The Characterization of *Phytophthora* Isolates Obtained from Diseased *Alnus glutinosa* in Poland

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Abstract

Since 2000 an increasing decline and dieback of alders (*Alnus glutinosa*) has been observed in Poland. Woodlots were monitored in 2004 and 2005, and sixty-eight isolates of *Phytophthora* were obtained from diseased alder trunks and from the surrounding rhizosphere. Ten *Phytophthora* species were identified: seven isolates *P. alni* subsp. *alni*, five *P. cactorum*, one *P. cambivora*, one *P. cinnamomi*, eight *P. plurivora*, four *P. gonapodyides*, seven *P. megasperma*, seven *P. pseudosyringae*, seven *P. syringae* and twenty-one *P. lacustris*. *P. alni* subsp. *alni* and *P. cambivora* were only found in samples of diseased alder trunks, whereas *P. cactorum*, *P. plurivora*, *P. megasperma*, *P. pseudosyringae*, and *P. syringae* was isolated from both tree and soil samples, and *P. gonapodyides*, *P. cinnamomi* and *P. lacustris* only from the soil samples. All mentioned above species colonized leaves and young shoots of alder but with different levels of aggressiveness. *P. alni* subsp. *alni* was the most aggressive in its colonization of shoots, and *P. plurivora* from alder trunks caused the largest necrosis on leaves. The isolates of *P. plurivora* from alder trunk tissues colonised leaves more rapidly than the isolates from the rhizosphere. *P. gonapodyides*, *P. megasperma*, *P. pseudosyringae* and *P. syringae* were the weakest colonisers of excised leaves and shoot segments of *A. glutinosa.*

Key words: *Phytophthora,* pathogen; identification, decline and dieback alder; pathogenicity

Introduction

Alders are important components of forest stands as well as riparian ecosystems. Alders [mostly *Alnus glutinosa* (L. Gaertn.) and less common *A. incana* (L. Moench] are the dominant species growing principally along watercourses and near lakes and ponds in substantial areas of natural Polish woodland. There has also been a recent increase in commercial interest in alder timber. Symbiosis of roots with *Actinomycetes* in the genus *Franckia*, which is able to fix atmospheric nitrogen, makes it the tree of choice for planting in areas to be reclaimed (Jung and Blaschke 2004). Moreover, as a light-demanding pioneer species, it can grow up 90 cm per year (Kremer 1995).

The appearance of *Phytophthora* root and collar rot among European alders is thus of great concern. This disease was first noted in 1993 in the United Kingdom (Brasier et al. 1995) and in the following years in Germany (Jung and Blaschke 2004), Austria (Cech 1998), Czech Republic (Černy et al. 2003, Černy et al. 2008). During the International Union of Forest Research Organizations (IUFRO) conference devoted to Phytophthoras, held in 2004 in Freising, Germany, numerous examples of alder dieback were reported. The alder tree dieback has been noted mainly along rivers but also in nursery seedlings and alders growing on former agricultural land (Thoirain et al. 2007). The disease has spread to the whole of Europe, from Italy to Sweden (Nagy et al. 2003). In Poland, common alder trees dieback intensified in the last decades (Orlikowski et al. 2003). The causal agent isolated from several symptomatic *Alnus* trees showing root and collar rot at their stem-bases or on the entire trunk has been described as *Phytophthora alni* Brasier & SA Kirk (Gibbs et al. 2003, Brasier et al. 2004). Initially it was

thought, that *P. alni* is an interspecific hybrid between *P. cambivora* and a species related to *P. fragariae*. Within *P. alni* three subspecies were distinguished: *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis* and *P. alni* subs. *multiformis* (Brasier et al. 2004). Later studies clarified the genesis of *P. alni* subsp. *alni* as an interspecific hybrid between *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis* (Ioos et al. 2006). The hybrid *P. alni* subsp. *alni* is more aggressive than its progenitors and it is responsible for disease spread. However, it is not known when and where hybridization took place. Moreover, the origin of the parental species, *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*, remains unknown. *P. alni* subsp. *alni* is widespread in Europe and has not been identified elsewhere. Although *P. alni* subsp. *multiformis* has been isolated exclusively in Europe, *P. alni* subsp. *uniformis* has been isolated both in Europe and, recently, in the North America. The results of Aguayo et al. (2013) suggest that the European *P. alni* subsp. *uniformis* population is most likely alien and originates from the introduction of a few individuals, whereas the North American population probably is an indigenous.

In 2000, studies on the decline of alder trees in Poland were begun. The main pathogens isolated from the diseased trees were *Phytophthora*, *Armillaria* and *Fusarium* species (Orlikowski at al. 2003, Orlikowski and Oszako 2005, Oszako 2005).

The aim of this study is to determine species of *Phytophthora* associated with symptomatic alder trees in natural stands on the waterfronts of rivers and ponds. This information could be essential for the management and control of spread of the disease.

Material and Methods

Isolation of Phytophthora spp. from symptomatic trees and surrounding soil

Natural stands of *Alnus glutinosa* along watercourses in the south-eastern Poland (Figure 1) were surveyed between spring and autumn in 2004 and 2005. In total, 160 trunk samples (2-3 per tree) from 57 trees with symptoms typical for *Phytophthora* spp. were collected. Inner bark pieces of approx. 10 × 4 × 5 cm were excised from the boundaries between the necrotic and healthy tissues of the trunks and transferred to the laboratory individually in plastic bags. The bark tissues were washed thoroughly in running tap water, flamed, trimmed into pieces of 3-5 mm^2, placed on potato-dextrose agar (PDA, Difco, Detroit, USA) in 90 mm Petri dishes, with 8 pieces per dish, and incubated in the dark at 24ºC. The cultures were checked daily and presumptive *Phytophthora* colonies growing from the tissue pieces were cleaned by subsequent transfer onto PDA medium (Orlikowski at al. 2003).

Figure 1. The positions of natural stands of symptomatic alder trees on embankments of rivers or ponds, from which the samples were taken: 1: Pulawy (a tributary of the Kurówka river), 2: Bialobrzegi (the Pilica river), 3: Morawica (the Morawka river), 4: Koszyce (the Szreniawka river), 5: Sosnowica (a forest pond), 6: Kijany (the Wieprz river), 7: Naleczow (a pond in the city park); 8: Siedliska (the Prutnik river), 9: Kornie (fish ponds)

Soil samples were taken from at least 4 points around each diseased tree at a depth of 10-15 cm. The samples from below one tree (approx. 2 kg) were mixed thoroughly, and 500 g sub-samples flooded with 1.5 l tap water. Leaves of 1-year old alder seedlings (*Alnus glutinosa*) and *Rhododendron catawbiense* cv. 'Nova Zembla' grown in the greenhouse were placed on the water surface 10-12 per vessel as baits for *Phytophthora* isolation, and incubated at 20ºC in the dark for 3-4 days, according to Erwin and Ribeiro (1996) and Orlikowski and Oszako (2005). Fragments (5 × 5 mm) of the necrotic tissues that appeared on the leaf baits were plated onto PDA medium. After 24 to 48 hours of incubation in the dark at 24ºC, colonies growing from the diseased leaf fragments were stored on PDA slants.

Identification of species on the basis of morphology and sequencing

The identification of *Phytophthora* species based on morphological traits of sexual and asexual structures and growth characteristics of colonies according to Nechwatal et al. (2013), Jung and Burgess (2009),

Brasier et al. (2004), Erwin and Ribeiro (1996). In addition, nucleotide sequences of the internal transcribed spacer regions of the ribosomal RNA gene repeats within the primers ITS 4 and ITS 6 (Cooke and Duncan 1997) were compared with those available in GenBank (NCBI) and served as decisive for the identification of species.

Colony growth patterns were described in 7-day old cultures grown at 23ºC in the dark on V8A (Erwin and Ribeiro 1996) and PDA (Merck). Sporangia and gametangia were measured on V8A as described by Jung and Burgess (2009).

DNA was extracted from fresh mycelia using the method described by Wiejacha et al. (2002). The PCR reactions were performed in a Gene*Amp* PCR System 9700 (PE Applied Biosystems). The reaction mixture of 25 µl in volume contained 25 ng DNA, 0.5 U *Taq* polymerase (Fermentas), 0.5 µM of each primers, 50 µM of each DNA nucleotide, and 1.5 mM $MgCl_2$. The PCR parameters were as follows: 3 min of initial DNA denaturation at 94ºC and 35 cycles of amplification (30 sec of denaturation at 94ºC, 30 sec of annealing at 55°C, 60 sec of elongation at 72ºC), and 5 min of final elongation at 72°C. The PCR products were cleaned using Clean-up (A&A Biotechnology), and sequenced using Roche GS FLX/Titanium. All of the sequences were compared against those available in GenBank (NCBI) using nucleotide-nucleotide BLAST (blastn) software.

Ability for infection/colonization of Alnus glutinosa leaves and shoots

Selected isolates of ten *Phytophthora* species obtained from the diseased alder trunks and their rhizospheres were used in the colonization test, which was performed on the leaves and 8 cm long cuttings from the middle shoot parts of 1-year old alder seedlings of 25 cm height and 1 cm trunk diameter grown in the greenhouse. Detached leaves and cuttings were punctured with the pointed end of a sterile scalpel and laid on wet cellulose paper. The place of puncture was covered with 3.5 mm diameter agar disks with mycelium and incubated at 22ºC, and a photoperiod 12/12 h light/dark. The size of appearing necrotic spots was measured on the 6th and 9th day by ruler after removing agar disk. After 9 days, re-isolation of *Phytophthora* from the lesions in alder leaves and shoot segments was made using the method described above, and their identity was confirmed by morphological characters, and in PCR reactions with species-specific primers according to Boersma et al. (2000) for *P. cactorum*, Minerdi et al. (2008) for *P. cinnamomi* and Nechwatal and Kurt (2006) for *P. lacustris*.

The experimental design was completely randomised, with four replications. Ten leaves and 10 shoot fragments were used per replication. Analysis of variance was performed to test differences in lesion size after the previous of Box-Cox transformation. The mean values were compared using Duncan's test at $P = 0.05$ (Statistica 10 package).

Results

Identification of Phytophthora spp. isolates

In total, 68 isolates of *Phytophthora* were obtained: 22 from diseased alder trunk tissues (32%) and 46 from soil samples (68%). The results of classification of isolates to *Phytophthora* species using microbiological methods (Table 1) were fully confirmed by sequencing of DNA fragments obtained from PCR amplification (Table 2). Ten *Phytophthora* species were identified. *P. alni* and *P. cambivora* were isolated only from trunk samples. *P. cinnamomi*, *P. gonapodyides* and *P. lacustris* were isolated exclusively from soil samples. The remaining species, *P. cactorum*, *P. megasperma, P. plurivora*, *P. pseudosyringae*, and *P. syringe,* were obtained from the trunk and soil samples. The highest number of trunk isolates belonged to *P. alni* subsp. *alni* (7), *P. plurivora* (5) and *P. cactorum* (4). From trunks were also obtained 2 isolates of *P. megasperma*, 2 of *P. syringae*, and by 1 of each *P. cambivora* and *P. pseudosyringae* (Table 2).

In all cases, only one species was identified from samples of a given tree. On the alder trap leaves, *P. cinnamomi*, *P. pseudosyringae*, *P. lacustris* and on rhododendron trap leaves *P. cactorum*, *P. plurivora*, *P. gonapodyides*, *P. megasperma*, and *P. syringae* had been caught from soil extracts.

Colonization of leaves and shoots

All of the selected isolates of ten *Phytophthora* species were able to colonize alder leaves and shoots, although with a different strength (Figures 2, 3). After 6 days, *P. alni* subsp. *alni*, *P. plurivora* obtained from tree and *P. cinnamomi* produced the largest necrotic spots on the shoots (26.9, 15.9 and 17.4 mm), while leaves were most susceptible to the *P. plurivora* isolated from trunks (17.6 mm), followed by *P. alni* subsp. *alni* and *P. cactorum* (Figure 2). This trend was also maintained after 9 days after inoculation with one exception, that isolate *P. plurivora* from soil produced almost the same size necrotic spots on leaves like *P. cactorum* (17.4 and 17.0 mm respectively) (Figure 3). The isolates of *P. cactorum* and *P. plurivora* obtained from the trunk were more aggressive for shoots and leaves than the isolates of the same species obtained from the rhizosphere soil. Contrary to this, isolates of *P. megasperma*, *P. pseudosyringae* and *P. syringae* from the trunks and soil were equally aggressive but

Table 1. Morphological traits and dimensions (µm) of *Phytophthora* species

	P. alni sus. *alni*	*P. cactorum*	*P. cambivora*	*P. cinnamomi*	*P. gonapodyides*
No. of isolates investigated	7	5	1	1	4
Sporangia (on V8 medium)					
shape/type of sporangia	ellipsoid, non-papillate	ovoid, papillate	ovoid, non-papillate	ovoid, non-papillate	ovoid to obpyriform, non-papillate
length × width mean	50.7 ± 7.9[1] × 35.7 ± 5.6	35.1 ± 7.3 × 25.8 ± 6.2	42.6 ± 7.0 × 32.2 ± 7.0	46.6 ± 8.0 × 33.9 ± 7.2	50.7 ± 3.7 × 36.8 ± 3.8
length × width range	35 – 65 x 24 – 50	20 – 54 x 16 – 42	30 – 58 x 20 – 46	35 – 63 x 24 – 50	43 – 59 × 29 – 45
range of isolate means	49.3 – 51.1 x 35.1 – 36.2	34.2 – 36.8 x 24.8 – 27.2	-	-	50.4 – 51.2 × 36.5 – 37.3
Oogonia (on V8 medium)	Homothallic	Homothallic	A2 mating type	A2 mating type	Sterile
type of oogonium	ornamental	smooth	ornamental	smooth	
diam range	36 – 56	23 – 35	40 – 52	26 – 46	
range mean	45.2 ± 4.8	27.8 ± 2.7	46.3 ± 3.7	37.05 ± 4.4	
range of isolate means	44.5 – 46	27.0 – 28.2	46.3	37. 05	
Oospores (on V8 medium)					
mean diam	35.5 ± 4.5	22.1 ± 1.7	40.6 ± 3.8	33.9 ± 4.5	
diam range	27 – 47	18 – 25	36 – 46	25 – 42	
Antheridia (on V8 medium)					
type of antheridia	amphigynous	paragynous	amphigynous	amphigynous	
length × width mean	22.8 ± 2.8 x 18 ± 1.4	11.9 ± 2.3	24.0 ± 2.8 × 17.1 ± 2.3	18.5 ± 2.8 × 17.2 ± 3.2	
length × width range	18 – 31 × 15 – 21	9.1 – 17.0	15 – 33 × 11 – 23	13 – 22.5 × 14 – 23.2	
Type of mycelium on PDA	aerial	little aerial	rosaceous	rosa or camellia	rozette
Type of mycelium on V8A	aerial	radial	aerial	aerial	rozette

	P. lacustris	*P. megasperma*	*P. plurivora*	*P. pseudosyringae*	*P. syringae*
No of isolates investigated	6	4	4	4	4
Sporangia (on V8 medium)					
shape/type of sporangia	ovoid to obpyriform, non-papillate	ovoid, non-papillate	ovoid, obpyriform, semi-papillate	limoniform, semi-papillate	ovoid-ellipsoid, semi-papillate
length × width mean	45.6 ± 4.2 × 32.2 ± 3.8	42.2 ± 6.1 × 30.0 ± 5.8	47.1 ± 6.0 × 33.4 ± 8.3	47.5 ± 4.6 × 32.7 ± 3.7	43.1 ± 6.7 × 33.1 ± 6.5
length × width range	36 – 54 × 25 – 43	30 – 58 × 20 – 46	32 – 62 x 20 – 48	37 – 57 × 27 – 42	30 – 58 × 20 – 47
range of isolate means	45.0 – 46.1 × 31.5 – 32.6	41.0 – 42.9 × 28.7 – 31.0	46.5 – 47.6 x 32.3 – 34.0	46.7 – 48.3 × 32.3 – 33.4	42.4 – 44.1 × 31.9 – 34. 2
Oogonia (on V8 medium)	Sterile	Homothallic	Homothallic	Homothallic	Homothallic
type of oogonium		smooth	smooth	smooth	smooth
diam range		40 – 50	16 – 35	17 – 41	20 – 36
range mean		45 ± 2.8	27.8 ± 4.3	30.1 ± 5.6	28.7 ± 4.1
range of isolate means		44.5 – 45.4	26.8 – 28.8	29.6 – 31.2	28.0 – 29.5
Oospores (on V8 medium)					
mean diam		24.2 ± 4.3	24.2 ± 4.3	26 ± 4.8	25.5 ± 3.9
diam range		14 – 32	14 – 32	15 – 37	18 – 33
Antheridia (on V8 medium)					
type of antheridia		paragynous	paragynous	paragynous	paragynous
length × width mean		16.7 ± 2.3 × 13.2 ± 2.1	10.8 ± 3.2 × 7.9 ± 3.1	13.5 ± 3.8 x	9.8 ± 2.9 × 8.7 ± 2.6
length x width range		14.3 – 20.0 × 10.4 – 18.0	8.2 – 10.5 × 5.8 – 15.0	11.3 – 15.8 × 9.3 – 12.9	8.7 – 13.0 × 6.5 – 10.6
Type of mycelium on PDA	chrysanthemum	aerial	chrysanthemum	aerial	aerial
Type of mycelium on V8A	radial	aerial	radial	stellate	stellate

[1] ±SD (standard deviation)

their ability to colonize *Alnus* leaves and shoots was low. Black spots in the control treatment were about two times smaller than with any *Phytophthora* isolates. All of the re-isolation samples confirmed their identity with the inocula. No presence of *Phytophthora* was found in control leaves and shoots.

Discussion and Conclusions

In the last decades, several plant-pathogenic *Phytophthora* species have caused huge damage to crops, landscape plants, forests, and ecosystems, for instance *P. ramorum* in the North America and Europe and *P. alni* in European forests and riparian stands. Also in Poland, the alder trunk bleeding and accompanying symptoms are now widespread with severe damages particularly in south-eastern part of the country (Orlikowski et al. 2003, Orlikowski and Oszako 2005). Despite the strong symptoms in the studied trees, species of *Phytophthora* were isolated only from 32% of the trunks. The success in isolation may depend on the year, month, and current weather. Streito et al. (2002) isolated alder *Phytophthora* from 9.9% of trunk necrosis samples in 1997 whereas from 62.5% samples in 1998.

Preliminary classification of the obtained *Phytophthora* isolates to species based on colony growth pattern on PDA and V8 medium, and on the morphological characters of sexual and asexual structures produced on V8A was fully confirmed by the results of sequencing. However, the identification of *Phytophthora* species using microbiological techniques is time

Table 2. *Phytophthora* species isolated from diseased alder trunk tissues and rhizosphere samples surrounding the trees

Phytophthora species	Isolate number	Source of isolate	Gen Bank Accession No	% of identity
P. alni subsp. *alni*	PAA1	T	AY689131	100
P. alni subsp. *alni*	PAA2	T	AY689133	100
P. alni subsp. *alni*	PAA3	T	AY689133	100
P. alni subsp. *alni*	PAA4	T	AY689133	100
P. alni subsp. *alni*	PAA5	T	AY689131	100
P. alni subsp. *alni*	PAA6	T	AY689131	100
P. alni subsp. *alni*	PAA7	T	AY689131	100
P. cactorum	PCAC1	T	GU111587	100
P. cactorum	PCAC2	T	GU111587	100
P. cactorum	PCAC3	T	GU111587	100
P. cactorum	PCAC4	T	GU111587	100
P. cactorum	PCAC5	S^{Rh}	GU111587	100
P. cambivora	PCAM1	T	EU000145	100
P. cinnamomi	PCIN1	S^{Al}	AY964101	100
P. gonapodyides	PGON1	S^{Rh}	DQ176010	99
P. gonapodyides	PGON2	S^{Rh}	DQ176010	99
P. gonapodyides	PGON3	S^{Rh}	EU000162	99
P. gonapodyides	PGON4	S^{Rh}	EU000162	99
P. lacustris	PLAC1	S^{Al}	EU240177	99
P. lacustris	PLAC2	S^{Al}	EU240124	99
P. lacustris	PLAC3	S^{Al}	EU240138	99
P. lacustris	PLAC4	S^{Al}	EU240177	99
P. lacustris	PLAC5	S^{Al}	EU240177	99
P. lacustris	PLAC6	S^{Al}	EU240067	99
P. lacustris	PLAC7	S^{Al}	EU240089	99
P. lacustris	PLAC8	S^{Al}	EU240124	99
P. lacustris	PLAC9	S^{Al}	EU240180	99
P. lacustris	PLAC10	S^{Al}	EU240177	99
P. lacustris	PLAC11	S^{Al}	EU240177	99
P. lacustris	PLAC12	S^{Al}	EU240138	99
P. lacustris	PLAC13	S^{Al}	EU240124	99
P. lacustris	PLAC14	S^{Al}	EU240067	99
P. lacustris	PLAC15	S^{Al}	EU240138	99
P. lacustris	PLAC16	S^{Al}	EU240177	99
P. lacustris	PLAC17	S^{Al}	EU240177	99
P. lacustris	PLAC18	S^{Al}	EU240089	99
P. lacustris	PLAC19	S^{Al}	EU240089	99
P. lacustris	PLAC20	S^{Al}	EU240067	99
P. lacustris	PLAC21	S^{Al}	EU240177	99
P. megasperma	PMEG1	T	EU106587	100
P. megasperma	PMEG2	T	EU106587	100
P. megasperma	PMEG3	S^{Rh}	EU098125	100
P. megasperma	PMEG4	S^{Rh}	EU106587	100
P. megasperma	PMEG5	S^{Rh}	EU000152	100
P. megasperma	PMEG6	S^{Rh}	EU098125	100
P. megasperma	PMEG7	S^{Rh}	EU098125	100
P. plurivora	PPLU1	T	GU120199	100
P. plurivora	PPLU2	T	FJ665232	100
P. plurivora	PPLU3	T	GU120199	100
P. plurivora	PPLU4	T	GU120199	100
P. plurivora	PPLU5	T	FJ665232	100
P. plurivora	PPLU6	S^{Rh}	FJ665232	100
P. plurivora	PPLU7	S^{Rh}	GU120199	100
P. plurivora	PPLU8	S^{Rh}	GU120199	100
P. pseudosyringae	PSEU1	T	AY366463	100
P. pseudosyringae	PSEU2	S^{Al}	EF490383	100
P. pseudosyringae	PSEU3	S^{Al}	AY366463	100
P. pseudosyringae	PSEU4	S^{Al}	AY366463	100
P. pseudosyringae	PSEU5	S^{Al}	EF490383	100
P. pseudosyringae	PSEU6	S^{Al}	EF490383	100
P. pseudosyringae	PSEU7	S^{Al}	AY366463	100
P. syringae	PSYR1	T	AA054895	99
P. syringae	PSYR2	T	AA054895	99
P. syringae	PSYR3	S^{Rh}	AA054895	99
P. syringae	PSYR4	S^{Rh}	AA054895	99
P. syringae	PSYR5	S^{Rh}	AA054895	99
P. syringae	PSYR6	S^{Rh}	AA054895	99
P. syringae	PSYR7	S^{Rh}	AA054895	99

[T] diseased alder trunk, [S] soil samples surrounding the trees, [Rh] *Rhododendron* 'Nova Zembla' baits, [Al] *Alnus glutinosa* baits

consuming and may be not accurate because of lack of reliable morphological markers and considerable morphological plasticity occurring in some taxa (Jung and Burgess 2009, Trzewik and Orlikowska 2011, Grünwald et al. 2011). In addition, this method requires both theoretical and practical knowledge of the organisms. Identification based on sequences of rDNA ITS fragments are commonly used for systematic classification *Phytophthora* species, although in the case of some species, for example *P. fragariae* and *P. rubi* (formerly *P. fragarie* var. *fragarie* and *P. fragarie* var. *rubi*), it is impossible due to their ITS sequence similarity (Cooke and Duncan 1997). In those cases, isozyme analysis (Man in 't Veld 2007) or dissimilarity within sequences of other nuclear or mitochondrial genes (Martin and Tooley 2003, Kroon et al. 2004) used single or in combination may be used. Therefore a use both morphologic and molecular techniques seems to

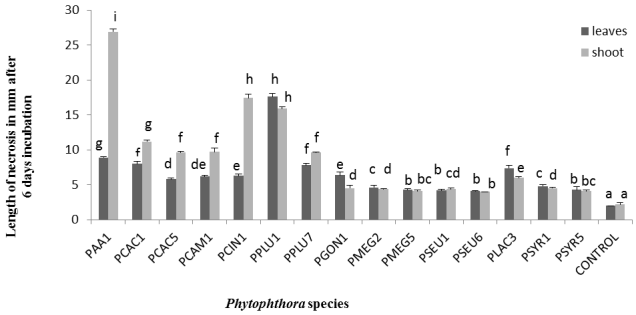

Figure 2. Colonization of leaves and shoot segments of *Alnus glutinosa* by isolates of *Phytophthora* species 6 days after inoculation; number of tested leaves and shoot segments (n = 40)

[1] means in the columns followed by the same letter that the values were not significantly different ($P < 0.05$; one-way ANOVA, Duncan's multiple range test), ± *SD* (standard deviation)

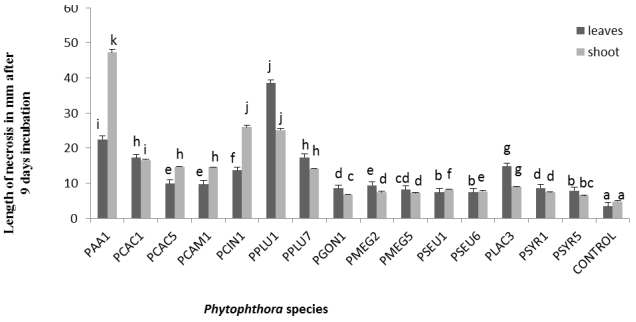

Figure 3. Colonization of leaves and shoot segments of *Alnus glutinosa* by isolates *Phytophthora* species 9 days after inoculation; number of tested leaves and shoot segments (n = 40)

[1] means in the columns followed by the same letter that the values were not significantly different ($P < 0.05$; one-way ANOVA, Duncan's multiple range test), ± *SD* (standard deviation)

be the optimal way for identification of some *Phytophthora* species.

Currently, in addition to *P. alni*, nine *Phytophthora* species were reported from alder trees (Gibbs 1995, Brasier and Kirk 2001, Streito et al. 2002, Brasier et al. 2003, Jung et al. 2003, Ioos et al. 2006, Jung and Blaschke 2004, Nagy et al. 2003). This paper is the first one to report in Poland *P. cactorum*, *P. plurivora*, *P. megasperma*, *P. pseudosyringae*, and *P. syringae* isolated from alder.

P. alni subsp. *alni* and *P. cambivora* were isolated only from diseased trunks, while *P. gonapodyides*, *P. cinnamomi* and *P. lacustris* were obtained only from soil samples. *P. lacustris* was originally isolated from *Salix* root in the UK and *Alnus* debris in Denmark (Nechwatal and Kurt 2006, Nechwatal et al. 2013). Inoculation of leaves with *P. lacustris* proved to be more aggressive towards *Salix alba* than *P. gonapodyides* (Nechwatal and Kurt 2006). In our investigation, this species was more aggressive to *Alnus* tissues not only than *P. gonapodyides* but also than *P. megasperma*, *P. pseudosyringae* and *P. syringae*. According to Erwin and Ribeiro (1996) and Hansen and Delatour (1999), *P. gonapodyides* has mainly saprotrophic nature.

P. alni was isolated by us only from diseased trunks but not from the surrounding soil samples, which contradicts with the report of the Hungarian authors Nagy et al. (2003). A lack of *P. alni* in the soil surrounding diseased trees may result from the low survival rate and poor germination of zoospores of this species (Nagy et al. 2003, Brasier et al. 2004). Černy at al. (2012) reported that isolates of *P. alni* subsp. *alni* are frost sensitive and die after 21 days of incubation at -7.5°C and after 2 days at -10°C. Moreover, the viability of the pathogen decreased significantly if the temperature of -5°C was maintained for at least 1 week and -7.5°C for least 4 days. In the current investigation, all of the tested isolates of ten species obtained from diseased trunks and rhizosphere were able to colonize alder leaves and shoots segments. *P. plurivora* was the most aggressive species on the leaves, whereas *P. alni* was more aggressive on the shoots of alder. This relationship may be because *P. plurivora* may cause both aerial canker and root rot, while *P. alni* mainly infects fine or adventitious roots, from which it extends to the trunk where destroys phloem and cambial tissues (Jung and Blaschke 2001). Otherwise, this study also showed that the isolates of *P. plurivora* and *P. cactorum* obtained from the trunks were more aggressive for shoots and leaves than the isolates of the same species obtained from the rhizosphere soil. This proves that isolates of the same *Phytophthora* species can vary in colonization activity of plant tissues. Brasier and Kirk (2001) shown that different isolates of P. *cambivora* caused leaves necroses ranging from 18.3 to 85.7 mm in length and from 12.6 to 17.3 mm in width. *P. gonapodyides*, *P. megasperma*, *P. pseudosyringae* and *P. syringae* were the weakest colonizers of alder shoots. *P. cinnamomi* was a relatively strong colonizer of alder shoots in our investigations, although Brasier and Kirk (2001) did not find it to be very aggressive to pieces cut from the trunks of living alder trees.

The results of our and other authors' studies show that in addition to *P. alni* other *Phytophthora* species are able to infect/colonize alder tissues (Haque and Diaz 2012, Santini et al. 2003). A key issue in a better understanding the infection process is to determine the role of factors stimulating pathogen virulence and dispersion, e.g. environmental conditions and their influence on the host plant capacity to counteract pathogens.

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