawionych badań było przeprowadzenie inwentaryzacji pod kątem obecności patogenów z rodzaju *Phytophthora* w glebie ryzosferowej zamierających olszy. W tym celu wytypowano 8 powierzchni zlokalizowanych w centralnej i północno-wschodniej Polsce, na których pobrano glebę do analiz: 4 powierzchnie zlokalizowane były na terenie Nadleśnictwa Dobrocin (RDLP w Olsztynie), a kolejne 4 w Nadleśnictwie Kutno (RDLP w Łodzi) oraz w Nadleśnictwie Radom (RDLP w Radomiu). Na wszystkich powierzchniach od dłuższego czasu obserwowano proces zamierania olszy objawiający się przerzedzeniem koron, przebarwianiem liści oraz wysiękami u podstawy pni. Szczegółowy opis powierzchni oraz podstawowe informacje na temat cech drzewostanów przedstawiono w tabeli 1. Badania z zastosowaniem tradycyjnej metody baitingu, gdzie jako pułapek roślinnych użyto młodych liści dębu szypułkowego *Quercus robur* i rododendronu *Rhododendron* sp., wykazały obecność 4 gatunków z rodzaju *Phytophthora*: *P. plurivora*, *P. polonica*, *P. gallica* oraz *P. pseudocryptogea*. Ponadto potwierdzono

obecność *Globisporangium megasporum* i *Phytopythium citrinum*. Najliczniej występującymi organizmami były *Globisporangium megasporum* i *Phytopythium citrinum*. Z patogenów należących do rodzaju *Phytophthora* najliczniej izolowano *P. pseudocryptogea* (6 izolatów), *P. plurivora* oraz *P. polonica* (po 4 izolaty), a także *P. gallica* (3 izolaty) (tab. 2). Wszystkie wymienione organizmy zostały już wielokrotnie opisane jako sprawcy zamierania drzew leśnych, w tym również olszy czarnej. Ponadto 2 z wymienionych gatunków (*P. gallica* i *P. pseudocryptogea*) zostały po raz pierwszy odnotowane w ryzosferze zamierających olszy. Warto jednak mieć na uwadze, że nie zawsze obecność *Phytophthora* w glebie musi wiązać się z zamieraniem drzew. W związku z tym konieczne są dalsze badania mające na celu spełnienie postulatów Kocha oraz potwierdzenie, czy wymienione patogeny mogą być odpowiedzialne za proces zamierania olszy czarnej na wybranych powierzchniach.