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Sudden *Phytophthora* Dieback of Wild Cherry Trees in Northwest Hungary

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Abstract – During a regular survey of declining forests in 2011, sudden dieback symptoms were observed on scattered wild cherry trees (*Prunus avium*) in a mixed deciduous forest stand, located in the flood plain area of the Rába River, in northwest Hungary. In this study, we correlated both soil conditions and presence of *Phytophthora* spp. to dieback of cherry trees. Two *Phytophthora* species, *P. polonica* and *P. plurivora*, were isolated from the rhizosphere soil of the dying trees. By contrast, only *P. polonica* was recovered from the necrotic tissues of symptomatic roots. Stem and root inoculation tests on cherry seedlings showed pathogenicity of both species, although *P. polonica* proved to be more virulent. This is the first report of natural infections of *P. polonica*.

soilborne pathogens / forest protection / *Phytophthora polonica* / *Phytophthora plurivora*

Kivonat – **Madárcseresznye fák fitoftórás pusztulása Északnyugat-Magyarországon.** Egy 2011-ben, pusztuló erdőállományokban végzett egészségi állapot-felmérés során egy elegyes erdőállományban a madárcseresznye fák pusztulására figyeltek fel a szerzők. Az erdőállomány a Rába folyó egy holtága mentén terül el. Egy két éves esettanulmány során, talajvizsgálatot és a talaj és a talált *Phytophthora* fajok hatását vizsgáló patogenitásteszteket végeztünk. Eredményeinket statisztikailag értékeltük. Míg az erdőállomány talajából *Phytophthora plurivorá*t és *Phytophthora polonicá*t tenyésztettünk ki, a pusztuló fák tüneteket mutató gyökereiből csak *P. polonicá*t sikerült izolálni. Madárcseresznye-csemetéken elvégzett törzssebzési és gyökérfertőzési kísérletek egyaránt kimutatták mindkét izolált faj patogenitását. agresszivitását statisztikai elemzések bizonyítják. Ez az első olyan alkalom, amikor természetes körülmények között a *P. polonica* kórokozónak bizonyult.

talajlakó kórokozó / erdővédelem / *Phytophthora polonica* / *Phytophthora plurivora*

1 INTRODUCTION

Wild cherry (*Prunus avium* L.) is present in Hungarian mixed deciduous forests as scattered trees. Besides fungal and bacterial pathogens, several *Phytophthora* species are known to cause dieback of cherry trees under natural or experimental conditions (Santini et al. 2006, Kurbetli 2014, Vettraino et al. 2008). Among *Phytophthora* species, *P. alni* Brasier & S. A. Kirk, *P. cactorum* (Lebert & Cohn) J. Schröt., *P. cambivora* (Petri) Buisman, *P. cinnamomi* Rands, *P. citricola* Sawada, *P. citrophthora* (R. E. Sm. & E. H. Sm.) Leonian, *P. cryptogea*

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Pethybr. & Laff., *P. drechsleri* Tucker, *P. megasperma* Drechsler, *P. nicotianae* Breda de Hahn and *P. syringae* (Kleb.) Kleb. are known to cause dieback of cherry trees under natural or experimental conditions (Mircetich – Matheron 1976, Thomidis – Sotiropoulos 2003, Santini et al. 2006, Thomidis et al. 2008, Vettraino et al. 2008). In this paper we describe the sudden death of wild cherry trees in northwest Hungary and evaluate the role of the combined impact of soil texture and soilborne *Phytophthora* spp. in the etiology of the disease.

2 MATERIALS AND METHODS

2.1 Isolation

In spring 2012 sudden dieback of twelve-year-old wild cherry trees was surveyed in a 1.84 ha mixed deciduous forest stand (pedunculate oak: 60%, common ash: 25%, wild cherry: 5%, eastern black walnut: 5%, other broadleaved tree species: 5%) partly surrounded by a backwater of the Rába River, near Sárvár (150 m above sea level) in northwest Hungary. Six symptomatic trees were randomly selected and necrotic root and rhizosphere soil samples were collected in a total amount of 500 g/sample. Both sample types were processed as previously described (Szabó et al. 2013). To recover *Phytophthora* isolates from the soil samples, cherry laurel (*Prunus laurocerasus* L.) and *Rhododendron* sp. leaves were used as baits. The infected leaf segments were placed onto selective PARPNH media (Jung et al. 2000). Morphological characterization was carried out as described by Szabó et al. (2013). All of the collected isolates were examined. For molecular identification, pure mycelial cultures were used for direct PCR with the PHIRE Plant Direct PCR Kit (Thermo Scientific) according to the manufacturer's user's guide. The ITS1-5.8S-ITS2 regions of the rDNA of selected isolates were amplified using the ITS6 and ITS4 primer pair (Cooke – Duncan 1997) in an Eppendorf Mastercycler Personal PCR machine. PCR conditions were as follows: 5 min initial denaturation at 98 °C, 5 s denaturation at 98 °C, 5 s annealing at 55 °C, 20 s extension at 72 °C (1 min for the final cycle) with 39 cycles in total. The amplified DNA fragments were sequenced in both directions in the Eurofin Laboratory (Ebersberg, Germany). All of the six collected isolates were sequenced and their sequence homologues were identified using Blast searches against the NCBI GenBank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Accession numbers: KT693123; KT693124; KT693125; KT693126; KT693127; KT693128).

2.2 Pathogenicity tests

Pathogenicity tests were performed using two different soil conditions to compare the effect of the local sandy alluvial soil versus the control soil type typical of the forests of the region. Both soil types proved to be free of *Phytophthora* species prior to infestation according to the result of leaf baiting controls. Soil analyses were performed in the WSL soil laboratory (Swiss Federal Research Institute, Birmensdorf, Switzerland). The pathogenicity of one *P. plurivora* (Jung – Burgess 2009) and one *P. polonica* (Belbahri – Moralejo – Lefort 2011) isolate (both 14 days old, grown on 39g/l PDA at 20°C in the dark) was assessed in both soil types (sandy alluvial and control soils). Stem inoculation and root infection tests were carried out on two-year-old, container-grown wild cherry seedlings, using 10 replicates per *Phytophthora* species and 10 replicates as control (not infected). For root infection, four equal aliquots of mycelial culture were put into the planting medium in four directions around the stem of the seedlings (altogether two cultures/seedling). Stems were wound inoculated by inserting an infested PDA plug (5-mm in diameter) under the bark using a sterile scalpel. Control seedlings were inoculated with sterile PDA. Wounds were sealed with Parafilm (Pechiney, Chicago, IL). Seedlings were maintained under outdoor natural conditions and were watered when

necessary. Lesion sizes were measured and the health condition of the shoots and roots was evaluated based on a five-point scale 13 weeks after inoculation (see *Table 1*). Necrotic tissues from the stem and roots of infected seedlings, and soil samples from the container of infected seedlings were collected for the re-isolation of *Phytophthora* species in order to fulfill Koch's postulates.

2.3 Statistical analyses

Statistical analyses were performed with STATISTICA 12 software (StatSoft, Inc. [2014]). Kruskal-Wallis and Mann-Whitney U tests were used for the comparison of the treatment groups. The effects of the two contributing factors (pathogen versus soil type) were evaluated both separately and together. Pearson Chi-square tests were performed to evaluate whether there is a significant correlation between treatments applied and the parameters of the root system. Boxplots were created and edited using SPSS (IBM, v. 22).

Table 1: The five-point scale used for the evaluation of pathogenicity tests

Stage	Crown symptom	Root symptom
1	Asymptomatic sapling	Healthy root system with plentiful healthy fine roots
2	Smaller leaves with yellowish discolouration	Dead root tips and fine roots occurring locally (less than 30%)
3	30%–50% of the crown is dead	30%–50% of the root system is already dead
4	More than 50% of the crown is dead	More than 50% of the root system is already dead
5	Completely dead sapling	Completely dead sapling

3 RESULTS

3.1 Isolation and identification

Sudden dieback of young wild cherry trees was clearly observed in a forest stand at the inundation area of the Rába River at the time of budburst in spring 2012. Approximately 70% of the wild cherry trees showed severe wilting symptoms or were already dead. Bark cracking and stripping, gummosis and wood discoloration under necrotic bark on the lower stems and also necroses on the main roots were observed. Six *Phytophthora* isolates were obtained from five from of the six soil samples using leaf baiting and a single isolate from the necrotic tissues. Based on the comparison of morphological traits and homology (99-100%) to other ITS sequences, four *P. polonica* (three from the soil and one from necrotic tissues) and two *P. plurivora* isolates (both from the soil) were identified. The isolates of the two species could be easily distinguished based on the daily growth rate, the shape and amount of sporangia produced and the presence or absence of hyphal swellings. While the two *P. plurivora* isolates had a daily growth rate 6.50–6.83 mm, *P. polonica* colonies grew more slowly (4.50–4.67 mm/day). The colonies of *P. plurivora* isolates formed readily and abundantly sporangia with variable shapes but hyphal swellings were absent. In contrast, *P. polonica* isolates produced abundantly single or catenulate hyphal swellings either with globose or irregular shapes and only two isolates formed a few, ovoid, non-papillate sporangia.

The analysis of the soil types illustrated that the base saturation values were nearly identical and only soil textures were different (*Table 2*).

Table 2: Soil types used for the pathogenicity tests

		Sárvár	Control soil
Texture	(% sand, silt, clay)	*61, 24, 15*	*12, 54, 34*
pH	(0.02 M $CaCl_2$)	*5.06*	*7.35*
Ca_{exch}	(mg kg^{-1})	3819.39	6983.97
Mg_{exch}	(mg kg^{-1})	604.76	700.45
K_{exch}	(mg kg^{-1})	269.37	431.95
Na_{exch}	(mg kg^{-1})	< 5.20	14.83
Mn_{exch}	(mg kg^{-1})	67.81	6.95
Fe_{exch}	(mg kg^{-1})	2.75	< 1.60
Zn_{exch}	(mg kg^{-1})	1.27	< 1.20
Al_{exch}	(mg kg^{-1})	3.52	< 2.00
CEC	($mmol_c$/kg)	*125.13*	*209.03*
Base saturation	(%)	98.80	99.94

3.2 Pathogenicity tests

In the case of the uninfected seedlings, the root system remained healthy and rich in fine roots. In contrast, altogether six *P. polonica*-, and four *P. plurivora*-infested seedlings died during the thirteen weeks of the root infection experiment. Thinned root system, dead fine roots and root tips, and extremely short main roots were observed (*Figure 1*). Significant differences in the health conditions of roots ($p = 0.000$) and in the root widths ($p = 0.000$) were identified according to the results of the Kruskal-Wallis tests. For the roots' health conditions (*Figure 2A*), both the effect of soil type ($p = 0.015$) and the pathogen ($p = 0.000$) were significant according to the results of the Kruskal-Wallis tests. There was a significant correlation between the applied treatment and the health condition of the root system ($p = 0.00000$) according to the Pearson Chi-square test. Altough, there wasn't any significant correlation between the applied treatment and the root width based on the Pearson Chi-square test. *P. polonica* seemed to be slightly more aggressive than *P. plurivora*. Both *Phytophthora* species were re-isolated from symptomatic roots and from the infected growth media.

In the stem inoculation experiment, sunken and dark necrotic lesions developed at the inoculation points of infected seedlings while only callus formation was observed on the control stems. Significant differences in the health conditions of shoots ($p = 0.002$) and necrotic areas ($p = 0.001$) were found between the treatment groups according to the results of the Kruskal-Wallis tests. For shoot health conditions (*Figure 2B*), only the effect of soil type was significant ($p = 0.000$) and the soil type did not have a significant impact on the size of the necrosis on the stems (*Figure 2C*). There was no significant difference in the aggressivity of the two species. Both *Phytophthora* species were re-isolated from the border of the lesions.

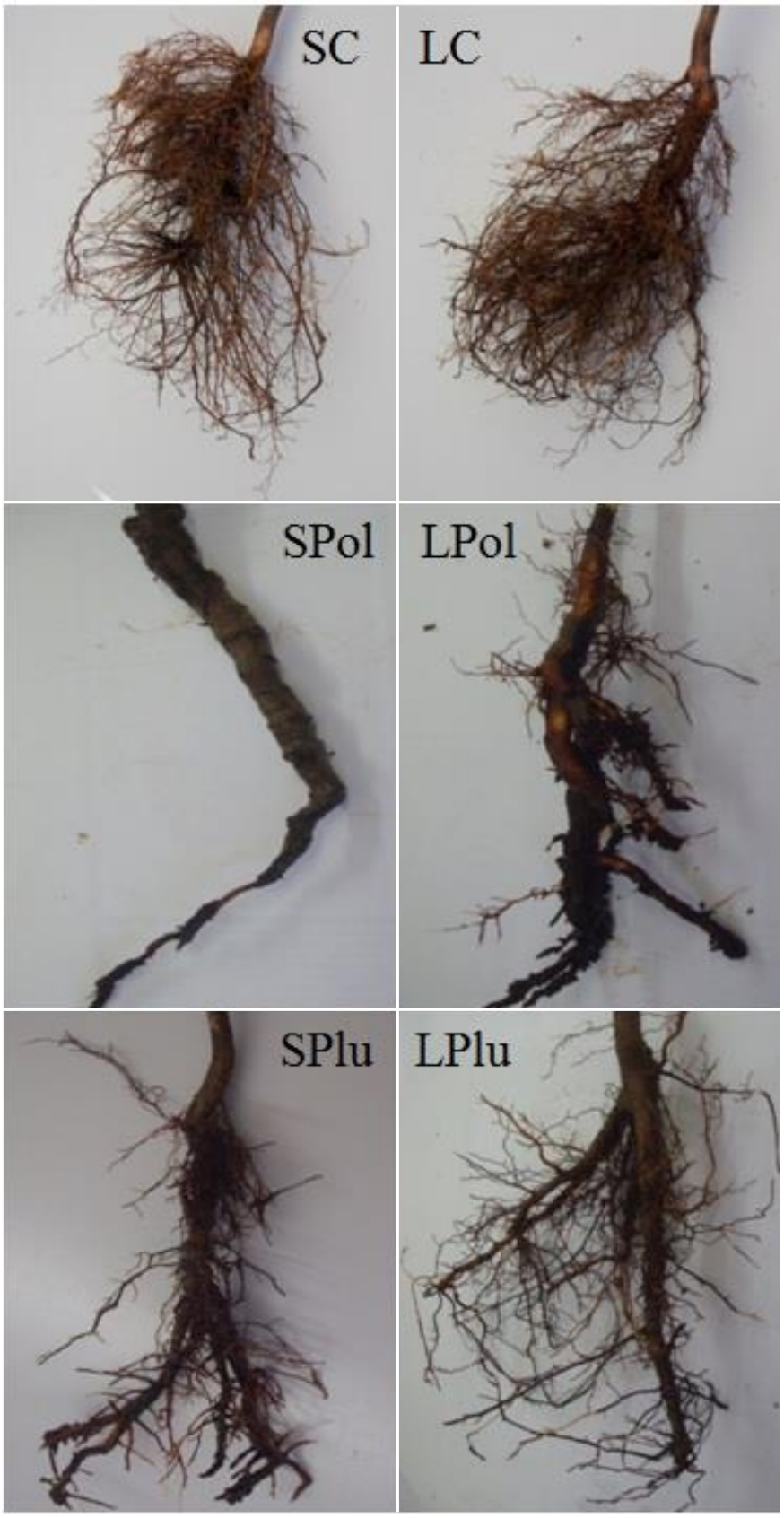

Figure 1. Root development in soil infection test (Abbreviations: L: control soil type, S: local alluvial soil type, Pol: P. polonica, Plu: P. plurivora, C: non-infected control)

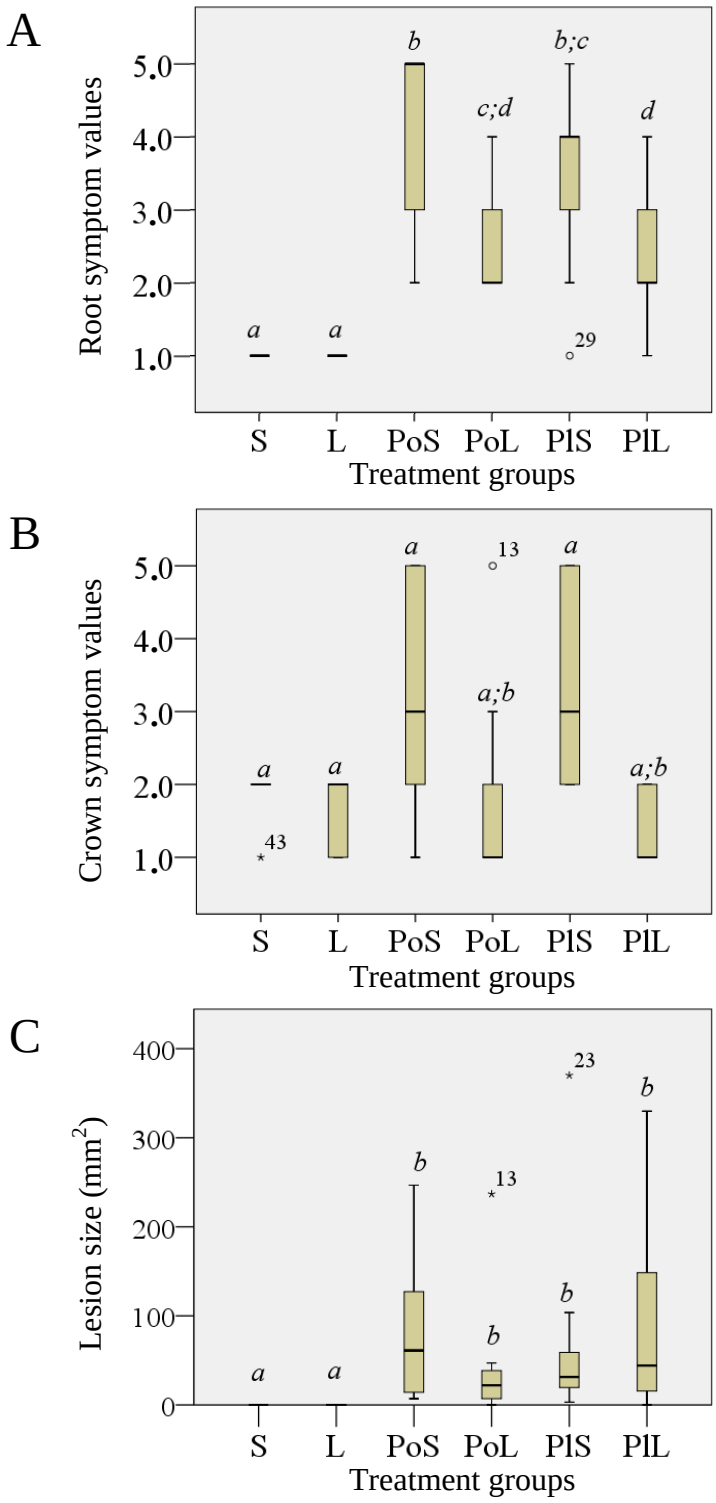

Figure 2.

A: Root system health conditions in the different treatment groups (mean values; different labels mean significantly different groups based on the Mann-Whitney U test).

B: Health conditions based on crown symptoms in the different treatment groups (mean values; different labels mean significantly different groups based on the Mann-Whitney U test).

C: Lesion sizes in the different treatment groups (mean values; different labels mean significantly different groups based on the Mann-Whitney U test).

4 DISCUSSION

The main goal of our study was to determine the causal agents of the selective wild cherry mortality in a mixed deciduous forest stand growing in a flooded riverside area. Initially, the sandy alluvial soil and the impact of the fluctuating water levels were suspected as they represent poor growth conditions for cherry trees. However, various bark cracking and necrosis symptoms in the trees that were still alive indicated a possible *Phytophthora* infection. At the same time, we did not observe any visible symptoms of a potential fungal or bacterial attack, neither in the dead, nor in the dying trees. The recovery of *P. polonica*, from both the necrotic tissues and the rhizosphere soil of the dying trees, suggested a primary role of this *Phytophthora* species in the observed dieback. *P. plurivora*, regarded as a pathogenic species (Oßwald et al. 2014), was also isolated from the soil and was considered as a potential contributing factor to the rapid decline of the trees.

Stem and root infection tests demonstrated the pathogenicity of both *Phytophthora* species, with *P. polonica* showing slightly more virulence against wild cherry seedlings. The significant differences in the root infection between the two different planting media used suggest that the poor site conditions may have led to weakened immunity in the wild cherry trees. The unusually high precipitation in the region, which occurred in August 2011 (112 mm), combined with the loose sandy soil texture, may have caused ideal environmental circumstances that exposed the vulnerable cherry trees to a rapid *Phytophthora* invasion.

Although all cherry trees were planted ones, suggesting that the pathogen may have emerged and been introduced to the forest site from a nursery, the seedlings were indeed grown from local seed resources in a nearby nursery. Therefore, the origin of the pathogen remains still unclear and a recent colonization of the forest site cannot be ruled out.

While *P. plurivora* has previously been associated with the decline of various forest stands in Hungary (Szabó et al. 2013), this is the first report of *P. polonica* being directly involved in rapid tree mortality. Since 2012, no similar decline of wild cherry trees has been reported in Hungary. Nevertheless, our results provide a warning of a potential threat to *Prunus* species and highlight the importance of considering site conditions when planning plantations or reforestations.

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