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Four different *Phytophthora* species that are able to infect Scots pine seedlings in laboratory conditions

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Abstract

To investigate susceptibility of young Scots pine seedlings to four *Phytophthora* species: *Phytophthora cactorum*, *Phytophthora cambivora*, *Phytophthora plurivora* and *Phytophthora pini*; seven-day-old seedlings of Scots pine (15 seedlings per experiment) were infected using agar plugs of the respective species. Control group also consisted of 15 seedlings and was inoculated with sterile agar plugs. Results unambiguously show that after 4.5 days, all seedlings show clear signs of infection and display severe symptoms of tissue damage and necrosis. Moreover, three and two seedlings in the *P. cactorum* and *P. cambivora* infected seedlings groups, respectively, collapsed. The length of largest necrosis measured 13.4±3.90 mm and was caused by *P. cactorum*. To rule out any putative contamination or infection by secondary pathogens, re-isolations of pathogens from infection sites were performed and were positive in 100% of plated pieces of infected seedlings. All re-isolations were, however, negative in the case of the control group. Detailed microscopic analyses of infected tissues of young seedlings confirmed the presence of numerous *Phytophthora* species inside and on the surface of infected seedlings. Therefore, our results suggest *Phytophthora* spp. and mainly *P. cactorum* and *P. cambivora* as aggressive pathogens of Scots pine seedlings and highlight a putative involvement of these species in the damping off of young Scots pine seedlings frequently observed in forest nurseries.

Key words

pathogenicity, *Pinus sylvestris*, *Phytophthora cambivora*, *Phytophthora cactorum*, light microscopy

Introduction

Scots pine (*Pinus sylvestris* L.) is the most common woody species in Polish forests and is present in around 58.8% of total forest stands managed by State Forests in Poland (Milewski 2015). Unfortunately, Scots pine is susceptible to several pathogenic organisms in natural stands, planted forests and nurseries, including *Heterobasidion annosum* (Fr.) Bref. (Sierota 1996; Małecka and Sierota 2003). Different needle diseases, such as red band needle blight, caused by the fungus Dothistroma septosporum (Dorog.) Morelet also play an important role in the decline of this relevant tree species (Mańka 1998; EFSA 2013).

Phytophthora species are fungal-like organisms that nest within the SAR supergroup (Lara and Belbahri, 2011; Adl et al. 2012). Till now, more than 140 species and *Phytophthora* taxa are known (Abad 2014). These species are able to infect different tissues (fine roots, bark, stems, leaves and shoots) on different host species in nurseries, ornamental plantings and forest stands (Erwin and Ribeiro 1996; Pérez-Sierra and Jung 2013; Jung et al. 2013). Previously, several *Phytophthora* spp. have been recorded as pathogens of different conifer species, including *Phytophthora cactorum* (Leb. and Cohn) Schröeter (Hudler 2013), *Phytophthora cambivora* (Petri) Buisman (Vannini and Vettraino 2011), *Phytophthora lateralis* Tucker and Milbrath (Hansen 2011), *Phytophthora pini* Leonian (Hong et al. 2011), *Phytophthora pinifolia* (Duran et al. 2008; Hansen 2012), *Phytophthora plurivora* Jung and Burgess (Jung and Burgess 2009) and *Phytophthora citrophthora* (R.E. Smith et E.H. Smith) Leonian (Oszako and Orlikowski 2004).

Occurrence of these pathogenic *Phytophthora* spp. in nurseries and their subsequent introduction into semi-natural and natural ecosystems is the main driver of their dispersal and causes huge damage to forest ecosystems (Moralejo et al. 2009; Jung et al. 2015). In Poland, several species of *Phytophthora* were recorded in forest nurseries, including *Phytophthora citricola* Sawada on ash (Orlikowski et al. 2004), *Phytophthora cinnamomi* Rands on pedunculate oak (Oszako and Orlikowski 2005), *P. plurivora* on European beech and silver fir (Orlikowki et al. 2004; Stępniewska 2005), *P. cactorum* on European beech (Stępniewska 2003) and *Phytophthora gonapodyides* on a wide range of hosts (Oszako et al. 2007). *Phytophthora* spp. were also previously recorded on Scots pine in forest and ornamental nurseries in Poland, and the presence of *P. cinnamomi* (Duda et al. 2004), *P. cactorum, P. citrophthora* and *P. plurivora* (Orlikowski et al. 2012) was confirmed.

Owing to widespread and importance of Scots pine in Polish forests, our main goal was to determine whether the *Phytophthora* species can threaten Scots pine plants grown in nurseries and in young stands. Pathogenicity test with four selected *Phytophthora* species were, therefore, conducted, to determine the susceptibility levels of the Scots pine seedlings with selected species. Results and implications of the study are discussed in this paper.

Material and methods

Phytophthora species and isolates used in the experiment

Isolates of *Phytophthora* species used in the colonisation test were obtained from the Forest Research Institute-IBL *Phytophthora* culture collection. All isolates originated from different declining forest tree hosts. They were all morphologically identified, with molecular confirmation of morphological findings. Four different *Phytophthora* species were used in this experiment (Tab. 1).

Table 1. Description of isolates used in the experiment

Species	Host	Collection number	GenBank
P. cactorum	*Acer pseudo-platanus*	IBL325	JX276090
P. cambivora	*Fagus sylvatica*	IBL340	JX276088
P. pini	Poplar clone I214	IBL482	KF234656
P. plurivora	*Quercus robur*	IBL213	JX276023

Pathogenicity tests

In the different pathogenicity tests, non-stratified seeds of Scots pine were used (Zwoleń provenience). Seeds were incubated at 25°C under light for 24 h in glass chambers moisturised with sterile cotton wetted with sterile distilled water (Załęski et al. 1998). *Phytophthora* species used in the assay were transferred onto V8A media, prepared with 800 ml·L^{-1} distilled water, 200 ml·L^{-1} V8 juice (Tymbark, Poland), 18 g·L^{-1} agar-agar (BTL, Poland) and 3 g·L^{-1} of $CaCO_3$. The inoculum of *Phy-*

tophthora spp. was recovered from the growing edges of 3–4 days old colonies incubated at 22–25°C in the dark (Orlikowski et al. 2004; Milenković et al. 2012). Agar plugs with mycelium (~1 × 1 cm in size) were placed in sterilised, 90-mm glass Petri dishes containing sterile filter paper. Tips of seven-day-old Scots pine seedlings with a radicle length of 15–20 mm were placed on the top of agar plugs with mycelium in Petri dishes. The design of the experiment was completely randomised with a total of five replicates. For each replication, seedlings were used in individual Petri dishes (N = 15 seedlings per group). Control group also consisted of 15 seedlings and was placed on the sterile agar plugs.

Filter paper in the dishes was moisturised with 5 mL of sterile distilled water, and dishes were incubated under daylight at approximately 20–22°C. Dishes were monitored for every 8 h until the first seedlings collapsed, and measurements of the total lengths of seedlings and the necrosis length were performed.

To re-isolate the *Phytophthora* spp. from declining tissues, small necrotic parts of the seedlings (0.1–0.2 mm in size) were cut using sterile razor blade and plated on selective media (V8A-PARPNH), prepared according to Jung et al. (1996). Tissue fragments from control group were also plated onto V8A-PARPNH media. Observations were made under light microscope ZEISS Axioskop 2, equipped with Nikon Ds-fi1 camera, and NIS Elements AR4® software.

Statistical analyses

Data from the pathogenicity test were subjected to Kruskal–Wallis nonparametric test. The mean values of total length of plant, length of necrosis and percentage of necrosis were compared between the experimental groups using R software (PMCMR library).

Results

Pathogenicity tests and necrosis lengths

After 4.5 days of incubation when the first seedlings collapsed, measurements of necrosis lengths as well as total length of the seedlings were performed and are presented in Table 2. Three and two seedlings, respectively, from the *P. cactorum* and *P. cambivora* groups collapsed. Photos of necrotic lesions of these seedlings are presented in Figure 1.

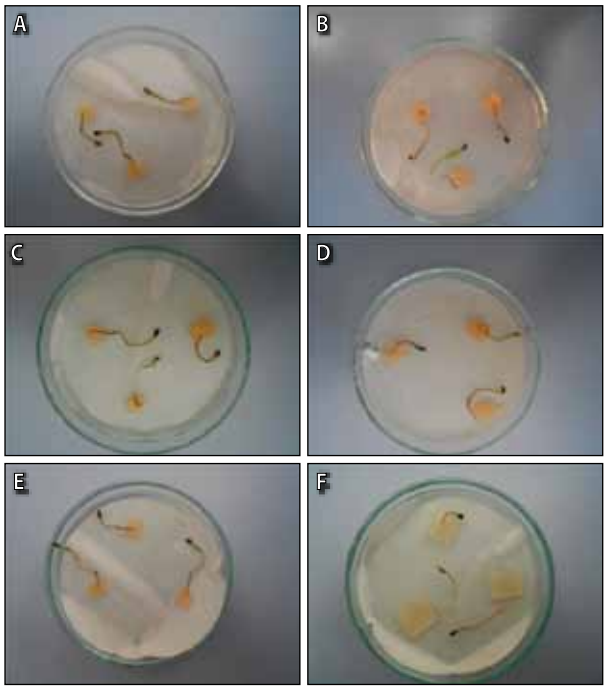

Figure 1. Necrotic lesions on seedlings caused by different *Phytophthora* species: A – *P. cambivora*; B – *P. plurivora*; C – *P. pini*; D–E – *P. cactorum*; F – control

Table 2. Pathogenicity of the different *Phytophthora* species inoculated on seedlings of Scots pine

Species	Number per experimental group	Number of collapsed seedlings	Total length of seedlings	Necrosis length	Percentage of necrotic length
			mean ± SD (mm)		% ± SD
Control	15	0	43.06±10.51	0	0
P. cactorum		3	37.00±4.14	13.4±3.90	37.49 ±14.15
P. cambivora		2	36.73±9.67	11.80±3.36	32.81±7.59
P. pini		0	43.46±12.87	10.93±3.89	27.09±10.85
P. plurivora		0	39.53±10.44	9.66±3.10	26.41±10.59

Re-isolations were successful in 100% of plated necrotic parts of the seedlings onto selective agar media, whilst the parts from control group showed no growth and were negative for the presence of any pathogenic or saprotrophic organisms.

Nonparametric test was applied to check statistical significance of seedling lengths between the tested experimental groups. No statistically significant difference between the tested experimental groups could be observed ($p = 0.337$). However, necrosis length between the different experimental groups was statistically significant ($p = 0.000$). To determine which experimental groups were statistically different, the post hoc test was applied. Results clearly suggest statistical support for the difference between each *Phytophthora*-infected experimental and the control group. We could also provide statistical support for *P. cactorum* causing the largest necrosis length than all the other tested species (Fig. 2).

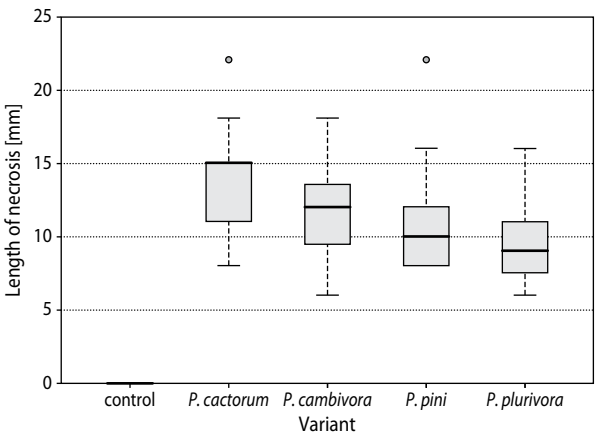

Figure 2. Necrosis lengths observed on Scots pine seedlings infected with the different *Phytophthora* spp.

Percentage of necrosis length (estimated as the percentage of observed necrosis per total plant length) was calculated for the control and experimental groups. The comparison between percentage of necrosis length amongst the different groups showed statistically significant difference between control and experimental groups infected by the respective *Phytophthora* species ($p = 0.000$). Between variants in which the *Phytophthora* was tested, no statistically significant difference was observed. The highest value was recorded for the *P. cactorum* experimental group (Fig. 3).

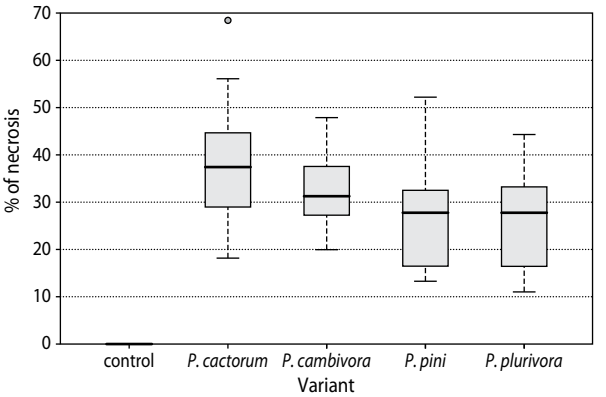

Figure 3. Percentages of necrosis length across the different control and *Phytophthora* infected experimental groups

Microscopic analyses

All tested *Phytophthora* spp. in the time course of this experiment were able to colonise the tissues of young seedlings. Observation under light microscope showed the presence of numerous *Phytophthora* structures in the tissues of all the infected seedlings. In most of the cases solely, oogonia with oospores were recorded (Fig. 4), whilst in some other cases, both oogonia and sporangia were recorded (Fig. 4C, D and H). No *Phytophthora* structures were recorded in the control group in which the tissues remained healthy.

Discussion

Results obtained in the course of this study unambiguously document the ability of the four tested *Phytophthora* species to infect the young Scots pine seedlings. Severe seedlings symptoms and damage to infected tissues reflected by necrosis length that differ in size between the respective pathogens were observed. The most aggressive species in our experimental conditions was *P. cactorum,* according to all monitored parameters. This homothallic species with papillate sporangia was one of the first described species from the *Phytophthora* genus (Erwin and Ribeiro 1996). This pathogen is causing damages on a wide range of hosts worldwide in nurseries, forest stands, ornamental and amenity plantings (Erwin and Ribeiro 1996), including Scots pine (Hudler 2013). This species belongs to *Phytophthora* Clade 1 (Martin et al. 2014) and is able to operate dif-

ferent hybridisation events (Érsek and Man in 't Veld 2013), giving rise to more aggressive and destructive pathogens (Man in't Veld et al. 2007, 2012).

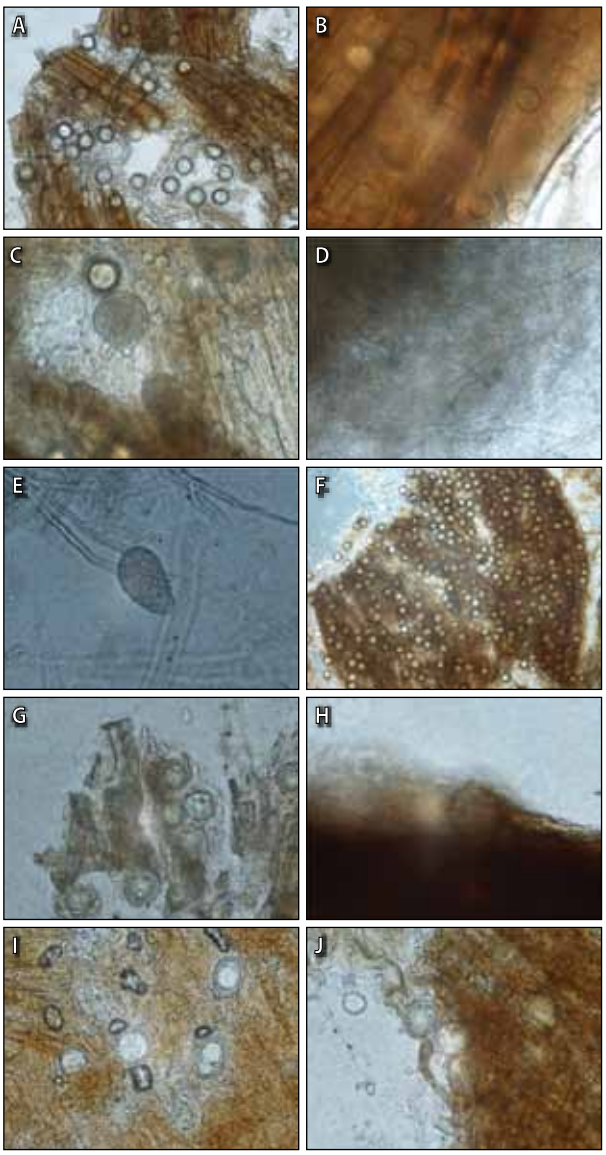

Figure 4. Microscopic analyses of the necrotic length of Scots pine tissue

P. cactorum: A – numerous oogonia in the necrotic tissue at ×200 magnification, B – oogonia in the tissue at ×400 magnification, C – oogonia and papillate sporangia in the broken necrotic tissue at ×400 magnification; ***P. cambivora***: D – hypha and young sporangia at ×200 magnification, E – non-papillate, mature sporangia on the surface of infected seedlings at ×400 magnification; ***P. pini***: F – numerous oogonia in the tissue at ×100 magnification, G – oogonia in the broken necrotic tissue at ×400 magnification, H – semi-papillate sporangia on the surface of necrotic tissue; ***P. plurivora***: I and J – oogonia in the necrotic tissue.

The second more aggressive species in our experiment was *P. cambivora*. This is heterothallic species with non-papillate sporangia, easily recognised by ornamented oogonia and two cells antheridia. It is known worldwide as the causative agent of ink disease (Erwin and Ribeiro 1996).

The third aggressive species in this experiment is *P. plurivora*, one of the most widespread species in different ecosystems worldwide. This species was previously lumped in the *P. citricola* Sawada species complex but was recognised as a new species in the study of Jung and Burgess (2009). It can parasitise a wide range of plant hosts, causing different damages and thriving in different ecological niches (Jung and Burgess 2009).

All listed species were often previously reported from different Polish nurseries (Duda et al. 2004; Orlikowski et al. 2012). However, there is no much data concerning *P. pini*. *P. pini* was described for the first time by Leonian (1925) and resurrected by Hong et al. (2011) as a part of the *P. citricola* complex. According to these authors, this species is a pathogen of at least seven genera in Europe and North America and many past damages that were assigned to *P. citricola* were actually caused by this species and *P. plurivora*. In Europe, this species was recorded in Finnish nurseries (Rytkönen 2011) and in poplar plantations in Serbia (Milenković I., unpublished data).

During the analyses of necrotic tissue using light microscopy, numerous oogonia were recorded inside and on the surface of the tissue of infected seedlings. Hyphae and sporangia of tested *Phytophthora* species were also recorded in congruence with multicyclic nature of these pathogenic organisms (Erwin and Ribeiro 1996).

Results obtained in this experiment highlight the potential risk caused by the presence of these species in Scots pine nurseries and in the first years after transferring them to the forest. On the basis of our preliminary results, a large-scale study involving sampling, isolation and species diversity determination of *Phytophthora* species in different young conifer stands and nurseries of Scots pine is warranted. Soil-infestation pathogenicity tests, as the closest experimental conditions to natural way of infection, are also required in the future to evaluate the potential of *Phytophthora* species to infect and damage mature Scots pine roots in field conditions.

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