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SHORT COMMUNICATION

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Molecular detection of oomycetes species in water courses

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Abstract

In Poland, about 20% of forest nurseries use irrigation water coming from natural superficial reservoirs, presumed to be the first source of infection caused by harmful pathogens belonging to the Oomycota class, especially *Phytophthora* genus and *Pythium* genus. The forest nursery is the only place where forest managers can react before pathogens leave it with asymptomatic plants or soil attached to their roots. The aim of this research was detection and identification phytopathogens in water samples. In order to recognise genus *Phytophthora* or *Pythium* in water collected from 33 places in five different forest districts in Poland, two DNA-based approaches of identification were applied: (i) the TaqMan probes, and (ii) sequencing of the ITS6/4 region.

The genomic DNA was obtained from 17 of 33 investigated water samples. TaqMan probes helped to identify 8 oomycetes present in 17 water samples. Based on ITS rDNA sequencing data, pathogens were identified in 17 cases, and this to the genus level (6 cases) and to the species level (11 cases). In total five Oomycetes species were identified, i.e. 3 *Pythium* species (*Py. citrinum*, *Py. angustatum*, *Py. helicoides*) and two *Phytophthora* species (*P. lacustris* sp. nov. – former taxon Salixsoil, *P. gallica* sp. nov.).

Key words

ITS, forest nurseries, *Phytophthora*, *Pythium*, sequencing, TaqMan probes, water courses

Introduction

Many forest nurseries (ca. 20%) in Poland take water from superficial sources like rivers or lakes. Such water is contaminated with plant pathogens, especially those with biological cycle related to water-like oomycetes. The most harmful organisms belong to *Phytophthora* and *Pythium* genus. Their occurrence in water was already identified by horticulturists (Orlikowski 2006; Orlikowski et al. 2007, 2009). In this paper, we wanted to check whether pathogenic oomycetes are present in small streams passing through forests and fields in eastern part of Poland. To this aim, two approaches were adopted: (i) TaqMan probes were applied in order to recognise genus *Phytophtora* and/or *Phytium*, and (ii) sequencing of the ITS4/6 region helped to identify oomycetes to the species level.

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Methodology

Water samples (Tab. 1, Fig. 1) of 1.5 l volume each were filtered through several membrane filters in order to collect mycelium and spores of oomycetes presumably present in streams. In the first round, water was filtered through 8 µm diameter pores filter; filters were then collected and kept at 4°C until further processing. In the second round, water was filtered through 5 µm in diameter pores filter; collected filters were handled as described above.

Table 1. Characteristics of sites where water samplings were preformed

No.	Forest directorate	Water origin	Coordinates
1	2	3	4
1	Borki	Lake in nursery	54° 5’18.02”N, 21°54’41.18”E
2	Borki	Litygajno lake	54° 6’5.02”N, 22° 9’43.63”E
3	Borki	Lake in nursery	54° 5’18.02”N, 21°54’41.18”E
4	Borki	Lake in nursery	54° 5’18.02”N, 21°54’41.18”E
5	Borki	Litygajno lake	54° 6’5.02”N, 22° 9’43.63”E
6	Borki	Wolisko lake	54° 5’52.75”N, 22° 5’18.92”E
7	Borki	Litygajno lake	54° 6’5.02”N, 22° 9’43.63”E
8	Białowieża	Narewka river	52°41’43.03”N, 23°51’47.86”E
9	Białowieża	Leśna river	52°44’37.00”N, 23°34’41.00”E
10	Hajnówka	Orlanka river	52° 44’ 46’’ N, 23° 19’ 33’’ E
11	Białowieża	Krynica river	52°44’8.15”N, 23°45’50.14”E
12	Rudka	Forest stream	52°44’25.00”N, 22°52’54.00”E
13	Rudka	Forest stream	52°44’14.42”N, 22°51’57.59”E
14	Borki	Wolisko lake	54° 5’52.75”N, 22° 5’18.92”E
15	Żednia	Lake Siemianówka	52°54’17.22”N, 23°50’8.54”E
16	Rudka	Nurzec river	52°44’20.00”N, 22°49’47.00”E
17	Białowieża	Narewka river	52°41’24.67”N, 23°52’44.61”E
18	Borki	Wolisko lake	54°5’52.75”N, 22°5’18.92”E
19	Rudka	Nurzec river	52°44’21’’N, 22°49’48’’E
20	Bielsk Podlaski	Biała river	52°45’42.00”N, 23°11’37.00”E
21	Rudka	Forest stream Brok	52°42’10.00”N, 21°54’21.00”E
22	Rudka	Forest stream Turka	52°42’1.00”N, 21°52’20.00”E
23	Rudka	Forest stream Truchełka	52°41’24.00”N, 21°42’5.00”E
24	Przasnysz	Forest nursery	53°1’29.42”N, 20°52’49.56”E
25	Przasnysz	Forest nursery	53° 1’29.42”N, 20°52’49.56”E
26	Koło	Forest nursery	52°19’46.19”N, 18°45’0.31”E
27	Koło	Forest nursery	52°19’46.19”N, 18°45’0.31”E
28	Koło	Forest nursery	52°19’46.19”N, 18°45’0.31”E
29	Koło	Forest nursery	52°19’46.19”N, 18°45’0.31”E
30	Koło	Forest nursery	52°19’46.19”N, 18°45’0.31”E
31	Białowieża	Narewka river	52°42’5.19”N, 23°51’1.56”E
32	Białowieża	Lake in Białowieża park	52°42’6.35”N, 23°50’45.03”E
33	Białowieża	Teremiski- Białowieża, Narewka river	52°42’21.21”N, 23°49’35.98”E

In order to obtain pure cultures of investigated water oomycetes, filter discs (5 µm) were placed on selective medium PARPNH (unclarified V8-agar amended with 10 µg ml^{-1} pimaricin, 200 µg ml^{-1} ampicillin, 10 µg ml^{-1} rifampicin, 25 µg ml^{-1} pentachloronitrobenzene (PCNB), 50 µg ml^{-1} nystatin, and 50 µg ml^{-1} hymexazol) and left for 24 h at 22°C. After 24 h, fil-

ter discs were removed, and plates with medium were incubated for next 5 days. Every day, plates were investigated, and pure isolates were subcultured on fresh PARPNH medium. Further cultivation of pure cultures was done on V8 agar medium at 22°C.

Figure 1. Alder decline along Krynica river in Białowieża forest district

Pure cultures, resistant to selective antibiotics present in medium, were obtained from samples: 10, 11, 12, 13, 19, 22, 23, 28, 29, 30, and subjected to further molecular analyses.

DNA extraction

Prior to DNA extraction, pure cultures of isolated organisms were cultivated in V8 liquid medium for 5 days at 22°C. Mycelium was ground with mortar and pestle in the presence of liquid nitrogen, and nucleic acids extraction was performed using Qiagen kit according to the manufacturer's instructions (QIAGEN, Warsaw, Poland). Quality and quantity of extracted DNA were analysed through agarose gel electrophoresis and with NanoDrop® ND-1000 (Wilmington, USA).

TaqMan PCR

TaqMan PCR amplification of the internal transcribed spacer 1 (ITS-1) regions of selected Oomycetes isolates was performed with FITS_15Ph; RITS_279Ph primers and so called All_Phytophthora probe as described by Kox et al. (Tab. 2). Amplification of gDNAs was followed by Chromo4 Real Time PCR System (BioRad) and the iQ®Supermix (Biorad). Reaction mixture of 30 µl contained 15 µl of 2x, 0.6 µl of 50x ROX Reference Dye II, 83 nM TaqMan probe, 250 nM of each primers and 2 µl of genomic DNA (in concentration 1 ng/µl). The results of TaqMan PCR (Ct value) are described in Table 3.

Table 2. Sequences of oligonucleotides used in the study

Name	Sequence (5'–3')	Modifications
FITS_15Ph	TGCGGAAAGGATCAT-TACCACACC	–
RITS_279Ph	GCGAGCCTAGACATC-CACTG	–
All_Phytoph-thora probe	TTGCTATCTAGTTA-AAAGCA	FAM/MGB
ITS6	AAGGTGAAGTCGTAA-CAAGG	
ITS4	TCCTCCGCTTATT-GATATGC	

Table 3. Results on TaqMan PCR amplification of ITS1 region of rDNA

Sample	Position	Dye	Ct value
10/1	A7	FAM	18.97
10/2	B7	FAM	17.94
11	C7	FAM	18.11
12/1	D7	FAM	N/A
12/2	E7	FAM	N/A
13/1	F7	FAM	N/A
13/2	G7	FAM	16.90
19/1	H7	FAM	18.06
19/2	A8	FAM	23.31
22/1	B8	FAM	N/A
22/2	C8	FAM	N/A
23	D8	FAM	23.89
28/1	E8	FAM	N/A
28/2	F8	FAM	21.37
29/1	G8	FAM	N/A
29/2	H8	FAM	N/A
30	A9	FAM	N/A

DNA sequencing and oomycete species identification

rDNA region (ITS1-5,8SrRNA-ITS2) of all DNA samples, regardless of TaqMan PCR results, were sequenced using primers ITS6/ITS4 as described by White et al. (1990) and Cooke et al. (2000). Sequencing was performed in CEQ™ 8000 sequencer (Beckman Coulter Inc., Fullerton, USA) and data analysis performed with BioEdit software v 7.1.3 (http://www.mbio.ncsu.edu/Bioedit/bioedit.html). Consensus sequence was compared with sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov) and the species was identified based on 100% similarity at the nucleotide level. Phylogram of sequences obtained from analysed DNA samples was built with MEGA 6 software (Tamura et al. 2013).

Results and discussion

Analysis of DNA extracted from pure cultures with TaqMan PCR, revealed that 8 of total 17 sequenced samples were recognized by *Phytophthora*-specific probe (Tab. 3). Even though mycelium growth was observed on selective medium in rest of the samples, they were not recognised as *Phytophthora* by the TaqMan probe.

Based on sequencing data, three *Pythium* and two *Phytophthora* species were identified in the investigated water samples (Tab. 4). *Phytophthora lacustris* sp. nov. (former taxon Salixsoil) is considered to be typical organism existing natural water ecosystems (Nechwatal et al. 2013). *Phytophthora gallica* sp. nov. was isolated from rhizosphere soil of a declining oak in Northeast France, and from the rhizosphere of Phragmites australis in south-west Germany in 1998 and 2004 (Jung and Nechwatal 2008). It is first report of this species in Poland. *Pythium citrinum* is closely related to *Py. sterilum* sp. nov., which was 10 years ago already found in Poland (Belbahri et al. 2006). To our knowledge, there were no records about the presence of *Py. citrinum* in Polish ecosystems, so far. *Pythium angustatum* n. sp. has been reported as parasitic in green algae (Sparrow 1931). The last mentioned Pythium species found in water courses, and *Pythium helicoides* causes root rot of miniature roses (Kageyama et al. 2002).

Tentative identification to species level of oomycetes in other water samples failed.

In four cases of oomycetes identification, there were no concordance between two molecular methods used (Tab. 4). The mentioned cases concerned *Py. citrinum* and *Py. sterilum*. Since *Phytophthora* and *Pythium* genus are closely related in taxonomy, a high similarity in ITS sequences, especially in regions close to 18S and 5.8S rRNA gene, was observed (Fig. 2). Therefore, some of the *Pythium* species may be improperly recognised by TaqMan probe. Sequencing of the DNA from all isolates, regardless the TaqMan PCR result, revealed that *Py. citrinum* and *Py. sterilum* are recognised by TaqMan probe (Kox et al. 2007).

Table 4. Oomycetes species identified on ITS-based sequences recognized with 100% of similarity with NCBI database

Sample number	Species	Accession number to NCBI	Concordance with TaqMan detection
10/1	*Phytophthora lacustris*	JX271790	+
10/2	*Phytophthora lacustris*	JX271791	+
11	*Pythium citrinum*	JX271792	–
12/1	*Pythium* sp.	JX271793	+
12/2	*Pythium angustatum*	JX271794	+
13/1	*Pythium* sp.	JX271795	+
13/2	*Phytophthora lacustris*	JX271796	+
19/1	*Pythium sterilum*	JX271797	–
19/2	*Pythium sterilum*	JX271798	–
22/1	*Pythium angustatum*	JX271799	+
22/2	*Pythium* sp.	JX271800	+
23	*Pythium sterilum*	JX271801	–
28/1	*Pythium helicoides*	JX271802	+
28/2	*Phytophthora gallica*	JX271803	+
29/1	*Pythium* sp.	JX271804	+
29/2	*Pythium* sp.	JX271805	+
30	*Pythium* sp.	JX271806	+

„+" – two methods confirmed the genus identification.

„–" – only ITS region sequencing identified genus or species.

The occurrence of plant pathogens in water courses (especially plant destroyers as phytophthoras) is an important information for nursery managers responsible for the health of plants for plantings. In Poland, about 20% of forest nurseries use irrigation water coming from natural superficial reservoirs. The only filters

they use (sand filters working under high pressure of water) are not able to stop all microscopic organism quickly passing through the sand particles, including plant pathogens. The filtration process is quite efficient in purifying water from weed seeds but not from propagules of *Phytophthora* and *Pythium* species, which are often found in water (also in investigated samples). Orlikowski et al. (2008) found *Phytophthora* species in rivers and confirmed pathogenicity of *P. citricola* toward alder, rhododendron and thuja (now *P. plurivora*, Jung and Burgess 2009). Apart from *P. plurivora*, he also listed *P. cambivora*, *P. citrophthora* and *P. cryptogea*, as well as two quarantine species: *P. cinnamomi* and *P. ramorum*. The last one is not only a cause of Sudden Oak Death in USA but also very harmful organism of Japanese larch in Great Britain. *P. cinnamomi* has large number of potential hosts and is devastating forest ecosystems in Australia.

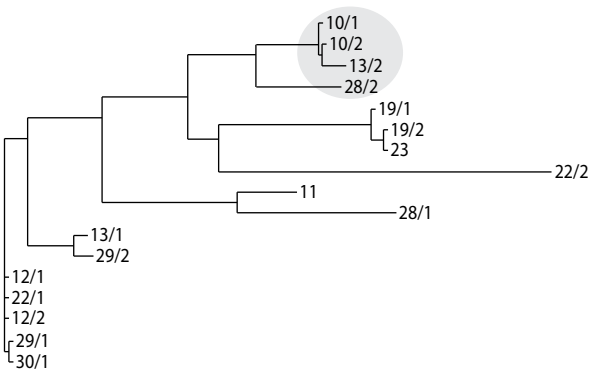

Figure 2. Phylogram of sequences obtained from analysed DNA samples. With circle a *Phytophthora* clade is marked

On the other hand, nursery is the only place where forest managers can react before pathogens leave it with asymptomatic plants or soil attached to their roots. Our report of quick DNA-based diagnosis is an important tool that can be supplied to end-users.

In addition, more detailed studies based on DNA analysis allow to detect and identify not only organisms present in water but also in soil or asymptomatic plant tissues. Knowing the exact pathogen species and their potential hosts forest allows managers to design preventive methods e.g. sowing of resistant or tolerant plants. Such approach allows to avoid infection and use of pesticides in nurseries. The use of so-called slow sand filters can be an another example of avoiding problems and coping with pathogens to reach their hosts. Finally, based on voluntary DNA tests, investigated nurseries can get certificate „free of pests", which help plant producers to be more competitive on the national, European or international market.

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