PHYTOPHTHORA GENUS PATHOGENS ISOLATED FROM RHODODENDRONS IN LITHUANIA

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Abstract
Rhododendron spp. plants were surveyed for *Phytophthora* infection in Lithuania during 2010 – 2016. This study aims to identify *Phytophthora* genus pathogen which infects rhododendrons in Lithuania. Samples were taken from young sick plants with visible infection symptoms. Soil sampling was performed from the rhizosphere of sick plants. DNA from soil and plant was tested for the presence of *Phytophthora* genus pathogens. Data showed positive results of *Phytophthora* genus specific probe during real-time PCR. All tested diseased leaves and soil samples have indicated *Phytophthora* sp. infection during Alert-LF® *Phytophthora* spp. analysis. The extracted DNA concentrations were not very high for *Phytophthora* species identification, but in most cases, it was high enough for further researches.
Key words: *Phytophthora* genus, *Rhododedron*, Lithuania.

Introduction

Rhododendrons are popular between professional and hobbyist abreast because of the great variety (ca. 12000 cultivars) and exceptional decorativeness (Malciūtė & Naujalis, 2010). In Lithuania, rhododendrons were first introduced in the Vilnius Botanical Garden in 1814 (Skridaila, 1996). They were seldom cultivated in Lithuania until the second half of the 20th c. However, rhododendron plants have spread rapidly in Lithuania during the last thirty years (Navasaitis, 2004). Intolerance to the low and negative temperatures is one of the most important limiting factors for rhododendrons in Lithuania (Malciūtė, Naujalis, & Šiaulienė, 2011). However, there is a shortage of information on the phytosanitary state of rhododendrons in Lithuania. Several fungi were isolated from rhododendrons in Lithuania, e.g. *Erysiphe azaleae* (U. Braun) U. Braun & S. Takamatsu and *Exobasidium japonicum* Shirai causing rhododendron mildew and leaf blisters (Grigaliūnaitė & Pribušauskaitė, 2006; Lygis *et al.*, 2010).

Phytophthora is plant pathogen genus belonging to *Oomycetes* which cause many plant species diseases. The first *Phytophthora* infections isolated from rhododendrons in other countries were documented ca. sixty years ago. At least ten *Phytophthora* species have been reported to be connected with *Rhododendron* roots, twig, and leaf pathogenesis worldwide: *Phytophthora cactorum* (Leb. and Cohn) Schröet., *P. cinnamomi* Rands, *P. citricola* Sawada, *P. citrophthora* (R.E. Smith and E.H. Smith) Leonian, *P. cryptogea* Pethybridge and Lafferty, *P. gonapodyides* (Petersen) Buisman, *P. lateralis* Tucker and Milbrath, *P. megasperma* Drechsler, *P. nicotianae* Breda de Haan, *P. palmivora* (Butler) Butler, *P. ramorum* Werres, De Cock & Man in 't Veld, and *P. syringae* (Klebahn) Klebahn (Benson & Jones, 1980; Erwin & Ribeiro, 2005; Hoitink & Schmitthenner, 1974; Nienhaus, 1960; Werres *et al.*, 2001). *P. ramorum* first isolated from rhododendrons in Europe in 2002 (Orlikowski & Szkuta, 2002; Morajelo & Werres, 2002), the pathogen quickly spread to rhododendron nurseries and outdoor plantations in many European countries.

In Lithuania, the increasing number of rhododendrons infected by *Phytophthora* genus fungi has been reported (Table 1).

Phytophthora citricola was first isolated in Lithuania from drying rhododendron branches and top of twigs in 2002. It was isolated in *Rhododendron*

Table 1

***Phytophthora* species isolated from rhododendrons in Lithuania**

Rhododendron species	Year, authors	*Phytophthora* species	Location	Injuries
R. catawbiense 'Grandiflorum'	2002; Jovaišienė, Lane	*P. citricola*	Marijampolė district	Branches and top of twigs
R. sp.	2004; Jovaišienė, Lane	*P. cactorum*	Kaunas, private collection	Leaf and twigs
R. sp.	2004; Jovaišienė, Lane	*P. cactorum*	Kaunas and Šiauliai Botanical Garden	Leaf and twigs
R. catawbiense 'Grandiflorum'	2006; Jovaišienė, Lane	*P. ramorum*	Marijampolė district	Leaf and twigs

catawbiense 'Grandiflorum' in the Marijampolė district. *Phytophthora cactorum* was first isolated from the collection of rhododendrons in Kaunas and Šiauliai Botanical Gardens in 2004 (Jovaišienė & Lane, 2006). *P. ramorum* was first isolated on fifty shrubs of *Rhododendron catawbiense* 'Grandiflorum' imported from Poland in the market centre of ornamental plants in Marijampolė district (Jovaišienė & Lane, 2006). This species was described at first on oranges in Taiwan as a disease agent of brown rot in 1927 (Sawada, 1927). At present, *P. citricola* is spread in Europe and it is a disease agent of collar roots and stem canker in many economically important crops. Therefore, it is considered as an aggressive pathogen. The investigation on *Phytophthora* genus fungi started in Lithuania State Plant Protection Service in 2002. Several years later, the investigation has started in the Kaunas Botanical Garden of Vytautas Magnus University (VMU).

This study was aimed to identify *Phytophthora* genus pathogen infecting rhododendrons in Lithuania.

Materials and Methods

During 2010 – 2016 at least 6 samples were taken from each place which had sick *Rhododendron*. Samples were taken from young sick plants with visible infection symptoms, e.g., top and leaf wilting, leaf blotch and longitudinal twisting, leaf browning along the main vessel, and twig necrosis. Soil sampling was performed from the rhizosphere of sick plants. The sampling place are Kaunas Botanical Garden of Vytautas Magnus University and Alytus Park.

The taken samples are stored in the zip bags. They could be stored under +4 °C for a longer period. Leaves are washed with a tap water one time, branches and pieces of the stem are washed two times. Dry parts of plants are soaked into the tap water for 24 hours, washed two times. Samples, cut from a necrosis border are cut into 5x5 mm pieces and put into at least four Petri dishes with growing medium under sterile environment (Jung, Blaschke, & Neumann, 1996).

The soil bating is performed in a bath, which is poured with distilled water for 2/3 of its volume. On the water surface fresh oak or rhododendron leaves are placed. The bath is left for 3 – 5 days maintaining light/dark schedule and 18 °C temperature. The leaves are removed from the bath, washed out with tap water, divided into two parts and placed into the growing media. The isolation and identification of *Phytophthora* genus fungi is much more difficult in comparison to the other microscopic fungi (Werres *et al*., 2001). Therefore, various laboratory tests should be performed for the identification at the species level.

Malt extract agar (MEA) was used for the identification of *Phytophthora* species. MEA medium is produced with chloramphenicol. The prepared medium is autoclaved under 120 °C for 20 min. (Erwin & Ribeiro, 2005).

The incubation time was 1 – 3 days in darkness maintaining 24 °C. On the third day, *Phytophthora* hives are usually visible from the bottom side of the plate. Hives with medium pieces are transferred to water agar (WA) medium with capsicum or hemp seeds, which stimulate the formation of sporangia, which are formed within a few days (Jung, Blaschke, & Neumann, 1996).

The *Phytophthora* genus fungi identification at the species level is performed according to descriptors (Erwin & Ribeiro, 2005; Gallegly & Hong, 2008).

Soil and plants samples before DNA extraction were tested for the presence of *Phytophthora* sp. using Alert-LF® *Phytophthora* spp. ELISA devices (Neogen Corporation). The soil probes were taken around roots from sick *Rhododendron* plants.

The DNA from leaves was extracted using NucleoSpin® Plant II kit (Mecherey-Nagel). The soil samples were prebaited four days in a PeaBroth PARP media and DNA was extracted using PowerSoil® DNA Isolation Kit (MoBio). The PB-PARP (1000ml) was prepared by autoclaving 100g of frozen peas and using the following amendments: 0.25 g. of ampicillin, 0.01g of pimaricin, 0.01 g. of rifampicin, 0.05 g. of hymexazol, and 0.05 g. of PCNB. Extracted DNA quality was checked using spectrophotometer NanoDrop (Thermo Fisher Scientific) and electrophoresis on TAE buffer. The presence of *Phytophthora* genus DNA in samples was confirmed using real time PCR (tests were performed at the Polish Forest Research Institute) (Vitas *et al*., 2012).

Results and Discussion

In 2013 – 2015 we found sick rhododendrons in Kaunas Botanical Garden nursery and Alytus Park. The common symptoms of infected rhododendrons – top and leaf wilting, leaf spots and twig necrosis.

All ELISA tests with soil and plants samples were positive for *Phytophthora* spp. infection.

DNA extracted from soil and plant (leaves) was tested for *Phytophthora* genus pathogens. All analyzed samples showed positive results of *Phytophthora* genus specific probe during real-time PCR.

The DNA concentrations of the extracted leaf samples varied from 2.75 to 5.10 ng ml^{-1} (Table 2). The DNA concentrations from soil samples were from 5.78 to 10.02 ng ml^{-1}. The ratio of sample absorbance at 260 and 280 nm varied from 1.08 to 2.90 and the ratio of sample absorbance at 260 and 230 varied from 0.50 to 1.41. The results above show the positive use of commercial kits for DNA extraction from symptomatic leaves and soil.

All 6 samples were recognized by *Phytophthora*-specific probe. Based on this all samples can be

Table 2

DNA concentrations, quality characteristics of extracted samples and results of *Phytophthora* genus specific real time PCR

Rhododendron species	City, place	Part of the plant or soil	DNA [ng ml^{-1}]	Ratio of absorbance 260/280	Ratio of absorbance 260/230	Ct
R. catawbiense	Kaunas Botanical Garden	leaves	2.75	2.42	0.92	17.70
R. sp.	Alytus Park	leaves	5.10	1.16	0.52	24.50
R. catawbiense	Kaunas Botanical Garden	soil	6.96	1.57	0.50	25.07
R. catawbiense	Kaunas Botanical Garden	soil	10.02	1.83	0.79	25.91
R. catawbiense	Kaunas Botanical Garden	soil	7.11	1.08	0.75	20.63
R. sp.	Kaunas Botanical Garden	soil	5.78	2.90	1.41	25.62

considered as containing *Phytophthora* DNA. The amount of DNA in the sample is expressed by the Ct value. Lower value means that more *Phytophthora* DNA is present in the sample. The lowest (Ct=25.91) amount of pathogen DNA was found in soil of Kaunas Botanical Garden rhododendron nursery (Table 2).

The extracted DNA concentrations were not very high for *Phytophthora* species identification, but in most cases, it was high enough for further researches.

Phytophthora genus pathogens spread is favoured by environmental conditions: soil flooding and excess moisture, droughts, and temperature extremes (Erwin & Ribeiro, 2005). Also, an intensive international trade of living plants accelerates the spreading of alien species over long distances (Jung *et al.*, 2005).

Conclusions

1. The common symptoms of *Phytophthora* infected rhododendrons – top and leaf wilting, leaf spots and twig necrosis.
2. All tested diseased leaves and soil samples have indicated *Phytophthora* sp. infection during Alert-LF® *Phytophthora* spp. analysis.
3. All tested samples can be considered as containing *Phytophthora* DNA. The extracted DNA concentrations were not very high for Phytophthora species identification, but in most cases, it was high enough for further researches.

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