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The present state of knowledge on *Phytophthora* spp. diversity in forest and ornamental woody plants in the Czech Republic

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Abstract

Issues concerning Phytophthora diseases in woody plants and Phytophthora diversity were overlooked in the Czech Republic until 2000. The investigation of a number of important problems concerning Phytophthora diseases of woody plants was initiated in the past decade, including problems related to alder decline caused by *Phytophthora alni*, the identification of the spectrum of *Phytophthora* species affecting forest and amenity trees, and *Phytophthora* spp. diversity in ericaceous plants (especially rhododendron) as an infection reservoir in nurseries and ornamental greenery.

Between 2006 and 2010, parasitic oomycetes were isolated from more than 20 host taxa, particularly from *Rhododendron* spp., *Alnus* spp., *Fagus sylvatica*, *Fraxinus excelsior*, *Quercus* spp., *Acer* spp., and *Tilia cordata*. In total, more than 360 isolates of pythiaceous oomycetes have been acquired and deposited in our culture collection. Sixteen *Phytophthora* species have been found thus far: *P. alni*, *P. cactorum*, *P. cambivora*, *P. cinnamomi*, *P. citrophthora*, *P. gallica*, *P. gregata*, *P. gonapodyides*, *P. megasperma*, *P. multivora*, *P.* taxon oaksoil, *P. plurivora*, *P. polonica*, *P. ramorum*, *P.* taxon raspberry, and *P.* taxon salixsoil. *Phytophthora alni*, *P. plurivora*, and *P. cactorum* are the most frequently detected species in the country.

Phytophthora-induced alder decline is the most important of the problems caused by *Phytophthora* species in the Czech Republic, and it has become a devastating epidemic. To date, this disease has been detected in approximately 300 sites throughout the Czech Republic. The severity of the disease and its impact on riparian alder stands in the western part of the Czech Republic is comparable to the situations in Great Britain, northeastern France and Bavaria.

Keywords: diversity; *Phytophthora*; *Phytophthora* alder decline; woody plants.

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Introduction

Issues concerning Phytophthora diseases in woody plants and Phytophthora diversity were overlooked in the Czech Republic until 2000. Phytophthora diseases of woody plants have occasionally been found in nurseries, gardens, urban greenery and in woodlands and forests. The most frequently reported Phytophthora disease is damping-off in beech seedlings (Prochazkova & Jancarik, 1991). The type of damage to alders that is most likely connected with contemporary Phytophthora alder decline was

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reported in the 1990s (Jančařík, 1993; Struková et al., 1996). However, records of *Phytophthora* species in woody hosts have not been reliably documented. No isolate from a woody host species was deposited in a Czech microorganism culture collection prior to 2000. More serious investigations into *Phytophthora* spp. and the diseases that they cause in woody plants began to be implemented in the past decade, with four specific topics being investigated: (1) Phytophthora alder decline; (2) Phytophthora diversity in natural stands (e.g. forest and riparian stands); (3) Phytophthora diversity in urban areas (e.g. parks and alleys); and (4) Phytophthora diversity in ornamental locations (ornamental nurseries, garden centres, and ornamental gardens). This article reviews the main outcomes of these investigations.

Materials and Methods

Sample collection

Ornamental and forest plants were surveyed for symptoms caused by *Phytophthora* spp. in randomly selected regions throughout the whole area of the Czech Republic in the years 2006 – 2010 (Figures 1 & 2). The variability of stands from natural to highly utilised was taken in to account. Symptoms observed were: root and collar necroses; bleeding canker, followed by yellowing and thinning of foliage and crown wilting due to root and collar rot; and/or foliar, twig and branch necroses due to crown infections.

Symptomatic specimens were usually sampled in the growing period (May – October). Samples were collected in 285 localities and from 49 host plant taxa (including more than 20 different rhododendron species and cultivars). Tissues exhibiting active lesions and damage (as detailed above) were identified and sampled. In the case of bark lesions on stems and collars of individual trees, the bark from the apical part of these lesions was removed to uncover the subcortical tissues. Samples (100 – 200 cm^2) of tissues (including cambium) were stripped from the wood using a wood chisel and placed into sterile polyethylene bags. The samples were kept in a dark, cool place or box and processed immediately. When root rot was identified, three soil samples (each of approximately 1 – 2 L) including damaged roots were usually collected with a shovel from depths of approximately 10 – 20 cm from different locations within the root zone of the declining tree. When ornamental shrubs with symptoms of dieback were found, the damaged plant parts (leaves, twigs, shoots, and whole branches) or whole small plants were sampled. Where a stand of trees was affected, a number of samples were taken from different trees.

Sites

Each of the surveyed sites was classified into one of three categories: (1) natural stands (e.g. forests, riparian stands); (2) urban areas (e.g. parks, alleys, and highly utilised suburban forests); and (3) ornamental locations (e.g. nurseries, gardens and

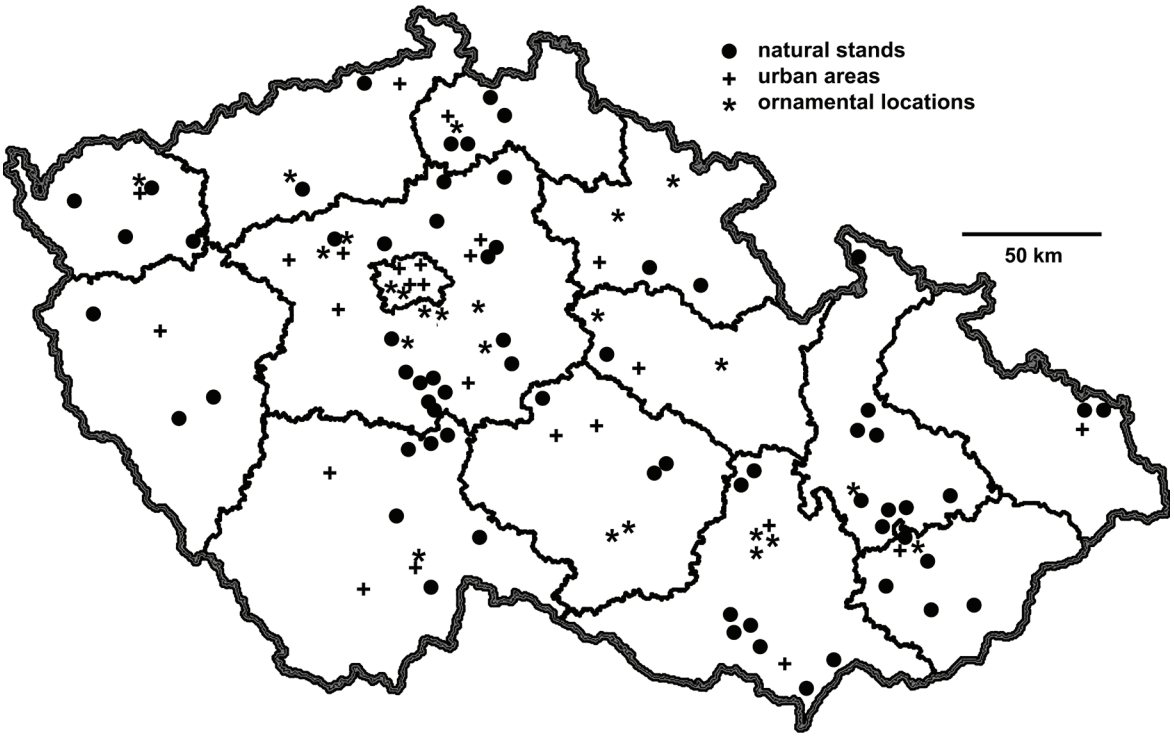

FIGURE 1: Distribution of investigated locations in the Czech Republic according to the type of sites (excluding *Phytophthora alni* riparian stands)

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garden centres). These three categories corresponded to the type and intensity of human activity, increasing in level from (1) to (3). For Category (1), a total of 172 riparian alder stands damaged mainly by *P. alni* subsp. *alni* and 64 forest and other natural tree stands were sampled. For Category (2), 25 stands were sampled, while 24 stands were sampled for Category (3). Two parks in Category (2) – one landscape park with relatively low human impact, and one city park with a high impact, and two nurseries in Category (3) – one large resale nursery, and one small local nursery were repeatedly (3× to 5×) sampled throughout the entire period of investigation. The aim of repeated isolation was to find the full diversity of oomycetes, and to identify differences in species composition in different sites within Categories (2) and (3). All other sites were sampled only once during the investigation.

Sample Preparation

The samples of above-ground tissues were carefully cleaned by hand in the laboratory under running tap water. Then, the margins of active lesions and freshly damaged plant parts were identified, and the tissues were cut into several dozen small segments (approximately 5 × 5 × 5 mm), repeatedly washed in sterile water and 95% ethanol, blotted on sterile filter paper or pulp and plated onto selective PARPNH V8-juice agar consisting of 100 mL V8 juice (Campbell Grocery Products Ltd., UK), 15 g agar, 3 g $CaCO_3$, 200 mg ampicillin, 10 mg rifampicin, 25 mg pentachloronitrobenzene (PCNB), 50 mg nystatin and 50 mg hymexazol per litre of deionised water (Jung et al., 1996).

Fine root samples were processed via a baiting method, and healthy rhododendron leaves served as bait (Erwin & Ribeiro, 1996). The colonised leaves usually showed brownish lesions after 3 – 5 days of incubation and were processed as described above.

Isolation of organisms

The PARPNH plates were examined for the presence of Phytophthora-like hyphae and colonies after 3 – 10 days. Single hyphae from the margins of growing colonies were transferred onto both V8-juice agar (100 mL V8 juice, 15 g agar, and 3 g $CaCO_3$ only per litre of deionised water) plates and carrot agar (50 g sliced carrot and 15 g agar per litre of deionised water) plates, and cultured for one to two weeks in the dark at 20 °C. The acquired isolates were preserved in tubes on oatmeal agar (50 g oatmeal, 15 g agar per litre of deionised water) (Erwin & Ribeiro, 1996).

Identification of Isolates

Morphological characteristics of each isolate were observed visually under a microscope. The morphological characteristics of mycelium, hyphal swellings, chlamydospores, and gametangia were observed on the solid carrot agar medium. The

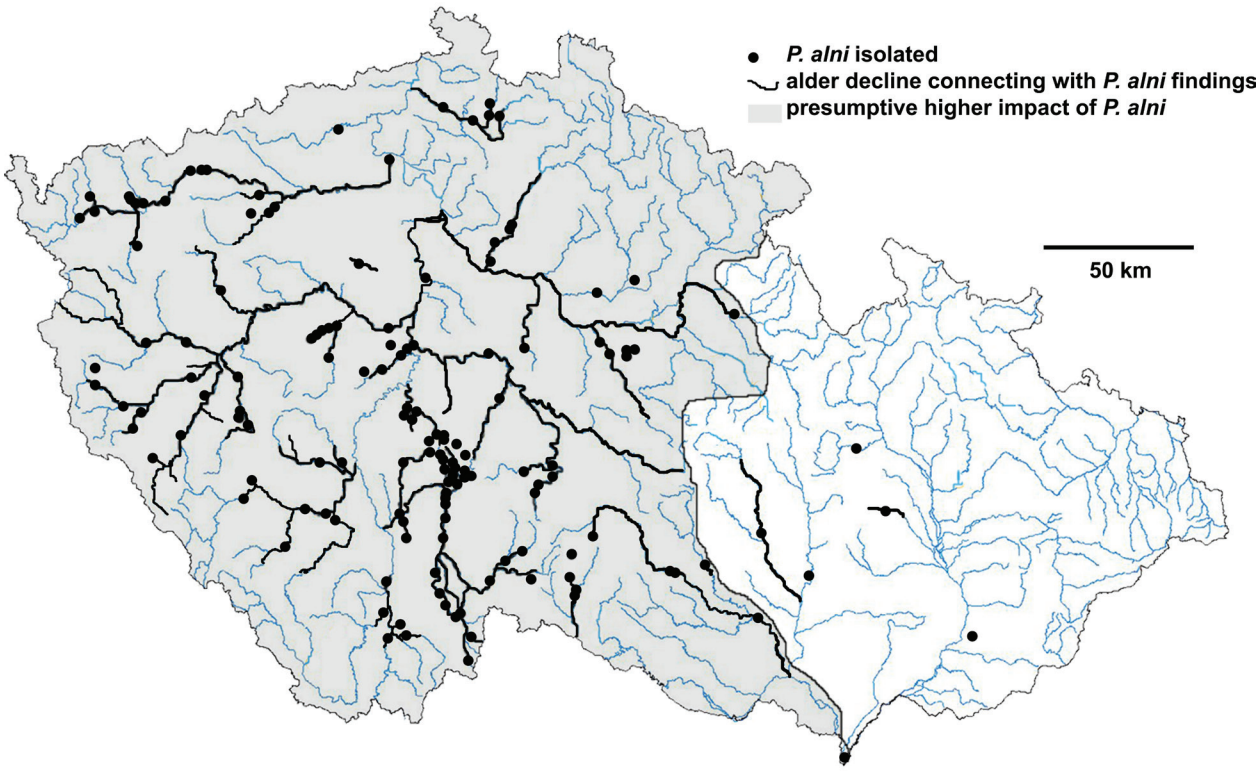

FIGURE 2: Distribution of *Phytophthora alni* and alder decline in the Czech Republic

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production of sporangia was induced by incubation of infested segments of carrot agar plates in soil extract or filtered pond water (Erwin & Ribeiro, 1996). Culture and growth characteristics were evaluated after one-week cultivation on V8A plates at 20 °C in the dark.

Isolates were also identified by sequencing the nuclear ribosomal DNA of the ITS region. For amplification of the ITS region, the primer pair ITS1/ITS4 was used. DNA was extracted from fresh cultures using a DNA extraction kit (DNeasy Plant Mini kit, Qiagen; Ultra Clean Microbial DNA Isolation Kit, Mo-Bio). The DNA was amplified by PCR using a Mastercycler® ep thermocycler (Eppendorf, Germany). Polymerase chain reaction (PCR) amplifications were conducted in a 25 µL reaction volume; the PCR mixture contained 50 ng of DNA, 20 pmol of each primer, 0.2 mM dNTPs, and 1 U of DynaZyme™ polymerase with the appropriate buffer (Finnzymes, Finland). PCR amplifications were performed under the following conditions: 94 °C for 3 min., 50 °C for 30 s, 72 °C for 1 min. (1×); 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s (33× cycle); and 94 °C for 30 s, 50 °C for 30 s, 72 °C for 5 min. (1× cycle). The PCR products were sequenced using the services of Macrogen Inc., Korea. The obtained sequences were analysed with BLAST algorithms in the GenBank database.

In general, identification by microscopy was confirmed by DNA sequencing. When this was not the case, DNA sequencing only was used for identification (in the case of newly identified species such as *P*. taxon raspberry, *P*. taxon salixsoil etc.).

Classified isolates were deposited in culture collection of our institute (RILOG, Průhonice). In addition, some of these isolates are also deposited in the Culture Collection of Fungi in Prague (CCF).

Results

The isolation medium used in this study is widely used in Europe (e.g. Balci & Halmschlager, 2003; Jönsson et al., 2005; Jung et al., 2005; Vettraino et al., 2002). However, we note that selective growth media can suppress certain *Phytophthora* species, e.g. *P. lateralis* so the range of *Phytophthora* species detected in our surveys could have been expanded if we had used other media. To date, *P. lateralis* has been recorded in two European countries, the Netherlands and France (Hansen et al., 1999; Robin et al., 2010).

Using the selective growth media detailed above, we have acquired more than 420 isolates to date. These have been classified into more than 20 species of pythiaceous oomycetes. Sixteen known *Phytophthora* species or genetically characterised, but taxonomically undefined, groups, were identified. These were: *P. alni* Brasier & S.A. Kirk, *P. cactorum* (Leb. & Cohn) Schröeter, *P. cambivora* (Petri) Buisman, *P. cinnamomi* Rands, *P. citrophthora* (R.E. Smith & E.H. Smith) Leonian, *P. gallica* T. Jung & J. Nechwatal, *P. gregata* T. Jung, M.C.J. Stukely & T.I. Burgess, *P. gonapodyides* (Petersen) Buisman, *P. megasperma* Drechsler, *P. multivora* P.M. Scott & T. Jung, *P*. taxon oaksoil (Brasier et al., 2003; Jung et al., 2011), *P. plurivora* T. Jung & T.I. Burgess, *P. polonica* L. Belbahri, E. Moralejo & F. Lefort, *P. ramorum* Werres, De Cock & Man in´t Veld, *P*. taxon raspberry (Jung et al., 2011), and *P*. taxon salixsoil (Brasier et al., 2003; Jung et al., 2011). A list of the species isolated and the hosts on which they were found is given in Table 1. *Phytophthora alni*, *P. plurivora* and *P. cactorum* were the most frequently isolated species (Černý & Strnadová, 2010; Černý et al., 2008, 2009; Mrazkova et al., 2007, 2010). As expected, *P. alni* colonised both species of *Alnus* examined but other *Phytophthora* species isolated were found on a range of host species.

For Category (1) sites, oomycetes were isolated from 52 of the 64 forests/natural tree stands surveyed. We found that 14 native woody forest plants (most frequently *Alnus incana* (L.) Moench, *A glutinosa* (L.) Gaertn., *Fagus sylvatica* L., *Fraxinus excelsior* L., *Quercus robur* L., *Acer platanoides* L., *A pseudoplatanus* L., and *Tilia cordata* Mill.) hosted these oomycetes. A total of 9 *Phytophthora* species were identified (Table 2), and the most frequently detected species was *P. plurivora*.

For the 172 riparian alder stands sampled, *P. alni* subsp. *alni, P. alni* subsp. *uniformis*, *P. gallica*, *P. gregata*, *P*. taxon oaksoil, and *P*. taxon salixsoil were the most common taxa found. Very likely**,** many of our undetermined isolates assigned to *Phytophthora* spp. in Table 1 will fall into these newly described species.

The most frequently observed disease symptom at Category (1) sites was rot of fine roots (with corresponding crown thinning and withering) in several indigenous tree species, especially *Quercus robur, Acer* spp., *Alnus glutinosa*, *Fraxinus excelsior*, and *Tilia cordata* (Table 1).

In Category (2) sites, oomycetes were isolated on 13 hosts (both indigenous and exotic) at 14 of the 25 stands surveyed. A total of 8 *Phytophthora* species were identified (Table 1), and *P. plurivora* and *P. cactorum* were the most frequently detected of these. The most frequent disease symptom observed was root rot, caused by *P. plurivora* in *Quercus* spp. and in other woody hosts. Collar rot, caused by *P. cactorum* and *P. plurivora* (especially on *Fagus sylvatica*), was found in certain localities (for instance, in Prague´s largest public park, Kralovska Obora).

The repeated sampling in the two different parks (landscape park, and city park) resulted in quite different spectra of *Phytophthora* species. In the landscape park the detected spectrum was near to

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TABLE 1: *Phytophthora* species isolated from indigenous (*) and alien woody hosts in the Czech Republic and symptoms associated with their hosts.

	P. alni	*P. cactorum*	*P. cambivora*	*P. cinnamomi*	*P. citrophthora*	*P. gallica*	*P. gregata*	*P. gonapodyides*	*P. megasperma*	*P. multivora*	*P.* taxon oaksoil	*P. plurivora*	*P. polonica*	*P. ramorum*	*P.* taxon raspeberry	*P.* taxon salixsoil	*Phytophthora* spp.	*Pythium* spp.
*Acer campestre**												R						
*Acer pseudoplatanus**		R										R/C						R
*Acer platanoides**												R						
Aesculus hippocastanum		R/C	R															R
*Alnus glutinosa**	R/C					R		R	R/C		R	R/C				R	R	R
*Alnus incana**	R/C																	
*Betula pendula**							R										R	
Castanea sativa			R/C															
*Fagus sylvatica**		R/C	R/C									R/C						R/C
*Fraxinus excelsior**						R	R					R				R		R
Pieris floribunda												D						
Pieris japonica														R/D				
*Populus alba**		R/C																
Populus balsamifera		R/C																
*Quercus pubescens**																		R
*Quercus robur**						R				R	R	R				R	R	R
Quercus rubra								R/C				R						R
Rhododendron spp.		C/D	R/D	R/C/D	D				R/C	D		R/C/D		R/C/D	R		R/C	R/C
*Salix fragilis**						R				R	R	R				R	R	
*Tilia cordata**		R	R				R					R	R					R
*Ulmus glabra**																		R
*Ulmus laevis**												R						
Vaccinium corymbosum				R/C								D						
Water (control)								+			+					+	+	
Total number of hosts§	2	18	6	13	1	4	3	3	2	3	4	16	1	2	1	4	7	19
Number of affected locations†	156	16	8	2	1	3	3	4	2	5	7	32	2	2	1	7	12	27

* = indigenous species
R = root rot and rhizosphere
C = collar rot
D = dieback

blank cell = not found
Frequent occurrence is highlighted in grey
§ = number of host taxa including number of *Rhododendron* species and cultivars (bulked together in the table)
† = number of locations with confirmed occurrence of the particular pathogen

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TABLE 2: *Phytophthora* species isolated from woody hosts in the Czech Republic according to the type of stand: natural stands (forest and riparian stands), urban areas (parks, alleys, highly affected suburban forests etc.), and ornamental locations (nurseries, gardens, garden centres, etc.).

	P. alni	*P. cactorum*	*P. cambivora*	*P. cinnamomi*	*P. citrophthora*	*P. gallica*	*P. gregata*	*P. gonapodyides*	*P. megasperma*	*P. multivora*	*P.* taxon oaksoil	*P. plurivora*	*P. polonica*	*P. ramorum*	*P.* taxon raspeberry	*P.* taxon salixsoil	*Phytophthora* spp.	*Pythium* spp.
Natural stands	+		+			+	+	+		+	+	+				+	+	+
Urban greenery	+	+	+				+	+		+		+	+				+	+
Ornamental nurseries and gardens		+	+	+	+			+	+	+		+		+	+		+	+

the spectrum in Category (1) sites (for instance *P. gonapodyides*, and *P. gregata* were found). On the other hand, in the highly affected city park *P. cactorum*, *P. plurivora* and *P. multivora* were frequent. Most likely, the differences in the species spectrum depended on the human activity level. The number of isolated species was nearly equal: 5 species were found in the landscape park, whereas 7 species were found in the city park.

In Category (3) sites, oomycetes were isolated from 28 exotic hosts at 18 of the 24 sites surveyed. *Rhododendron* was the most commonly infected genus with a total of 24 different species or cultivars affected. *Rhododendron catawbiense* Michaux, *R.* Cunningam White and *R.* Roseum Elegans specimens were most commonly infected. Ten *Phytophthora* species were detected at Category (3) sites, the most frequent being *P. plurivora* and *P. cactorum.* This result was similar to that found for Category (2) sites. These pathogens frequently caused both root and collar rot (as in Category (2) sites) but also caused the dieback of above-ground tissues (leaf anthracnose, twig and shoot blight and branch canker). Of particular note and concern is that one of the ten *Phytophthora* species detected at Category (3)-sites was *P. ramorum* (found on rhododendron in 2009 and pieris in 2011). This is the first detected occurrence of this quarantined organism *P. ramorum* in the Czech Republic.

The difference in the two repeatedly sampled ornamental nurseries (large resale and small local) were less important. The majority of isolates in both nurseries belonged to alien or cryptogenic species. The most frequent species obtained were *Phytophthora plurivora*, *P. cactorum*, and *P. cambivora* in both nurseries. *Phytophthora cinnamomi* was consistently isolated only in the large nursery. In this nursery, 12 species of oomycete were isolated, whereas in the small local nursery only 7 species were found.

The three site categories investigated differed markedly in *Phytophthora* species diversity (Table 2). The spectrum of *Phytophthora* spp. in Category (1) sites included potentially indigenous species (e.g. *P. gallica*, *P. gregata*, *P*. taxon oaksoil, and *P*. taxon salixsoil), cryptogenic species (*P. plurivora*, *P. cambivora*, and *P. multivora*), and the alder pathogens *P. alni* subsp. *alni* and *P. alni* subsp. *uniformis*. Category (3) sites were characterised by the presence of alien invasive (*P. cinnamomi*, *P. citrophthora*, and *P. ramorum*) and cryptogenic species (*P. cactorum*, *P. cambivora*, *P. plurivora*, *P. multivora*, and *P. megasperma*). The biota of *Phytophthora* spp. in Category (2) sites was intermediate and incorporated species from the other two categories.

Moreover, it was shown that repeated sampling was necessary for detection of full oomycete diversity. Total number of oomycetes in four repeatedly tested

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stands was 17 (although only 9 species were acquired after the first series of isolation). However the most frequent and important species (*P. gonapodyides*, *P. cactorum*, *P. cambivora*, *P. plurivora*, *P. multivora*, and *P. cinnamomi*) were caught in the first isolation series and consistently found later during the investigation.

Our study has found that a wide range of *Phytophthora* spp. infect various host species in a number of different habitats. However, the most important disease caused by *Phytophthora* spp. in woody plants is Phytophthora-induced alder decline. To date, the pathogen has been isolated in 156 sites and the disease has been detected according to the presence of characteristic symptoms in approximately 300 sites throughout the Czech Republic (Figure 2). Moreover, this pathogen has become a common component of native alder ecosystems, especially in the western part of the country (Černý & Strnadová, 2010). The severity of the disease and its impact on riparian alder stands in the western part of the Czech Republic is comparable to the situations in Great Britain, north-eastern France and Bavaria (Gibbs et al., 1999; Jung & Blaschke, 2004; Streito et al., 2002). More than 95% of the records of this pathogen and its associated disease in the Czech Republic are restricted to areas such as bankside alder stands, alder carrs (wetland overgrown with trees), mixed ash and alder stands along small and medium-sized watercourses, and alder stands in periodically flooded alluvial plains. The disease is distributed mainly in basins of the Vltava, Ohre, Labe, and Morava rivers. The occurrence of the disease in forest plantations is generally rare, and no record of it has been reported in forest nurseries to date. Watercourses are the primary routes of infection for the pathogen (Černý & Strnadová, 2010). Fish farming ponds can be important in the introduction of pathogen into new areas. Although introduction of the pathogen through infected alder saplings from nurseries has not been detected thus far, this mode of introduction may occur in the near future (Černý & Strnadová, 2010).

Conclusions

More than 420 isolates of oomycetes from diseased woody hosts were acquired in the Czech Republic during the period from 2006 to 2010. Sixteen *Phytophthora* species were identified. The species most frequently isolated were: *P. alni* (common in riparian alder stands of Category (1); *P. plurivora* (frequent in sites from all three Categories; and *P. cactorum* (the only species found in Category (2) and (3) sites but not in Category (1) sites).

Other, potentially dangerous invasive species (including *Phytophthora cambivora*, *P. cinnamomi*, *P. citrophthora*, *P. multivora*, and *P. ramorum*) were recorded, but only locally. Of these species, only *P. cambivora* and *P. multivora* were locally recorded in Category (1) sites. *Phytophthora cinnamomi*, *P. citrophthora* and *P. ramorum* were found only in Category (3) sites so it is possible that the distribution of these species is currently relatively restricted to ornamental and urban trees and shrubs. Other *Phytophthora* species (*P. gonapodyides*, *P. gregata*, *P. gallica*, P. taxon oaksoil, and *P.* taxon salixsoil) are probably frequent in Category (1) sites but are probably less pathogenic.

To date, the Phytophthora-induced alder decline has been determined to be the most important health problem caused in any woody host by *Phytophthora* species in the Czech Republic. Nevertheless, the potential impact of other identified invasive species should not be underestimated.

The main topics that require greater attention in the near future are the following: Phytophthora diversity in woody plants in the Czech Republic in general; modes of spread, and the ecology of *Phytophthora alni* and its impact in alder stands; the impact of *P. plurivora* in oak and beech forest ecosystems; and the development of control methods and quarantine measures.

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