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SHORT COMMUNICATION

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First report on the occurrence of *Phytophthoraobscura* on *Acerpseudoplatanus* in Slovakia

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Abstract

Sycamore maple is a valuable admixture species in many European countries, including Slovakia. As most species belonging to the genus *Acer*, it is susceptible to several pathogenic organisms including species of Oomycetes. To determine whether the decline of *Acer pseudoplatanus* in Slovakia is caused by pathogenic Oomycetes, soil samples were taken from the rhizosphere in three sycamore maple stands. Isolation from leaf baits and subsequent identification based on sequencing of the ITS showed the presence of three species: *Pythium intermedium*, *Phytophthora plurivora* and *Phytophthora obscura*. This is the first report on the occurrence of *P. plurivora* and *P. obscura* in the rhizosphere of declining sycamore maple stands in Slovakia.

KEYWORDS
Acerpseudoplatanus, inoculation experiments, *Phytophthoraplurivora*, rhizosphere, soilborne pathogens

1 | INTRODUCTION

Sycamore maple (*Acer pseudoplatanus* L.) occurs in Slovakia as an admixture species often found in forest communities together with beech, oaks and other valuable hardwood species. It is very desirable and widely used for landscaping, particularly as park plantings. However, like all species in the genus *Acer*, it is considered susceptible to infections by pathogens of the genus *Phytophthora* (Milenković et al., 2014). Infected trees show various symptoms, including root rot and loss of small roots, necrosis of the main roots, crown dieback, wilting of leaves, defoliation and bark lesions with dark exudates on the trunk. Data on the presence and diversity of pathogenic *Phytophthora* species in maple stands in Slovakia are not available.

The aim of this study was to identify species of the genus *Phytophthora* in the rhizosphere of dying sycamore maples in Slovakia.

2 | MATERIALS AND METHODS

Samples were collected in June 2019 from three forest stands where *Acer pseudoplatanus* occur. The first two areas were located in western Slovakia near Trstín, the third area was located in the vicinity of the Niżne Tatry National Park. At all sites, maple grows admixed with beech (*Fagus sylvatica*). Many of the trees showed damage typical of that caused by *Cryptostroma corticale* on the bark (dark necrosis on the stem at the level of approximately one metre and higher). In each stand, five trees were selected randomly. Four soil samples (from the four cardinal points) were collected from each tree at a distance of 50–150 cm from the base of the trunk and pooled. In the laboratory, from each sample 200 g of soil was flooded with 500 ml of distilled water. Young oak (*Quercus robur*) and *Rhododendron brachycarpum* leaves were used as baits. Eight to ten leaves were placed on the water surface in each soil and water container. After 3–7 days, brown spots appeared on the surfaces of some leaves. These were then cut into smaller pieces (approximately 5 × 5 mm) and placed on PARPNH-agar selective media (Jung et al., 1996). The Petri dishes were incubated for a minimum of 48 hours in the dark at 20°C.

Thereafter, all outgrowing colonies were transferred to V8 medium (200 ml of vegetable juice, 3 g of $CaCO_3$, 800 ml distil water). After approximately a week of incubation, the cultures were divided into morphotypes based on colony growth.

Genomic DNA from eight selected isolates representative of all morphotypes was extracted from young mycelia harvested from V8

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 was amplified using the primers ITS4 (White et al., 1990) and ITS6 (Cooke et al., 2000). 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 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 visualized on 1% agarose gel stained with the GelRed® dye (Biotium, USA) and purified using CleanUp Kit (A&A Biotechnology, Poland) prior sequencing with the use of BigDye™ Terminator v3.1 Cycle Sequencing Kit (ThermoFisher Scientific™, USA) according to manufacturer's protocol. Obtained products were analysed on an Applied Biosystems 3500 Genetic Analyzer (ThermoFisher Scientific™, USA). Raw sequences were checked manually and corrected if necessary in FinchTV v1.4.0 software and further aligned in BioEdit v. 7.0.5.3 (Hall, 1999) software. The nucleotide Basic Local Alignment Search Tool (blastn) was used to compare the obtained sequences with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov). Sequences of all isolates were submitted to GenBank, and the corresponding accession numbers are listed in Table 1.

Pathogenicity tests were performed using the soil infestation test according to Jung et al., (2017). Twelve maples were grown from seeds in mixture of sterile peat, sand and perlite (v:v:v = 1:1:1) in 20 l boxes (one box for control, and one per each *Phytophthora* species). The substrate for inoculum was made from mixture of 250 cm^3 fine vermiculite, 20 cm^3 of millet seeds and 175 ml of liquid V8 juice, and autoclaved at 120°C for 20 min (Jung et al., 1996). The substrate was inoculated with one fresh isolate of *P. plurivora* (MN255130) and *P. obscura* (MN228692). The one-year-old seedlings were infected by adding 20 cm^3 of inoculum per 1000 cm^3 potting mixture to the soil. Twelve control seedlings were inoculated with rinsed mixture of sterile vermiculite and V8 juice in the same ratio. The boxes with the seedlings were flooded immediately after inoculation for 72 hours, and the flooding was repeated every three weeks for 72 hr. When 70% of the seedlings showed symptoms (yellowing leaves and defoliation), the experiment was terminated. To confirm Koch's postulates, symptomatic seedlings were removed from the infested mixture and the roots were washed under running water. Pieces of symptomatic roots were thoroughly washed in tap water, superficially sterilized in 1% NaCl for 2 min, dipped in 70% EtOH for 30 s, rinsed in sterile distilled water, dried and plated on selective PARPNH V8-agar (Jung et al., 1996). Also, pieces of small roots from control variant plants were also plated on selective media.

3 | RESULTS AND DISCUSSION

A total of 31 isolates were recovered from soil samples collected in the three forest stands detected with declining sycamore maple (Table 1). Sequence analysis of the ITS region confirmed morphological analyses indicating the presence of three different species of Oomycetes, namely *Pythium intermedium*, *Phytophthora plurivora* and *Phytophthora obscura*. Most isolates were *Pythium intermedium* (18), while *Phytophthora plurivora* was isolated twelve times and *Phytophthora obscura* only once.

To determine pathogenicity of *P. obscura* and *P. plurivora* towards *A.* pseudoplatanus, inoculation experiments with one-year-old

TABLE 1 Isolation from rhizosphere soil samples in maple stands

Stand, Location	Tree No	Disease Symptoms	Species (No. Isolates)	GenBank Accession Numbers of the representative isolates
Stand No. 1 (Trstín)	1	Yellowing of leaves	*P. plurivora* (3)	MN255130
	2	Yellowing of leaves	*P. plurivora* (2)	
	3	Yellowing of leaves	*Py. intermedium* (7)	MN255128
	4	Yellowing of leaves	*P. plurivora*	
	5	No symptoms	-	
Stand No.2 (Trstín)	6	Yellowing of leaves	-	
	7	Yellowing of leaves	*P. plurivora* (5)	
	8	No symptoms	*Py. intermedium* (4)	MN255129
	9	No symptoms	*Py. intermedium* (5)	MN255126
	10	Yellowing of leaves	*P. plurivora*	
Stand No. 3 (Niżne Tatry)	11	Yellowing of leaves	*P. obscura*	MN228692
	12	No symptoms	-	
	13	No symptoms	-	
	14	No symptoms	*Py. Intermedium* (2)	MN255125
	15	No symptoms	-	
No. of positive samples			10	
No. of obtained isolates			31	

seedlings were conducted. The pathogenicity tests were finished after three months of incubation, when all seedlings showed symptoms of a *Phytophthora* infection (i.e. yellow leaves, wilting). The presence of the inoculated pathogens in the roots of the seedlings was confirmed by re-isolation (89.6% for *P. plurivora* and 69.7% for *P. obscura*). Of all the treated plants, necrosis was observed in a few plants (one seedling in a variant infected with *P. obscura* and two for *P. plurivora*). Necrotic root lesions were only observed on one seedling inoculated with *P. obscura* and two seedlings inoculated with *P. plurivora*. From the roots of control seedlings, no *Phytophthora* cultures could be recovered.

Several *Phytophthora* species were previously recorded on multiple hosts in Slovakia, including *Fagus sylvatica*, *Juglans regia*, *Castanea sativa* and *Quercus robur* (Jung et al., 2016). However, isolations from oaks were mostly performed from plants in nurseries, or amenity and ornamental plantings. Thus, Slovakia still remains a country where little is known about the occurrence of *Phytophthora* species in mature forest stands.

Phytophthora plurivora is a species with a wide geographic range of occurrence and a wide range of host plants causing serious damage to mature trees (Jung et al., 1996). In Europe, it has already been isolated from the rhizosphere of declining oaks, beeches, alders, ash trees, maples and many others tree species (Jung et al., 2016). Milenković et al., (2014) showed that *P. plurivora* is able to infect young *Acer* (including *A. pseudoplatanus*) and cause severe damage to their fine roots. *Phytophthora obscura* is a relatively new species which was first described in 2012 (Grünwald et al. 2012). It was detected in the foliage of *Kalmia latifolia* and in the rhizosphere of *Pieris* in the USA, as well as in the rhizosphere of *Aesculus hippocastanum* in Germany. *P. obscura* is a homothallic species with paragynous antheridia and semipapillate sporangia (Grünwald et al. 2012). It is morphologically and ecologically very similar to *P. syringae*, with which it has probably been confused in the past. This study provides the first evidence of the occurrence of *P. obscura* in forest stands in Slovakia (till now recorded only in USA and Germany). Based on inoculation experiments, both species seem to be pathogenic towards *A. pseudoplatanus*. However, additional investigations would be necessary to better understand the biology and ecology of the two species in Slovakia.

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