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SHORT COMMUNICATION

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Isolation and pathogenicity of *Phytophthora* species from sessile oak (*Quercus petraea* (Matt.) Liebl.) stands in Slovakia

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Abstract

During the monitoring of oak decline phenomenon in Slovakia, symptoms indicative of *Phytophthora* diseases were observed in sessile oak stands in western Slovakia. The study aimed to test the presence and diversity of *Phytophthora* species associated with declining oak stands. From rhizosphere soil samples, *Phytophthora plurivora*, *P. quercina* and *Pythium intermedium* were detected. Soil inoculation tests with *P. plurivora* and *P. quercina* on 1-year-old oak plants inflicted damage to the root system, and significant differences in measured root parameters between the inoculated and control treatments after 5 months. Although more wilting symptoms were observed in *P. plurivora*—infected seedlings (7 out of 12 inoculated seedlings), *P. quercina* caused the highest amount of damage to root systems, and for example, fine root length was 1.6 times shorter compared to the control. To our knowledge, this is the first report of *P. plurivora* and *P. quercina* in sessile oak stands in Slovakia.

KEYWORDS
pathogenicity, pathways, *Phytophthora plurivora*, *Phytophthora quercina*, *Quercus*, soilborne pathogens

1 | INTRODUCTION

During the 20th century, the phenomenon of oak decline was recorded in many European countries, and several keystone oak species are affected by the decline, including sessile oak (*Quercus petraea* (Matt.) Liebl.), pedunculate oak (*Quercus robur* L.), cork oak (*Q. suber* L.), holm oak (*Q. ilex* L.) and other *Quercus* species (Jung et al., 2018). Several studies have attributed species of *Phytophthora* as causal agents of the oak decline phenomenon (Jung, Blaschke, & Neumann, 1996; Jung et al., 2018).

In Slovakia, mixed oak–hornbeam forests with predominantly a larger share of oaks are the most common forest type covering large areas, from the lowlands to mountainous areas up to 600 m a. s. l. Historically, these forests have showed little damage; however, currently small fragments are under high pressure of abiotic, biotic and anthropogenic factors (Holecová et al., 2005). Symptoms observed in oak stands, for example yellowing of leaves and increased crown transparency of the trees, may indicate the loss of roots due to the infection of *Phytophthora* pathogens (Jung et al., 1996).

The aims of this study were to (a) determine the presence and diversity of *Phytophthora* species in declining sessile oak stands in western Slovakia, and (b) test the pathogenicity of isolated species in controlled conditions.

2 | MATERIALS AND METHODS

2.1 | Study sites and sample collection

Eleven mature sessile oak stands located in the western part of Slovakia were selected for the study (Table 1). All the selected stands are managed forests originating from artificial regeneration, and sessile oak was

TABLE 1 Isolation of *Phytophthora* species from rhizosphere soil samples in *Quercus petraea* stands in Slovakia

Stand, Location	Tree No	Age	Disease symptoms	Sample	*Phytophthora* species (No. of isolates)	GenBank Accession Numbers of the representative isolates
Stand No. 1 48°16′58.8″N 17°20′34.8″E	1	95	Yellowing of leaves	Rhizosphere soil	*P. plurivora*	MT299766
	2		Yellowing of leaves	Rhizosphere soil	–	
	3		Yellowing of leaves	Rhizosphere soil	–	
	4		Yellowing of leaves	Rhizosphere soil	–	
	5		Yellowing of leaves	Rhizosphere soil	–	
Stand No.2 48°16′56.8″N 17°20′10.0″E	6	80	Yellowing of leaves	Rhizosphere soil	–	
	7		Yellowing of leaves	Rhizosphere soil	–	
	8		No symptoms	Rhizosphere soil	*Py. intermedium* (2)	
	9		No symptoms	Rhizosphere soil	–	
	10		No symptoms	Rhizosphere soil	–	
Stand No. 3 48°22′33.6″N 17°21′39.6″E	11	65	No symptoms	Rhizosphere soil	–	
	12		No symptoms	Rhizosphere soil	–	
	13		No symptoms	Rhizosphere soil	–	
	14		Yellowing of leaves	Rhizosphere soil	–	
	15		No symptoms	Rhizosphere soil	–	
Stand No. 4 48°26′38.4″N 17°29′16.8″E	16	65	No symptoms	Rhizosphere soil	–	
	17		Crown transparency	Rhizosphere soil	*P. plurivora*	MT299767
	18		Crown transparency	Rhizosphere soil	*P. plurivora*	MT299768
	19		Yellowing of leaves	Rhizosphere soil	–	
	20		Yellowing of leaves	Rhizosphere soil	–	
Stand No. 5 48°33′36.0″N 17°26′09.6″E	21	105	No symptoms	Rhizosphere soil	*Py. intermedium* (4)	
	22		Crown transparency	Rhizosphere soil	*Py. intermedium* (2)	
	23		Yellowing of leaves	Rhizosphere soil	–	
	24		Crown transparency	Rhizosphere soil	*Py. intermedium* (4)	
					P. plurivora	MT299769
	25		No symptoms	Rhizosphere soil	*Py. intermedium* (5)	
	26		No symptoms	Rhizosphere soil	*Py. intermedium* (6)	

(Continues)

TABLE 1 (Continued)

Stand, Location	Tree No	Age	Disease symptoms	Sample	*Phytophthora* species (No. of isolates)	GenBank Accession Numbers of the representative isolates
Stand No. 6 48°38′09.8″N 18°47′08.3″E	27	95	Crown transparency	Rhizosphere soil	*P. plurivora* (3)	MT299770
	28		Crown transparency	Rhizosphere soil	*P. plurivora* (9)	
	29		Crown transparency	Rhizosphere soil	*P. plurivora* (8)	MT299771
	30		Crown transparency	Rhizosphere soil	*P. plurivora* (12)	MT299773
	31		Crown transparency	Rhizosphere soil	*P. plurivora* (7)	
Stand No. 7 48°40′15.2″N 18°04′00.2″E	32	85	High crown transparency	Rhizosphere soil	*P. quercina* (2)	MT299779
Stand No. 8 48°40′20.1″N 18°03′56.5″E	33	45	Yellowing of leaves	Rhizosphere soil	–	
Stand No. 9 48°40′40.3″N 18°04′06.7″E	34	45	No symptoms	Rhizosphere soil	–	
Stand No. 10 48°39′55.1″N 18°04′08.9″E	35	95	No symptoms	Rhizosphere soil	–	
Stand No. 11 48°41′02.8″N 18°05′26.4″E	36	65	No symptoms	Rhizosphere soil	–	

TABLE 2 Results of the soil infestation test of *Phytophthora* spp. on *Quercus petraea* after 5 months: number and per cent of symptomatic plants, and mean values with standard deviation (*SD*) of the measured root parameters

Treatment	No. of plants	No. (%) declining plants	Parameters				
			Total root length (cm)	Fine root length (cm)	Mother root length (cm)	Number of root tips	Fine root surface area (cm^2)
P. quercina	12	0 (0)	544 ± 78 **b**	506 ± 70 **b**	27 ± 7 **b**	2,678 ± 264 **b**	59 ± 10 **b**
P. plurivora	12	7 (58.3)	698 ± 174 **b**	664 ± 168 **b**	24 ± 6 **b**	2,681 ± 426 **b**	59 ± 19 **b**
Control	12	0 (0)	853 ± 190 **a**	801 ± 179 **a**	13 ± 6 **a**	3,378 ± 427 **a**	103 ± 22 **a**

Note: Different letters denote statistical significance at $p < .001$ (Duncan's multiple range test).

the dominant tree species. Although the stands were characterized by weakened conditions and contained symptomatic trees (e.g. yellowing of leaves, crown transparency), declining symptoms were not uniformly observed in all stands; seemingly healthy individual trees with no visible crown symptoms occurred throughout (Table 1).

In June 2019, one to six trees (Table 1) were selected from eight declining stands, and one tree was selected from each of three seemingly healthy stands. From each tree, two soil monoliths were taken at 0.5–1.5 m distance from the stem base and mixed thoroughly into one aggregate sample. Sampling was avoided where two or more trees grew closely to each other. In total, 36 samples were collected: 22 from symptomatic trees and 14 from apparently healthy trees (Table 1). Isolation was performed following the standardized baiting method (Jung et al., 1996). Plates with plated necrotic fragments on V8A-PARPNH selective media (Jung et al., 1996) were incubated at 20°C in the dark. After 24–48 hr, when the first emerging hyphae were observed, they were immediately subcultured onto fresh V8A media (Jung et al., 1996). Three- to four-week-old colonies were observed at ×400 magnification for the presence of typical *Phytophthora* sexual and asexual structures, using a light microscope (ZEISS Axioskop 2). Based on the appearance of the colony, all isolates were divided into morphotype groups.

2.2 | Molecular identification of isolates

Genomic DNA (from eight selected isolates representative of all morphotypes) was extracted from fresh mycelia harvested from V8A plates using the Plant DNA Mini Kit (Syngen, Poland), following the manufacturer's instructions. The internal transcribed spacer region (ITS) of the ribosomal RNA gene repeat was amplified using specific primers ITS4 and ITS6 (Grünwald et al., 2011). The PCR reactions (25 µl) contained 1 × PCR Buffer (Taq PCR Core Kit, QIAGEN), 1.5 mM $MgCl_2$, 0.4 mM of each dNTP, 0.2 µM of each primer, 1 U of Taq polymerase and 10–20 ng of template DNA. The PCR thermal protocol consisted of an initial 5 min denaturation step at 95°C, 35 cycles of amplification cycles of 95°C for 30 s, 56°C for 30 s, 72°C for 50 s and a final extension step of 72°C for 10 min. Amplicons were analysed by electrophoresis, visualized in a 1% agarose gel stained with the GelRed® dye (Biotium, USA), purified using CleanUp Kit (A&A Biotechnology, Poland) and sequenced on an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific™, USA). The nucleotide Basic Local Alignment Search Tool (blastn) was used to compare obtained sequences with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Sequences were deposited in GenBank under the accession numbers (Table 1).

2.3 | Pathogenicity test

Pathogenicity tests were performed using the standardized soil infestation protocol according to Jung et al. (1996). Sessile oak plants were grown from seeds in a mixture of peat, sand and perlite (v:v:v = 1:1:1) in individual one-litre pots. The substrate for inoculation was made from mixture of fine vermiculite, millet seeds and liquid V8 juice media autoclaved at 120°C for 20 min, and inoculated with fresh isolates of *P. plurivora* (MT299773; MT299770) and *P. quercina* (MT299779). The inoculated substrate was incubated for 4 and 6 weeks for *P. plurivora* and *P. quercina*, respectively, at 22–25°C in the dark. Soil was inoculated by filling the cavities prepared with glass tubes at the time of sowing around each plant with 20–25 cm^3 of inoculated substrate; 12 plants per treatment were inoculated. Twelve control plants were treated similarly, but the inoculum was substituted with a rinsed mixture of sterile vermiculite and vegetable juice. The boxes with potted plants were flooded immediately after inoculation for 72 hr, and the flooding was repeated every 3 weeks for 72 hr. The observations of above-ground symptoms were made once a week.

Five months after inoculation, the plants were removed from the inoculated soil and the roots were washed under running water. After washing, re-isolation from all plants (including control) was performed by plating 10 small root pieces and tips from each plant on selective V8A-PARPNH media, after drying on filter paper. If present, necrotic pieces were carefully cut, dried and plated as well. All the roots were scanned using the WinRhizo® software (Regent Instruments, Canada) and EPSON Perfection V700 Photo Scanner. After scanning, roots were dried for 48 hr at 65°C in dryer (Termaks Series 2000, Norway), and the dry biomass of fine roots (<2 mm in diameter) and mother roots (>2 mm in diameter) were measured using the analytical scale (Sartorius A200S, GMBH, Germany). All the obtained values were used for the calculation of root parameters (Table 2).

2.4 | Statistical analyses

For all the analysed parameters, assumptions for parametric tests (compliance with normal distribution and homogeneity of variance) were checked, followed by unidirectional analysis of variance at $p = .05$. Differences between average root parameters were tested using Duncan's multiple range post hoc test ($\alpha = 0.05$). All analyses were performed using STATISTICA® (ver. 13.1).

3 | RESULTS AND DISCUSSION

After the isolation tests, 16 out of 36 (44.4%) collected samples (from six of the 11 stands) were positive for Oomycetes (Table 1). Of the 22 samples collected under symptomatic trees, 11 (50%) were positive, and of the 14 apparently healthy trees, four were positive (28.6%) (Table 1). In total, 68 isolates were obtained, and after the morphological classification, the isolates were identified based on ITS sequence as *P. quercina*, *P. plurivora* and *Pythium intermedium* (Table 1). *Phytophthora plurivora* was the most frequently isolated species from a total of nine samples from four different stands. *Py. intermedium* was isolated from 6 samples from two stands, and *P. quercina* was isolated from a single sample (Table 1). The most severe crown damage was observed on the tree number 32 (Table 1), where the presence of *P. quercina* was confirmed in the rhizosphere soil. Also, under the tree number 24 in the oldest sampled stand, both *P. plurivora* and *Py. intermedium* were obtained (Table 1).

Five months post-inoculation, when 70% of plants showed the symptoms, the experiment was finished. *Phytophthora plurivora* and *P. quercina* were recovered from 81.7% and 90.8% of the harvested roots plated on media, respectively. Of all the treated plants, necrosis was observed in only one seedling in a variant infected with *P. plurivora*. Plants from the control treatment were negative after the re-isolation. The mean values for the five tested parameters were significantly lower for infested plants than for controls, with the exception of mother root length (Table 2). Also, there were no significant differences between treatments infested with *Phytophthora* species. However, lower mean values were observed for all the parameters in the treatment where the soil was infested with *P. quercina* (Table 2). Fine root length, as one of the most representative root parameters was 1.6 and 1.2 times shorter in the *P. quercina* and *P. plurivora* treatments compared to control, respectively (Table 2).

Before this study, very little was known about the presence of *Phytophthora* species in natural oak stands in Slovakia. Probably, these species have been present in the rhizosphere soil of oaks for many years, as in other parts of Europe. Another option is the introduction of the pathogen along with new propagation material from forest nurseries. In the present study, *P. plurivora* and *P. quercina* were detected from 10 of 36 rhizosphere soil samples from six sessile oak stands. Both species have been commonly reported throughout Europe, as causing serious damage to mature trees (Jung et al., 1996; Jung, Cooke, Blaschke, Duncan, & Oßwald, 1999). *Phytophthora plurivora*, the most frequently isolated species in this study, has been widely reported in Europe and has a broad host range, including oak trees (Jung & Burgess, 2009). Both, *P. quercina* and *P. plurivora*, were able to infect the roots of young inoculated oak plants, and our results are in congruence with previous findings (Jung et al., 1996). In conclusion, the presence of *P. quercina* and *P. plurivora* in the sampled oak stands and demonstrated aggressiveness to young oak plants suggest that these species are representing a serious threat to the health of sessile oak forests in Slovakia.

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