DOI: 10.2478/frp-2020-0017
Wersja PDF: www.lesne-prace-badawcze.pl

Leśne Prace Badawcze / *Forest Research Papers*
Grudzień / December 2020, Vol. 81 (4): 147–152

ORIGINAL RESEARCH ARTICLE

e-ISSN 2082-8926

Decline of Black Alder *Alnus glutinosa* (L.) Gaertn. along the Narewka River in the Białowieża Forest District

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Abstract. Black Alder *Alnus glutinosa* (L.) Gaertn. is an important tree commonly growing in Poland. Alders are actinorhizal plants that play an important ecological role in riparian ecosystems through atmospheric nitrogen fixation, filtration and purification of waterlogged soils as well as providing a refuge for terrestrial and aquatic organisms thus helping to stabilize stream banks. Black Alder used to be considered a very pest and disease resistant species but, the situation changed in 2000, when an unprecedented decline of Alders was observed in Poland. In the Białowieża Forest District, this decline has been observed on wet meadow habitats and along rivers or watercourses.

Currently, there are several hypotheses explaining Alder dieback, among them climatic changes and *Phytophthora* infections. In terms of climate, Black Alder requires a high atmospheric humidity during all phases of its reproductive cycle. It tolerates neither long-term summer flooding nor a significant decrease in the groundwater level. In terms of pests, oomycete pathogens of the genus *Phytophthora* are the most destructive plant pathogens known and many of them are present in forests and nurseries all over Europe.

The aim of this study was to evaluate the health of Black Alder along the Narewka River in the Białowieża Forest District. Selected areas were monitored in 2012 and 2018, but no relationship between drought and alder health was found. A preliminary analysis of soil and water samples by real time PCR revealed the presence of two *Phytophthora* species: *P. alni* and *P. cactorum*. Further and more detailed research is required to elucidate the role of these pathogens in Alder dieback.

Keywords: alder, dieback, drought, *Phytophthora*, PCR

1. Introduction

Common alder *Alnus glutinosa* (L.) Gaertn. is found in Europe, Siberia, Asia Minor and North Africa. In Poland, it is an important species from an ecological and economic point of view. It is a fast-growing tree, and thus valuable in the context of wood production (Jaworski 2011). Black alder is the only forest-forming tree in Poland whose roots can form actinorhizae with actinomycetes of the genus *Frankia*, ectomycorrhizae with large fruiting fungi and arbuscular mycorrhizae with fungi of the genus *Glomus*. The high adaptability of black alder allows it to be used for reclamation and afforestation on soils that are damaged and difficult to afforest (Pancer-Kotejowa, Zarzycki 1980; Jaworski 2011).

Of the native trees, this species tolerates high soil moisture best and thrives in areas where flowing water is present, although it can also occur in areas with stagnant

Received: 10.09.2020 r., accepted after revision: 12.10.2020 r.

water. It does not tolerate both long-term summer flooding and a significant reduction in groundwater levels (Orphanage 2001), but needs high air humidity for its maintenance in all phases of the reproductive cycle (Claessens et al. 2010). Until recently in Poland, black alder was considered to be a species with a low level of susceptibility to pests and diseases. This situation changed at the turn of the 21st century, when the dieback of alder stands was observed (Piętka, Grzywacz 2018). In this multifactorial phenomenon, abiotic factors, including weather anomalies, play an important role. The phenomenon of the mass dieback of alder stands was first confirmed in Great Britain in 1995 (Gibbs 1995). In a short period of time, it spread throughout Europe, also affecting grey and Italian alder in Germany, Austria, Hungary, Poland and Slovakia (Brasier et al 1995; Orlikowski et al. 2003; Jung, Blaschke 2004). One of the harmful biotic factors are oomycetes, which cause phytophthorosis in the root collar at the base of stems in both nurseries and forest stands. One of the *Phytophthora* species was identified as the cause of the disease. Gibbs (1995) described it as a taxon similar to *Phytophthora cambivora* (Petri) Buisman, which is a common pathogen of deciduous trees. Then, Brasier et al. (1999) demonstrated that this pathogen is an inter-species hybrid. Later studies proved that it is a hybrid of three species: *Phytophthora alni* Brasier & S.A. Kirk, *Phytophthora multiformis* and *Phytophthora uniformis* (Brasier & S.A. Kirk) Husson, Ioos & Aguayo. The latter *Phytophthora* is a diploid species, *Phytophthora multiformis* is an allotetraploid and *Phytophthora alni* is an allotriploid containing half the genome of each of its parents *Phytophthora multiformis* and *Phytophthora uniformis* (Husson et al. 2015). The species *Phytophthora alni* and *Phytophthora multiformis* are widespread in Europe, but have not been identified in other continents, while *Phytophthora uniformis* has been isolated both in Europe and North America (Navarro et al. 2015). So far, only the pathogen *Phytophthora uniformis* has been detected in Poland (Trzewik et al. 2008). Apart from the above-mentioned ones, other *Phytophthora* species have also been found in the rhizosphere and on the trunks of alder: *Phytophthora cactorum* (Lebert& Cohn) J. Schröt., *Phytophthora cambivora*, *Phytophthora cinnamomi* Rands, *Phytophthora plurivora* T. Jung and T.I. Burgess, *Phytophthora gonapodyides* (H.E. Petersen) Buisman, *Phytophthora megasperma* Drechsler, *Phytophthora pseudosyringae* T. Jung & Delatour, *Phytophthora syringae* (Berk.) Kleb. and *P. lacustris* Brasier, Cacciola, Nechwatal, Jung & Bakonyi (Trzewik et al. 2008). The source of infection can be both water and soil with seedlings, also asymptomatic, which do not yet exhibit any symptoms (Orlikowski et al. 2013).

Long-term droughts have often been indicated as the cause of weakening trees; therefore, the aim of this study was to assess the health condition of alders growing along watercourses in the Białowieża Forest District. The working hypothesis assumed that more trees died further away from the water source, which would indicate that the cause of death could be the shortage of water in the soil (drought).

2. Material and methods

In order to determine the causes of the phenomenon of black alder *Alnus glutinosa* dieback in the Białowieża Forest District, research was conducted along the banks of the Narewka River. Three observation sites were chosen in riparian ecosystems with a predominance of alder trees of coppice origin at the age of about 30–40 years:

1. Alder stand in alder carr habitat (52°69'N; 23°88'E), Białowieża Forest District, Stoczek Forest Unit, Section 476A;
2. Alders growing along the Narewka River (52°70' N; 23°84' E);
3. Alder stand in a flood plain (52°72'N; 23°74' E), Białowieża Forest District, Batorówka Forest Unit, Section 394 Ab.

River sections of 100 m long (Sites 1 and 2) and the alder stand (Site 3), which was periodically flooded by water overflowing the river, were selected for the survey.

During the fieldwork, healthy, sick and dead trees were counted, and their distance from the river bank was measured. Sick trees were considered to be those with sparse crowns resulting from dying shoots and smaller than usual leaves, which took on a lighter colour (light yellowish-green). The research was conducted in 2012 and from July to September 2018. In order to examine the presence of *Phytophthora*, a soil sample was taken in 2018 at each site from under three alders with symptoms of disease (as the most probable location of this pathogen) for analysis by real-time PCR. Soil samples were taken with a spade in two locations at a distance of about 1 m from the tree trunks at a depth of 20 cm (about 0.5 kg) and mixed together.

Analysis of the presence of *Phytophthora* spp.

DNA from the soil samples (0.5 g) was isolated with the NucleoSpinSoil Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The presence of species of the genus *Phytophthora* was determined by means of real-time PCR. As a positive control,

DNA isolated from the relevant *Phytophthora* species growing in Petri dishes was used. As a negative control, an appropriate amount of water was added to the reaction instead of DNA. Reactions took place in a mixture containing 2 µl DNA solution, 10 µl 2 × LuminoCt Master Mix (Sigma-Aldrich, St. Louis, MO, USA), 2 µl F and R starters (5 µM) and 0.2 µl TaqMan probe (5 µM). The probe and primer sequences used in the analysis are listed in Table 1. The Reaction Thermal Profile: 3 min predenaturation at 95°C; then 40 amplification cycles: denaturation at 95°C, 30 s; primer annealing at 55°C, 30 s; synthesis at 72°C, 30 s (Nowakowska et al. 2016). In order to identify *Phytophthora multiformis* (previous name *Phytophthora alni* sub sp. *multiformis*), probes and primers were used (Nowakowska et al. 2016), and for *Phytophthora cactorum* and *Phytophthora plurivora*, the probes and primers described in the publication (Nowakowska et al. 2017). The starters and probe to identify *Phytophthora alni* were designed in the Allele ID program (PREMIER Biosoft International, Palo Alto, CA, USA) according to the standard guidelines for TaqMan-type probes (Dorak 2006). Starters and the probe to detect *Phytophthora alni* were designed based on the ITS sequence (JF300250.1) available in the NCBI gene bank (www.ncbi.nlm.nih.gov). The probes were marked with the fluorescent dye JOE at the end of the 5' probe and an HBQ1 silencer at the end of 3' (Sigma-Aldrich, St. Louis, MO, USA). Real-time PCR was performed in the Rotor Gene 6000 cycler (Qiagen, Germany).

The Microsoft Excel 2019 program was used for the statistical calculations.

3. Results

Both inventories from 2012 and 2018 showed a similar number of dead trees (2012 – 11.4%; 2018 – 11.0%), while in 2018, there were 2.2 times more healthy trees (2012 – 27.8%; 2018 – 61.0%) (Table 2). The increase in the number of healthy trees was accompanied by a more than two-fold decrease in the average distance from water, from 23.3 m to 9.6 m. In the case of sick and dead trees, the average distance from the water source did not change. Trees growing 20 m from water did not show any symptoms of disease, while trees growing just along the banks were usually dead (Table 2).

Table 1. Sequence of primers and probes used to identify *Phytophthora* in the analyzed samples

Species	Primer F (5'–3')	Primer R (5'–3')	Probe F (5'–3')	Refeences
P. alni	ctgtcgatgtcaaagttg	atgggtttaaaagataagg	acccaaacgctcgccat	in this study
P. multiformis	ccgtatcaacccacttag	cacagtatgttcagtattcaa	cggcctggctgtcgatatca	Nowakowska et al. 2016
P. cactorum	acgtgaaccgtttcaaac	cagccgccaacaataaag	cagccgccaccagacaagac	Nowakowska et al. 2017
P. plurivora	ccgtatcaacccttttag	gcagtataatcagtattgtaga	ccccgcagtataatcagtattgtagaa	Nowakowska et al. 2017

Table 2. Number of healthy, sick and dead tress depending on the distance to the bank of the Narewka River

Trees	Mean distance from the bank of river [m] ± SEM in 2012	Number of trees	Mean distance from the bank of river [m] ± SEM in 2018	Number of trees
Healthy trees	23.3 ± 7.22	22	9.6 ± 3.38	50
Sick trees	9.3 ± 4.58	48	9.3 ± 4.06	23
Dead trees	3.4 ± 1.60	9	3.4 ± 2.67	9
Total		79		82

Healthy trees 2012–2018, $p<0,01$
Healthy trees – sick trees 2012, $p<0,01$
Sick trees – dead trees 2012, $p<0,05$
Sick trees – dead trees 2018, $p<0,01$

Real-time PCR allowed the presence of *Phytophthora* to be detected in the soil samples. The result of the reaction is expressed as the Ct (threshold cycle) value, which is the number of the PCR cycle in which the instrument detected the fluorescence emission of the probe in the tested sample. The lower the Ct value, the greater the amount of the pathogen. A Ct value above 40 indicated the absence of the tested species' DNA in the sample. The analysis showed the presence of two pathogens in the soil: *Phytophthora alni* (Ct=35.57) in Site 2 and *Phytophthora cactorum* (Ct=36.77) in Site 3 (Table 3). The analysis did not reveal the presence of *Phytophthora multiformis* or *Phytophthora plurivora* in the tested soil samples.

4. Discussion

It was initially thought that alder dieback may be caused by drought (Oszako 2005), but current research has shown that most of the dead trees are on the river bank (Table 2). The long drought occurring in Białowieża in 2015 (Nowakowska et al. 2020) may have been the cause of the observation that in 2018, healthy trees were located closer to the river than in 2012. In south-eastern Poland, ten *Phytophthora* species have been found, including *Phytophthora alni* and *Phytophthora cactorum*, on trunks and in the rhizosphere of alders (Trzewik et al. 2015). The occurrence of *Phytophthora cactorum* was recorded around the Siemianówka Reservoir, located in two countries – Belarus and Poland (Malewski et al. 2019). *Phytophthora alni* (Zviagintsev et al. 2015) was found in the alder forests of Belarus. The Narewka River flows out of the Dziki Nikor and Kuty wilderness areas in Belarus at an altitude of 159 m and flows into the Narew River at an altitude of 137 m above sea level. (Górniak 2006). Thus, *Phytophthora* can move with its waters (Cahill et al. 2008), and the difference in the levels ensures that the buoyant spores can move with the current and infect encountered trees. The occurrence of *Phytophthora cactorum* was also observed along the Polish-Ukrainian river section in the area of Włodzimierz Wołyński, Jarosław and Medyka (Matsiakh et al. 2016).

Phytophthora can be carried not only by water but also by birds. *Phytophthora cactorum* is especially often carried by: the Eurasian siskin *Carduelis spinus*, the greenfinch *Chloris chloris* L. and the blue tit *Paruscae ruleus* L. (Malewski et al. 2019). Taking into account the presence of *Phytophthora* along the Siemianówka Reservoir and the Polish-Ukrainian border, it can be expected that it also occurs in the natural forest communities of the Białowieża Forest. Confirmation of *Phytophthora*'s presence in the soil samples taken from under the alder trees suggests that this may be the cause of their dieback in the Białowieża Forest District.

Conclusions

The largest number of sick and dead alders is closest to the bank of the Narewka River, which allows us to reject the assumed hypothesis of water shortage in the soil as the cause of their dying.

Table 3. Analysis of the presence of *Phytophthora* in soil samples

Place	C_t value				
	Sample	*P. alni*	*P. cactorum*	*P. multiformis*	*P. plurivora*
Place 1	Sample 1	>40	>40	>40	>40
	Sample 2	>40	>40	>40	>40
	Sample 3	>40	>40	>40	>40
Place 2	Sample 1	>40	>40	>40	>40
	Sample 2	>40	36,77	>40	>40
	Sample 3	>40	>40	>40	>40
Place 3	Sample 1	35,57	>40	>40	>40
	Sample 2	>40	>40	>40	>40
	Sample 3	>40	>40	>40	>40

Two pathogens were found (using the real-time PCR method): *P. alni* and *P. cactorum* in soil samples taken from the Białowieża Forest District.

Conflict of interest

The authors declare the lack of potential conflicts of interest.

Funding sources

The study used the results of the research of the Forest Research Institute financed by State Forests.

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Authors' contribution

K.S. – concept, experiment, analysis of results, text editing; T.O. – concept, text editing; K.K. – experiment, analysis of results, text editing; J.A.N.– concept, analysis of results, text editing; T.M. – analysis of results, text editing.

Translated by: Barbara Przybylska