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ORIGINAL ARTICLE

Incidence of *Phytophthora* species in beech stands in Serbia

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Abstract

According to many surveys of pathogenic organisms in forest soils, the presence of the *Phytophthora* genus is very common in both dominant and mixed stands of European beech. In Serbia, *Phytophthora* species were isolated from rhizosphere soil in declining, as well as apparently healthy stands. After detailed morphological and molecular identification, several *Phytophthora* species were confirmed. The most common pathogen of fine roots in Serbian European beech stands was *Phytophthora plurivora* Jung and Burgess. This species was characterized as homothallic, semipapillate, produces sporangia of various shapes, and has an optimum temperature for growth at around 25ºC.P. *plurivora* occurred on 58% of positive samples, followed by *P. cambivora* (Petri) Buisman at 17%, *P. gonapodyides* (Petersen) Buisman at 8%, with other unidentified species accounting for the remaining 17%. A pathogenicity test performed with *P. plurivora* and young beech germinants from ten Polish beech provenances demonstrated the ability of this pathogen to colonize and cause deterioration of plant tissue.

Key words

Oomycetes, *Fagus sylvatica* L., PCR, ITS sequencing

Introduction

Phytophthora species are fungi-like organisms belonging to the kingdom *Chromista* (Kirk et al. 2008). These pathogens infect different tissues, e.g. fine roots, bark and cambium of woody roots and stems, and the shoots and leaves of a very wide range of host species in nurseries, ornamental and amenity plantings, and forest ecosystems (Erwin and Ribeiro 1996). The main reason for their almost ubiquitous distribution throughout the world is the growth of the international trade of living plants (Evans and Oszako 2006; Brasier 2008), and the introduction of *Phytophthora* with infected nursery stock into parks, forests, and natural ecosystems (Brasier and Jung 2006). According to Brasier (2009) about 110 species and informally designated *Phytophthora* taxa are known (Brasier 2009).

European beech (*Fagus sylvatica*) is one of the most widespread and most important tree species in Serbia, comprising around 29% of all woodland (Banković et al. 2009). The involvement of *Phytophthora* species in beech decline was previously reported in several stud-

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ies: Brasier et al. 2005; Jung et al. 2005; Jung 2009; Jung and Burgess 2009; Orlikowski et al. 2006; Schmitz et al. 2007; Munda et al. 2007; Weilend et al. 2010. Data on the occurrence of *Phytophthora* species living on European beech in Serbia are relatively poor to date. However, Milenkovic et al. (2011a, b) reported the isolation of *Phytophthora* spp. from several symptomatic trees, including *Fagus sylvatica*, in forests and parks in Serbia.

Due to the high risks posed by *Phytophthora* species to forestry and biodiversity, and to European beech in particular (Jung et al. 2005; Jung 2009), a study was performed with the aim of (i) determining the presence and the main species of phytophthoras occurring in beech stands in Serbia, and (ii) performing pathogenicity tests on ten Polish beech provenances with *P. plurivora* isolates from both Serbia and Poland and with *P. cactorum* as a control species, in order to determine the threat posed by Serbian strains to Polish Beech woodland.

Material and Methods

Study locations

For these studies, seven beech stands within five different beech forest types were selected, including both dominant and mixed stands. A list of study sites and the sample numbers collected is provided in tab. 1. In total, 28 samples were taken including three samples from water courses. Sampling was performed between the autumn of 2009 and spring 2012 (tab. 1).

Sampling and isolation methods

Sampling and isolation methods were performed according to Jung (1998, 2009), and Jung et al. (1996). Tissue samples were taken from necrotic parts and plated directly onto selective agar medium (V8-PARPNH). The soil and root systems were sampled via soil monoliths measuring ~ 25 × 25 × 25 cm, and isolation tests were performed using oak and beech leaves as baits (Jung et al. 1996, 2000; Jung 2009). Water was collected in 1l plastic bottles, which were sterilized with 70% ethanol, and washed with distilled water. Sampled water was processed in the lab, using baiting techniques.

Morphological classification of isolates

For the morphological screening of isolates, non-sterile soil extract mix was prepared according to Erwin and Ribeiro (1996). All isolates were transferred onto clarified carrot agar (12–15 cm³/Petri dish), prepared with 900 ml/l of distilled water, 100 ml/l of fresh, clarified carrot juice (Tymbark, Poland), 2 g/l of $CaCO_3$, 16 g/l of agar-agar (BTL, Poland), and left to grow in the dark at ~ 25°C. After 3–5 days, ~ 15 × 15 mm pieces from the growing edge were cut and covered with non-sterile soil extract mix in 9cm Petri dishes, which were incubated at ~ 20°C in daylight. After 6 hours the soil extract mix was replaced with distilled water; the pieces were washed again after a further 6 h, and once more

Tab. 1. Study locations and samples taken in beech stands in Serbia

Location	No.	Stands studied	Coordinates (if available)	Sampling date	Number of samples taken	Number of positive samples	
						N	%
Beograd, Avala	1	Avala mountain	44°41’48,49” 20°30’34,69”	4, 8 / 2011	3	2	66
Goc	2	Goc / Bela reka	43°33’51,12” 20°48’18,10”	5 / 2011	4	2	50
Trstenik	3	Trstenik/Trstenicke sume	43°35’39,49” 20°59’30,46”	11 / 2010 + 2, 4, 8 / 2011	6	3	50
	4	Trstenik/Ljubostinjske sume	–	5 / 2012	1	0	0
Novi Pazar	5	Novi Pazar/Turjak	–	3 / 2012	7	4	57
Boljevac	6	Boljevac/ Brezovica	–	6, 7 / 2011	5	1	20
	7	Boljevac/J.Kucaj II	–	6, 7 / 2011	2	0	0

after 12 h (Jung and Burgess 2009). After 24–36 hours, cultures were observed at ×400 magnification (ZEISS Axioskop 2, equipped with Nikon Ds-fi1 camera and NIS Elements AR 4® software) and the typical features of mature and empty sporangia recorded. Additionally, pure cultures were incubated at ~20°C in the dark on carrot juice agar in order to allow for the formation of chlamydospore, oogonia, and oospores.

Colony morphology, growth rates, and cardinal temperatures

To examine colony morphology patterns, cultures were transferred onto Malt Extract (Fluka Biochemica, Sigma Aldrich®, GmbH, France), Carrot and V8 (Tymbark, Poland), agar (BTL, Poland). Colony morphologies were observed and described based upon previous publications (Erwin and Ribeiro 1996; Brasier et al. 2003; Jung et al. 1999, 2002; Jung and Burgess 2009).

To determine the growth rates of isolates, inoculum (6 mm in diameter), was taken from 24-hour old cultures and sub-cultured on fresh clarified carrot agar and incubated at 22–25°C in the dark for 24 hours. Subsequently, three replicate dishes were placed at each of the following temperatures: 5, 10, 15, 20, 25, 30, and 35°C. After an additional 24 hours of incubation, growth in each culture was marked in four directions with a sterile steel needle. Over the next five days, or until the culture filled the Petri dish, growth was marked along the axes denoted by the steel pins. Cultures which failed to grow above 35°C were returned to 20°C to check viability.

DNA isolation, amplification and sequencing

Isolates of *Phytophthora* sp. were grown in liquid V8 media (100 ml clarified V8 juice in 900 ml distilled water, amended with 2 g of $CaCO_3$) for 3–5 days in the dark at 22–25°C. The mycelium was subsequently rinsed in sterile distilled water, then dried and disrupted in liquid nitrogen prior to the DNA extraction. Total DNA was extracted from mycelium by using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich® GmbH, Germany), following the manufacturer's protocol. Polymerase chain reaction (PCR) amplification of the ITS region of the template DNA was performed using primers ITS6 and ITS4 (White et al. 1990; Cooke et al. 2000) in a 50 µl reaction containing 50–100 ng genomic DNA, 250 nM of each primer, 200µM of each dNTP, 1 mM $MgCl_2$, 1U *Taq* polymerase, 1xQ solution, and 1xPCR buffer (Qiagen Ltd., Valencia, CA, USA). The reaction was performed in a PTC-200Ô Programmable Thermal Controller (MJ Research, Inc.) for 40 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s followed by extension at 72°C for 50 s, with an initial denaturation of 3 min at 94°C before cycling and a final extension of 10 min at 72°C after cycling. A portion of the amplified products was run on 1.5% TBE-agarose gel; the presence of a single band (ca. 800 bp) was used as a check for successful amplification. The PCR product was purified using the A&A Biotechnology (Gdynia, Poland) Clean-up kit, following the manufacturer's protocol. Sequencing was conducted on a CEQ™8000 9.0.25 automated sequencer (Beckman Coulter®, Fullerton, USA). The consensus sequences were made with complementary DNA strands and the results were aligned with the NCBI Nucleotide collection (http://www.ncbi.nlm.nih.gov) (Zhang et al. 2000).

Pathogenicity testing and statistical analyses

For the pathogenicity trial, ~1 × 1cm inoculum from two isolates of *P. plurivora*, one Serbian and one Polish (JX276086 and JX276027), and one additional isolate of *P. cactorum* as a control (JX276028), were sub-cultured from the growing edge of 3–4 day old colonies growing on carrot agar on sterile Petri dishes containing sterile, moist Ø 90mm filter paper (Filtrak, Germany) (Orlikowski et al. 2004), with three sub-cultures per Petri dish. Germinants of European beech (*Fagus sylvatica*)

Fig. 1. Germinating beech seedlings plated onto agar with a colony of *P. plurivora*, after 5 days of incubation

were transferred to the Petri dishes containing filter paper and inoculum, and placed with the young germinant tips in direct contact with the inoculum (fig. 1). Dishes were incubated in the dark at approximately 20–22°C, and lengths of necrosis in mm were measured after 1, 2, 5, and 7 days. This experiment was performed in a genetic laboratory at the Forest Research Institute – IBL, Sękocin Stary, Poland.

For each treatment, four Petri dishes containing beech germinants were prepared, and the process was replicated three times, for a total of 30 germinants per provenience. Ten Polish beech provenances were tested, including Iława, Jagiełek, Korpele, Kudypy, Miłomłyn, Młynary, Mrągowo, Susz, Wichrowo, and Wipsowo. Control germinants were mock-inoculated with sterile carrot agar, again using 30 germinants per provenience.

To account for variation in sample size, a non-parametric ANOVA Tuckey's test was applied (STATISTICA 8.0), α = 0.05.

Tab. 2. *Phytophthora* species on European beech in Serbia, origin of isolates, and GenBank access numbers

No.	Hosts	Disease symptoms	Origin of isolates			Country/Region/ Forest district/ Department/Year of detection	Age	*Phytophthora* spp.	GenBank accession number
			Soil and roots	Water	Tissue				
1	*Fagus sylvatica*	Increased crown transparency, stunting of shoots	X			Serbia/Avala mountain/64/2011	70–90	*Phytophthora plurivora*	–
2	*Fagus sylvatica* + *Quercus petraea*	Crown transparency, dying of branches	X			Serbia/Avala mountain/64/2011	80	*Phytophthora cambivora*	JX276088
3	*Fagus sylvatica*	Aerial cankers, crown transparency	X			Serbia/Goc mountain/B. reka/2011	~100	*Phytophthora* spp.	–
4	*Fagus sylvatica*	No symptoms		X		Serbia/Goc mountain/B. reka/ 2011	–	*Phytophthora gonapodyides*	JX276084
5	*Fagus sylvatica*	No symptoms	X			Serbia/Trstenik/ Trstenicke sume/2011	90–100	*Phytophthora plurivora*	–
6	*Fagus sylvatica*	Stunting of shoots, crown transparency	X			Serbia/ Trstenik/2010	90	*Phytophthora plurivora*	JX276086
7	*Fagus sylvatica*	No symptoms		X		SerbiaTrstenik/ 2010	90	*Phytophthora plurivora*	JX276087
8	*Fagus sylvatica*	Crown transparency	X			Serbia/Novi Pazar/ Turjak/2012	60–70	*Phytophthora plurivora*	–
9	*Fagus sylvatica*	No symptoms	X			Serbia/Novi Pazar/ Turjak/2012	60	*Phytophthora* spp.	–
10	*Fagus* sp./*Betula* spp./*Populus* spp. - small forest stream	Yellowing of leaves and crown transparency on beech trees		X		Serbia/Novi Pazar/ Turjak/2012	–	*Phytophthora plurivora*	–
11	*Fagus sylvatica*	Yellowing of leaves, dying of branches	X			Serbia/Novi Pazar/ Turjak/2012	70	*Phytophthora cambivora*	–
12	*Fagus sylvatica*	Crown transparency	X			Serbia/Boljevac/ Brezovica 41/ 2011	120	*Phytophthora plurivora*	–

Results

Isolation of *Phytophthora* species in Serbian beech stands

Phytophthora species were found in five out of the seven tested beech stands in Serbia, and twelve isolates were obtained (tab. 2). Isolations were successful in the periods from March to November across almost the entire natural range of beech in Serbia, which provides evidence of their abundance in natural ecosystems. Among the species found, *P. plurivora* was dominant, being present in 58% of positive samples. *P. cambivora*, *P. gonapodyides,* and two unidentified isolates were also present (tab. 3). Two isolates of *P. plurivora* and a single isolate of *P. gonapodyides* were obtained from water samples, taken from water courses flowing through beech stands (tab. 2). All stands examined were aged between 60 to 120 years, and most of them displayed symptoms of disease in the crown (crown transparency, dying of branches, stunting of shoots). However, bark necrosis and aerial cankers were absent, or very rarely recorded in these studies. Four out of twelve isolates were obtained from apparently healthy stands, including two water samples (tab. 2).

Morpho-physiological classification of isolates

Preliminary classification of isolates was made based upon established colony growth patterns. Most of the isolates possessed slight or limited aerial mycelium in the centre, and had petalloid growth patterns. Two of the isolates were mostly fluffy with variable growth patterns. Three isolates were appressed and velvety, with chrysanthemum or irregular shapes. After the development of sexual and asexual structures, and screening via microscope, seven isolates seemed to be homothallic (isolates Nos 1, 5, 6, 7, 8, 10, and 12). These produced paragynous antheridia and mostly globose oogonia with plerotic oospores, and semipapillate sporangia which varied in shape. The optimum temperature for growth was around 25°C, the minimum was around 5°C, and maximum between 30–35°C. The radial growth rate was 7.7 mm/day on carrot agar. These isolates were preliminary identified as *P. plurivora*. The other five isolates were heterothallic or sterile, producing nonpapillate sporangia. Two isolates with fluffy mycelium (Nos 2 and 11) showed ellipsoid, sometimes ovoid and obpyriform sporangia shapes, without a tapered base, and these also had an optimum growth temperature of around 25°C, a minimum of around 5°C, and a maximum of between 30–35°C. The radial growth rate at optimum temperature was 5.1 mm/day on carrot agar. These two isolates were preliminary identified as *P. cambivora.* The remaining three isolates were also nonpapillate, with ovoid, obpyriform or ampuliform shape, nested internal proliferation and a tapered base. The optimum growth temperature of one chosen isolate (isolate No. 4) was around 25°C, the minimum was around 5°C, and the maximum was between 25–30°C. The radial growth rate at optimum temperature was 2.8mm/day on carrot agar media. This isolate was preliminary identified as *P. gonapodyides.* Identification of last two isolates (Nos 3 and 9) is still ongoing at time of writing. Structures re-

Tab. 3. Occurrence of *Phytophthora* spp. in different forest types of European beech in Serbia

No.	Forests of	Soil types	Obtained *Phytophthora* isolates			
			P. cambivora	*P. plurivora*	*P. gonapodyides*	*P*. spp.
1	Sub-mountain beech (*Fagetum moesiacae submontanum-typicum*)	delluvium, brown, acid brown, and leached brown soils	–	+	–	–
2	Sub-mountain beech and sessile oak (*Querco-fagetum*)	cambisol, leached cambisol and, brown to acid brown soils	+	+	–	–
3	Mountain beech (*Fagetum moesiacae montanum-typicum*)	deep brown and acid brown soils on delluvium	+	+	–	–
4	Mountain beech with noble hardwoods (*Fagetum moesiacae montanum aceretosum*)	humus-silicate soils	–	+	–	+
5	Mountain beech and silver fir (*Abieti-Fagetum moesiacae montanum serpentinicum typicum*)	mull rankers, typical, and brownish skeletal brown soils	–	–	+	+

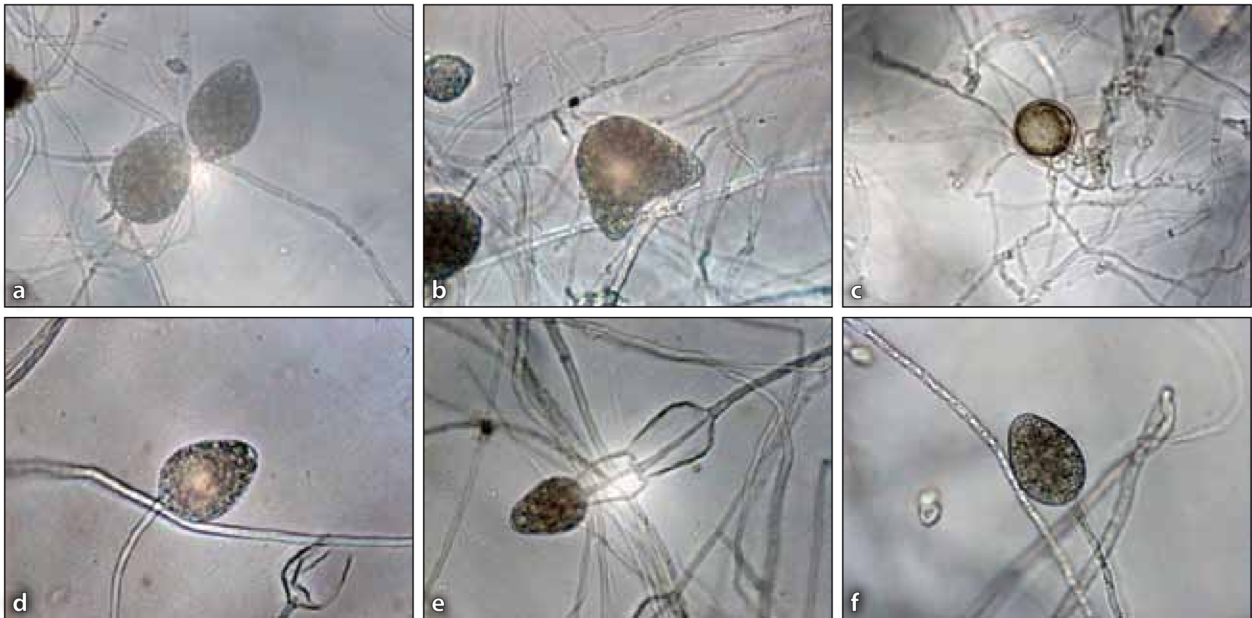

Fig. 2. Different *Phytophthora* spp. structures taken after 24–36 h from agar pieces flooded with nonsterile soil extract: a) *P. plurivora* – semipapillate sporangia on sympodial conidiophores; b) *P. plurivora* – semipapillate sporangia with two peaks; c) *P. plurivora* -globose oogonia with paragynous antheridia; d) *P. gonapodyides* – nonpapillate sporangia; e) *P. gonapodyides* – nested internal proliferation; f) *P. cambivora* – nonpapillate sporangia

corded at x400 magnification with the Zeiss microscope and Nikon camera, are shown in fig. 2.

Molecular analyses

The generic primers ITS6 and ITS4 were able to amplify the ITS sequence in all *Phytophthora* isolates. The sequence of the rDNA region under investigation allowed us to confirm the morphological identification of isolates examined. Two isolates were confirmed as *P. plurivora*, and another two as *P. cambivora* and *P. gonapodyides*. The sequences obtained were submitted to the NCBI database (tab. 2).

Pathogenicity trial

Phytophthora plurivora was found to be pathogenic in all ten Polish beech provenances tested. The Polish isolate of *P. plurivora*, seemed to be more aggressive compared to the Serbian strain, as represented by the longer infection lesions in nine out of the ten provenances (fig. 3). The Serbian isolate of *P. plurivora* was found to be more aggressive only in the samples taken from the Jagiełek provenance. The pattern of resistance/susceptibility was the same for both *Phytophthora* isolates and beech provenances. The most resistant was Mrągowo and the most susceptible were Wipsowo and Kudypy (significant ANOVA test: $p = 0.000008$; $\alpha = 0.05$).

The control isolate, *P. cactorum*, appeared to be much less aggressive in all ten provenances tested compared with the Polish isolate of *P. plurivora*, while it was more aggressive than Serbian isolate of *P. plurivora* only in the samples collected from the Mrągowo provenance (fig. 3). The most resistant provenance to *P. cactorum* was Susz, while the most susceptible was Wipsowo.

Discussion

Fagus sylvatica is an important tree species, and one of the major tree species in European forests on account of its tolerance to a wide range of climatic conditions. However, over the recent years, the health status of beech stands in Europe decreased dramatically.

The Beech decline phenomenon was considered to be the result of bark disease alone for many years, in which severe bark infestations by the insect *Cryptococcus fagisuga* Lind. facilitated trunk attack by the fungi genus *Nectria* (*N. coccinea* Desm., *N. dittisima* Tul. *and*

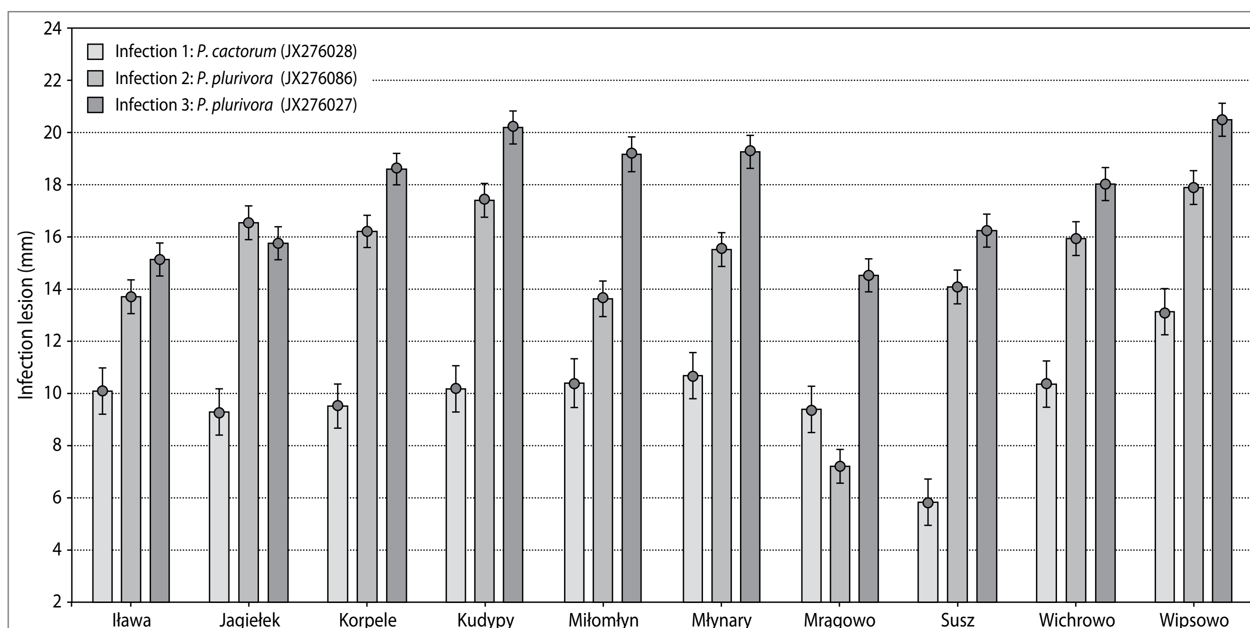

Fig. 3. Resistance of Common beech provenances against Serbian (infection 2) and Polish (infection 3) isolates of *Phytophthora plurivora,* and against *P. cactorum* (infection 1), expressed as an infection (lesion length in mm) in germinants, α = 0.05

N. galigena Bres.). However, besides harmful factors like insects and fungi, some pathogenic soil *Oomycetes*, such as pathogens from the *Phytophthora* genus, were also reported as an important factor involved in beech decline phenomenon (Jung et al. 2005; Jung 2009).

In Serbia this is the first record of *Phytophthora* species present in the rhizosphere soil of five seperate beech stands.

P. plurivora is currently the most common pathogen of fine roots in many deciduous species (such as beech, oak, or ash) in natural ecosystems, forest nurseries, and plantations (Jung and Burgess 2009; Orlikowski et al. 2011). The involvement of *P. plurivora* in the decline of several beech stands was previously reported (Jung et al. 2005; Jung and Burgess 2009; Jung 2009). Due to the persistence of resting spores (Jung and Burgess 2009), there is a risk that this organism could easily be transported (e.g. via roots and associated soil) with plants intended for shipment elsewhere. Another species isolated in Serbian beech stands, *P. cambivora*, is also a well-known and aggressive pathogen of deciduous trees, including European beech (Jung 2009; Hartmann et al. 2006; Orlikowski et al. 2006; Stępniewska and Dłuszyński 2010).

The isolation of *Phytophthora* species in Serbian beech stands was most successful in early spring and in autumn (tab. 1), while it was less efficient during the summer, probably due to the dormancy period of these pathogens. Additionally, they were isolated from different stand age classes, from 60 to 120 years old, and in different soil types, ranging from cambisol, brown, acid brown, and humo-silicate to rankers.

In the pathogenicity trial performed in this study, the Polish isolate of *Phytophthora plurivora*, seemed to be more aggressive toward beech germinants compared to the Serbian isolate in nine out of ten tested provenances. This means that if, in the future, it is brought inadvertently to Poland, we can expect no greater damage to beech trees than those caused by isolate already present in Poland. This is shown in Figure 3, with reference to the length of infection lesions. Conversely, in the event that the Polish isolate is translocated to Serbian beech forests, it could cause far more damage than the already existing and tested *P. plurivora* isolate.

Climate change is likely to affect the presence, distribution, and activity of pathogenic organisms. It is understood that the most problematic are those which are introduced from a colder to a warmer climate, where they become more virulent. Our data were consistent with this theory, as in Serbia the average annual temperature is much higher than that of Poland.

Furthermore, there is a risk of hybridization between species resulting in a new organism, which may have increased pathogenicity. This has already occurred in alder *Phytophthora*, now regarded as three subspecies: *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis*, and *P. alni* subsp. *uniformis* (Brasier et al. 2004). Ioos et al. (2007) claims that the second and third subspecies listed above are the parents of the first (*P. alni* subsp. *alni*), which is the most pathogenic of the three, and is particularly damaging to alder.

Intriguingly, until now *P. cactorum* was considered to be the most harmful to beech trees. The pathogenicity test showed that beech seeds germinants were damaged far more by the Polish isolate of *P. plurivora* than by *P. cactorum* in all ten provenances. Interestingly, *P. cactorum* was more aggressive than Serbian isolate of *P. plurivora* only in germinants from the Mrągowo provenance, which was at the same time the most resistant to both isolates of *P. plurivora* (fig. 3).

In this light, further nursery studies are needed in both countries. It is likely that *P. plurivora,* previously known as *P. citricola,* was introduced later than *P. cactorum*, or that *P. plurivora* had already been present for a long time, but was misidentified, as described in Jung and Burgess (2009). A good knowledge of species ecology, in combination with contemporary molecular biology tools, allow both species to be clearly distinguished.

The pathogenicity of other *Phytophthora* species towards beech seedlings has been examined in several studies, e.g. Fleischmann et al. (2002, 2004), and Portz et al. (2011). But nevertheless, the mechanism by which *F. sylvatica* develops resistance to infection by *Phytophthora* still remains unknown.

However, after this preliminary investigation, larger scale surveys for the presence of *Phytophthora* in beech stands within Serbian forest ecosystems are required in combination with further pathogenicity tests, due to the large area of coverage and high importance of this species for Serbian forestry.

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