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MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *Phytophthora* SPECIES FROM MAPLE TREES IN SERBIA

Ivan MILENKOVIĆ[1)], Justyna A. NOWAKOWSKA[2)], Tomasz OSZAKO[2)], Katarina MLADENOVIĆ[1)], AleksandaR LUČIĆ[1)], Ljubinko RAKONJAC[1)], Dragan KARADŽIĆ[3)]

[1]Institute of Forestry, Belgrade, Serbia
[2]Forest Research Institute-IBL, Sekocin Stary, Raszyn, Poland
[3]University of Belgrade-Faculty of Forestry, Belgrade, Serbia

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The paper presents the results of the study performed with aims to determine the presence and diversity of *Phytophthora* species on maple trees in Serbia. Due to high aggressiveness and their multicyclic nature, presence of these pathogens is posing significant threat to forestry and biodiversity. In total, 29 samples of water, soil and tissues were taken from 10 different localities, and six different maple hosts were tested. After the isolation tests, 17 samples from five different maple hosts were positive for the presence of *Phytophthora* spp., and 31 isolates were obtained. After the detailed morphological and physiological classification, four distinct groups of isolates were separated. DNA was extracted from selected representative isolates and molecular identification with sequencing of ITS region was performed. Used ITS4 and ITS6 primers successfully amplified the genomic DNA of chosen isolates and morphological identification of obtained isolates was confirmed after the sequencing. Four different *Phytophthora* species were detected, including *P. cactorum, P. gonapodyides, P. plurivora* and *P. lacustris*. The most common isolated species was homothallic, and with very variable and semipapillate sporangia, *P. plurivora* with 22 obtained isolates. This is the first report of *P. plurivora* and *P. gonapodyides* on *A. campestre*, *P. plurivora* and *P. lacustris* on *Acer heldreichii* and first report of *P. lacustris* on *A. pseudoplatanus* and *A. tataricum* in Serbia.

Key words: Acer spp., morphology, *Phytophthora plurivora,* PCR, Sequencing

Corresponding author: Ivan Milenković, Institute of foresty, Kneza Višeslava 3, 11030 Belrade Serbija, tel: +381 64 2031985; E-mail: ivan.milenkovic@sfb.bg.ac.rs

INTRODUCTION

Phytophthora species are fungi like organisms and according to KIRK *et al.* (2008) belong to kingdom *Chromista.* Together with other fungi like organisms, algae and protists, these organisms were classified to the super kingdom Chromalveolate (BEAKES *et al*. 2012). In their thick-walled, persistent and resting structures (sexual oospores and asexual chlamydospores), they can survive unfavorable conditions, such as drought, low and high temperatures, for a long period. With the period of favorable conditions these structures are germinating and form typical zoosporangia that release zoospores in the soil moisture. These zoospores can swim with their two flagella and they infect young roots, and soon after infection numerous new sporangia are formed at the root surface releasing new zoospores (ERWIN and RIBEIRO, 1996). Due to their multicyclic nature and persistence of resting structures, *Phytophthora* species seems to be among the most dangerous pathogens. According to ERWIN and RIBEIRO (1996), species from the *Phytophthora* genus can be specialized for particular host, but the most of the species are generalists, attacking the hosts from different genus and families. Climate changes and disturbances in precipitations and droughts periods supported infections with pathogens from *Phytophthora* genus, and reason for their widespread distribution is upgrowth of international trade with living plants and introduction of infected plant material from nurseries to natural and semi-natural ecosystems (BRASIER 2008; BRASIER and JUNG, 2006).

According to JOVANOVIĆ (1961), 13 species from the *Acer* genus were recorded in Serbia, including both domestic and the allochthonous. Domestic species from *Acer* spp. genus are usually building communities with beech, oaks and other noble hardwoods. Also, different *Acer* species are very useful and widely used as ornamental, amenity and park trees.

According to KARADŽIĆ (2010) and KARADŽIĆ *et al*. (2011, 2014) maple trees are susceptible on several infectious diseases, and among them *Verticilium albo-atrum* Reinke and Berthold, *Ganoderma* spp., *Nectria cinnabarina* (Tode) Fr. and *Rhytisma acerinum* Schwein. appeared to be the most common and the most aggressive pathogens affecting different maple trees in Serbia.

However, species from the *Acer* genus are considered to be susceptible to infections with *Phytophthora* species (ERWIN and RIBEIRO, 1996; BRASIER and JUNG, 2006; JUNG and BURGESS, 2009; GINETTI *et al*. 2013), and attacked trees are showing different symptoms, ranging from decay and loss of fine roots, necrosis and wounds of mother roots, crown transparency, wilting and stunting of shoots, up to collar rots and stem cankers and aerial cankers with dark exudates.

Data on the presence and diversity of pathogens from the *Phytophthora* genus on maple trees in Serbia are scarce or missing so far, although MILENKOVIĆ *et al*. (2011 a, b) reported on the isolation of *Phytophthora* species and types of symptoms on forest and park trees including maples.

Due to lack of information on the presence, diversity and distribution of *Phytophthora* species as pathogens of maple trees in Serbia, and their high susceptibility, as well as the high risks for forestry and biodiversity posed by the presence of pathogens from the *Phytophthora* genus, a study was performed with aims to determine: (i) the presence of *Phytophthora* species on maple trees in Serbia; (ii) the main hosts of *Phytophthora* spp. from the *Acer* spp. genus; and (iii) the main *Phytophthora* species based on morphological and molecular identification of obtained isolates.

MATERIALS AND METHODS

Studied localities

Sampling was performed in different forest and semi-natural ecosystems in Serbia, and five different hosts were sampled including *Acer campestre* L., *A. heldreichii* Orph., *A. negundo* L., *A. pseudoplatanus* L. and *A tataricum* L. Age of the sampled hosts was between 10 and 60 years (table 5). In total, 29 samples were collected from 10 different localities, and sampling was performed between the years 2010 and 2012. Most of the samples were collected in April and May, 10 samples were collected in June and July, and 3 samples were collected in November. Details about localities, hosts and samples taken are shown in table 2.

Sampling and isolation methods

Sampling was performed according to previously described methodology (JUNG, 2009; JUNG *et al*. 1996, 2000). Tissue samples were taken from necrotic zones and after washing in distilled water and drying out on filter paper, plated directly onto selective-agar-media (V8A-PARPNH (JUNG *et al*. 1996). Incubation lasted for 3-5 days at 22-25°C in the dark, and after appearance of first hypha they were transferred onto fresh unclarified carrot agar media-CA (900 ml/l distilled water, 100 ml/l fresh organic carrot juice (Biotta®, Swiss), 18 g/l agar (Torlak, Serbia) and 3 g/l $CaCO_3$ (JUNG and NECHWATAL, 2008). Soil and roots were collected in the form of soil monoliths, measuring ~ 25×25×25 cm, and two such monoliths per tree were taken, packed into 10 l plastic bags and transported to the lab. Both, symptomatic and apparently healthy trees were sampled. Isolation tests were performed with baiting method (NECHWATAL *et al*. 2012; JUNG, 2009; JUNG *et al*. 1996, 2000), Soil was mixed and ~ 500 g was placed in the 1-l-plastic containers at 22-25°C in the lab. Soil was flooded with deionized water, surface of water was cleaned with paper towels, and young leaflets of oak, beech and cherry laurel were plated on the surface of clean water as baits. Young leaflets were observed on every 12 h for the presence of necrotic spots, and after appearance of the first spots, they were examined under the light microscope for the presence of typical *Phytophthora* spp. sporangia. Necrotic spots were aseptically separated with scalpel, sterilized in 70% ethanol and burned on the open flame, and plated onto selective V8A-PARPNH media (JUNG, 2009; JUNG *et al*. 1996, 1999, 2000). Incubation lasted for 3-5 days at 22-25°C in the dark, and after appearance of first hypha they were transferred onto unclarified CA media.

All the obtained isolates were stored at 8-10°C in the Phytopathological laboratory at University of Belgrade-Faculty of Forestry, and transferred after every 6-8 months on the fresh CA media.

Identification of obtained isolates

Colony growth patterns and cardinal temperatures

For the purposes of determination of colony growth patterns, isolates were transferred onto five types of nutrient media, including: carrot agar media-CA; malt-extract-agar-MEA (48 g/l malt-extract-agar (MERCK, Germany); V8-agar (800 ml/l distilled water, 200 ml/l V8 juice (Biotta®, Swiss), 18 g/l agar (Torlak, Serbia) and 3 g/l $CaCO_3$ (JUNG *et al*. 1996); potato-dextrose-agar-PDA (39 g/l potato-dextrose-agar (MERCK, Germany); prune-juice-agar-PJA (800 ml/l of distilled water, 200 ml/l of water extract from dried organic prunes, 3 g/l $CaCO_3$, 18 g/l agar (PARTRIDGE-METZ and CHANDRA, 2011) and incubation was at 20ºC in the dark. Colony patterns were determined after seven and ten days of growth according to previous publications

(ERWIN and RIBEIRO, 1996; BRASIER *et al*. 2003; JUNG and BURGESS, 2009; JUNG *et al*. 2002, 2003; NECHWATAL *et al*. 2012).

The growth rates of isolates were determined in the way that the cultures firstly were transferred onto carrot agar media (CA), and incubation was at 20ºC in the dark. After three to five days of growth, from the edge of young colonies pieces 6 mm in diameter were transferred onto fresh CA media, by using the metal cork-borer. Three replicates per isolate per temperature were transferred and incubated for 24 h at 25ºC in the dark. After that, inoculated Petri dishes were placed onto different temperatures (5, 10, 15, 20, 25, 30 and 35ºC), and left to be adapted for the next 24 h. Petri dishes were marked from the bottom side with two cross lines and radial growth of the colonies was marked with steal needle after every 24 h of growing in the next five days.

Morphological identification

For the purposes of morphological identification, non-sterile soil extract was firstly prepared according to ERWIN and RIBEIRO (1996). Cultures were transferred onto CA, incubated at 22-25ºC in the dark, and after 3-5 days of growth from the edges from young colonies, pieces ~ 1×1 cm were taken with sterile scalpel and flooded with non-sterile soil extract. Pieces of agar with young colonies were washed with distilled water after 4-6 h, and distilled water was replaced after additional 6 and 12 h (JUNG and BURGESS, 2009). Different sexual and asexual structures typical for the species from the *Phytophthora* genus were observed under the light microscope (CETI®MAGNUM-T/Trinocular Microscope with Pl. Objectives, UK), at ×400 magnification, including presence or absence of antheridia and oogonia with oospores, position of antheridia, chlamydospores, hyphal swellings, sporangiophores branching, shape, clustering and proliferation of sporangia. Structures were recorded and measured by using the Si3000® camera (UK) and software XliCap® (UK), and 30-50 structures per analyzed isolate were measured. Recorded features were compared with known *Phytophthora* identification keys and with the species listed in ERWIN and RIBEIRO (1996), as well as with the data from original papers with recently described species (JUNG *et al*. 1999, 2002, 2011; JUNG and NECHWATAL, 2008; JUNG and BURGESS, 2009; HONG *et al*. 2011; NECHWATAL *et al*. 2012).

Molecular identification

After the detailed morphological classification, representative isolates were chosen and small pieces from the edges of young colonies were transferred in liquid V8 media (900 ml/l of distilled water, 100 ml/l of V8 juice (Tymbark, Poland), 3 g/l CaCO3), and incubated at 22-25ºC in the dark. After 3-5 days of incubation, or until mycelium filled approximately the half of 90 mm Petri dish, fresh mycelium was collected, washed in sterile distilled water and dried out on sterile filter paper. Mycelium was smashed in liquid nitrogen and rDNA was extracted by using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich® GmbH, Germany), according to manufacturer recommendations. ITS amplifications of *Phytophthora* isolates were carried out using previously described universal primers ITS4 and ITS6 that target conserved regions in the 18S and 28S rRNA genes (WHITE *et al*. 1990). The amplification reaction mixture contained 1 x PCR buffer [75mm Tris-HCl (pH 9.0), 50mM KCl, 20 mM $(NH4)_2$ SO4]; 1×Q solution, 0.2 mM dNTPs, 0.25 mM of each primer; 1 mM $MgCl_2$; 1U of *Taq* Polymerase (Qiagen Ltd., Valencia, CA, USA); and 1 mL of mycelial DNA in a total volume of 50 μL.

Reactions were performed in PTC-200™ Programmable Thermal Controller (MJ Research, Inc.) machine, and according to the PCR protocol (table 1).

Table 1. PCR protocol, time and number of cycles

PCR steps	Temperature (°C)	Time	*No* of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 s	
Annealing	55	30 s	35
Extension	72	50 s	
Final extension	72	10 min	1

Amplified products were analyzed by 1.5 % TBE-agarose gel electrophoresis, stained with ethidium bromide, and visualized under a UV transilluminator. The presence of a single band (ca. 800bp) was regarded as positive reaction. The PCR products were purified using the A&A Biotechnology (Gdynia, Poland) Clean-up kit, following the manufacturer's protocol. Sequencing was conducted on CEQ™8000 9.0.25 automated sequencer (Beckman Coulter®, Fullerton, USA), using ITS 4 and 6 primers separately (i.e. from 5' and 3' direction). The consensus sequences resulting from alignment of two-directional sequencing (ZHANG *et al*. 2000) were compared with sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the ClustalW program (THOMPSON *et al*. 1994) and MEGA5 software (TAMURA *et al*. 2011).

RESULTS

Symptoms

During the field inspection several different *Phytophthora* related symptoms were observed on maple trees, from parks as well as forests in Serbia. A range of symptoms included: stunting of shoots, crown transparency, yellowing of leaves, collar rot and stem necrosis with dark exudates, necrosis and wounds on mother roots and decay and loss of fine roots. Symptoms were the most visible in early spring and in the autumn when the isolations were the most successful. However, 11 out of 31 obtained isolates were recovered from soil under the symptomless trees (table 5).

Isolation of *Phytophthora* species from Maple trees in Serbia

Five out of six tested hosts were positive on the presence of several different *Phytophthora* species and in total 31 isolates were obtained (tables 2, 3, 4 and 5). The most of the isolates were obtained from the soil samples, 26 in total, utilizing baiting techniques. From water samples, two isolates were obtained using the baiting probes. In the isolations from necrotic host plant tissues by direct plating on selective agar media three isolates were obtained, and there was the lowest isolation frequency with only three isolates out of 17 performed probes (table 3 and 4). Host plant from which *Phytophthtora* spp. were the most frequently isolated was Sycamore maple (*Acer pseudoplatanus*) with 18 obtained isolates and three different *Phytophthora* species, followed with Field maple (*A. campestre*) with seven obtained isolates and two present species (table 5).

Table 2. Studied localities and samples taken from maple trees in Serbia

No	Studied localities	Hosts	Sampling date	Number of samples	Number of positive samples
1	Debeli Lug	*Acer campestre* L.	April 2010	2	0
2	Avala	*Acer pseudoplatanus* L.	May 2011	3	3
3	Bubanj Potok	*Acer pseudoplatanus*	May 2011	4	4
4	Jastrebac	*Acer heldreichii* Orph.	July 2011	5	1
5	Košutnjak	*Acer pseudoplatanus*	May, November 2011	3	2
6	Morović	*Acer campestre*	June 2012	1	1
7	Kupinovo	*Acer campestre, Acer tataricum* L.	May 2012	3	2
8	Beograd	*Acer negundo* L., *Acer pseudoplatanus*	November 2011	2	1
9	Južni Kučaj II	*Acer campestre, Acer platanoides* L., *Acer pseudoplatanus*	June 2011	4	1
10	Vršac	*Acer pseudoplatanus, Acer platanoides*	May 2012	2	2
	Total	6	-	29	17

Table 3. Isolation frequency of Phytophthora species from different collected material

Origin of samples	Soil and roots	Water and mold	Necrotic tissues	Total
Total samples taken	19	2	8	**29**
Total number of performed isolation tests	38	5	17	**42**
Number of successful isolations (all isolated species)	26	2	3	**31**

Table 4. Origin and the number of Phytophthora spp isolates from different collected material

Origin of samples	Soil and roots	Water and mold	Necrotic tissues	Total
Phytophthora plurivora	18	1	3	**22**
Phytophthora cactorum	2	0	0	**2**
Phytophthora gonapodyides	3	0	0	**3**
P. lacustris	3	1	0	**4**
Total number of successful isolations	26	2	3	**31**

Table 5. Phytophthora species on Acer spp. in Serbia, origin of isolates, and GenBank access numbers

No	Hosts	Symptoms	Origin of isolates	Localities and year of isolation	Age	Obtained *Phytophthora* spp.	GenBank accession number
1	*Acer campestre*	Necrosis on roots	Soil	Južni Kučaj II, 2011	40	*P. plurivora*	-
2	*Acer campestre*	No symptoms	Soil	Morović/ V.Ž. 17, 2012	20	*P. plurivora*	-
3	*Acer campestre*	Dying branches, crown transparency	Soil	Morović / V.Ž.P. 20 / 2012	30	*P. plurivora*	-
4	*Acer campestre*	No symptoms	Soil	Kupinovo / J.B. 23 /2011	15	*P. gonapodyides*	-
5	*Acer campestre*	Crown transparency	Soil	Kupinovo / J.B. 23/ 2011	20	*P. gonapodyides*	**JX276089**
6	*Acer campestre*	Crown transparency with dying branches	Soil	Kupinovo / J.B. 23/ 2011	20	*P. plurivora*	-
7	*Acer campestre*	Necrosis on roots	Soil	Kupinovo / J.B. 23/ 2012	20	*P. gonapodyides*	-
8	*Acer heldreichii*	Stem necrosis with dark exudates	Tissue	Jastrebac/ 2011	60	*P. plurivora*	-
9	*Acer heldreichii*	No symptoms	Soil	Jastrebac / 2011	60	*P. lacustris*	-
10	*Acer heldreichii*	Dieback and dead branches	Soil	Jastrebac / 2011	60	*P. plurivora*	-
11	*Acer pseudoplatanus*	Dying of branches, dieback	Soil	Avala, 2011	40	*P. plurivora*	**JX276067**
12	*Acer pseudoplatanus*	Necrosis on roots, stunting of shoots	Soil	Avala, 2011	25	*P. plurivora*	-
13	*Acer pseudoplatanus*	Нема симптома	Soil	Avala, 2011	25	*P. plurivora*	-
14	*Acer pseudoplatanus*	Dieback of the branches	Soil	Avala, 2011	40	*P. plurivora*	-
15	*Acer pseudoplatanus*	Dying of branches	Soil	Stepin Gaj 2011	40	*P. plurivora*	**JX276066**
16	*Acer pseudoplatanus*	No symptoms	Water	Stepin Gaj 2011	-	*P. plurivora*	-
17	*Acer pseudoplatanus*	No symptoms	Soil	Bubanj Potok 2011	15	*P. plurivora*	-
18	*Acer pseudoplatanus*	Increased crown transparency	Soil	Bubanj Potok 2011	40	*P. cactorum*	**JX276070**
19	*Acer pseudoplatanus*	Increased crown transparency	Soil	Bubanj Potok 2011	40	*P. plurivora*	-
20	*Acer pseudoplatanus*	No symptoms	Water	Bubanj Potok 2011	-	*P. lacustris*	**JX276080**
21	*Acer pseudoplatanus*	Bark necrosis with dark exudates	Tissue	Košutnjak 2011	30	*P. plurivora*	-

Table 5. (continued) Phytophthora species on Acer spp. in Serbia, origin of isolates, and GenBank access numbers

No	Hosts	Symptoms	Origin of isolates	Localities and year of isolation	Age	Obtained *Phytophthora* spp.	GenBank accession number
22	*Acer pseudoplatanus*	Bark necrosis with dark exudates	Tisuue	Košutnjak 2011	30	*P. plurivora*	**JX276071**
23	*Acer pseudoplatanus*	No symptoms	Soil	Košutnjak 2011	30	*P. plurivora*	-
24	*Acer pseudoplatanus*	Yellowing of leaves and wilting of shoots	Soil	Beograd 2010	50	*P. plurivora*	-
25	*Acer pseudoplatanus*	Yelowing of leaves and wilting of shoots	Soil	Beograd 2011	50	*P. cactorum*	-
26	*Acer pseudoplatanus*	No symptoms	Soil	Južni Kučaj II, 2011	40	*P. plurivora*	-
27	*Acer pseudoplatanus*	No symptoms, slightly yellowing of leaves	Soil	Južni Kučaj II, 2011	40	*P. plurivora*	-
28	*Acer pseudoplatanus*	No symptoms	Soil	Vršac, 2012	50	*P. plurivora*	-
29	*Acer platanoides*	Dying of shoots	Soil and roots	Južni Kučaj II, 2011	40	*P. plurivora*	**JX276075**
30	*Acer platanoides*	No symptoms	Soil	Vršac, 2012	40	*P. lacustris*	-
31	*Acer tataricum*	No symptoms	Soil and roots	Kupinovo/ J.B. 23/ 2012	10	*P. lacustris*	-

Morphological identification of isolates

After the morphological and colony growth patterns classification, the majority of the isolates formed regular colony, slightly aerial in the middle and pressed at the edges. Due to different type of included media, these colonies were more or less petalloid with chrysanthemum shape of colony, or they were cottony without specific shape. Optimal temperature for growth was at about 25°C, minimum at 5°C, and maximum between 30-35°C. Radial growth rate of those isolates on CA at optimal 25°C was 7,7 mm/day. All isolates were homothallic with globose or slightly subglobose oogonia with average dimensions of ten measured isolates 27.93±3.67 × 26.62±3.0 µm, and range 17.60-36.30 × 17.60-32.30 µm. Antheridia were paragynous and averaged 11.01±2.05 × 8.50±1.36 µm, with range 6.70-17.5 × 6.10-13.3 µm. Oospores were mostly regular and plerotic, and aplerotic oospores were also recorded. Oospores averaged 20.29±2.33 × 20.34±2.38 µm, and ranged 12.5-24.0 × 12.5-24.0 µm. Oospore wall thickness averaged 1.36±0.25 µm, with range 0.8-1.9 µm. Sporangia were typically semipapillate on sympodially branched sporangiophores, persistent and with very variable shape. Average dimensions of sporangia were 49.65±10.02 × 32.93±6.31 µm, and range 20.40-77.90 × 15.20-49.40 µm. Empty sporangia exit pore averaged 7.93±0.94 µm, with range 5.10-10.00 µm. Length/breadth ratio averaged 1.52±0.24, with range 1.15-3.0. These isolates were identified as *P. plurivora* Jung and Burgess, and morphological identification was confirmed after the ITS sequencing of selected isolates.

Two isolates formed woolly to cottony colonies, with sparse aerial mycelium and with regular edges on V8, PDA, CA and PJA, and irregular on MEA. Minimum temperature for growth was at 5°C, optimum at 25°C, and maximum at 30°C. Radial growth rate at 25°C on CA was 6 mm/day. After development and observation of sexual and asexual structures, these isolates were homothallic, forming globose oogonia that averaged 30.37±2.62 × 30.45±2.18 µm and range was 22.3-35.8 × 26.0-34.7 µm. Antheridia were paragynous, averaged 13.45±2.43 × 9.8±2.23 µm, with range 8.0-19.3 × 6.0-19.0 µm. Oospores were regular, plerotic or slightly aplerotic, averaged 21.93±2.01 × 22.0±1.31 µm, and ranged 16.0-28.3 × 16.3-27.4 µm. Oospore wall thickness averaged 1.11±0.25 µm, with range 0.60-1.70 µm. Sporangia were typically papillate, usually formed in clusters on short pedicels and often caduceus with average dimensions of 41.76±7.02 × 30.18±4.08 µm, and range 28.50-54.10 × 21.60-38.20 µm. Length/breadth ratio averaged 1.38±0.09, with range 1.13-1.62. Species produced plerotic or slightly aplerotic chlamydospores, formed both terminally and intercalary, with averaged dimensions 23.21±3.94 × 22.76±4.26 µm, and range 18.40-29.10 × 17.30-29.10 µm. These isolates were identified as *P. cactorum* (Lebert and Cohn) Schröeter, and the results of the ITS sequencing of one selected isolate corresponded to morphological identification.

The remaining seven isolates were sterile or heterothallic, not forming oogonia on agar media. Sporangia were non-papillate, non-caduceus with often internal, and very rare external proliferation. Three out of these seven isolates were with velvety to cottony colony, immersed into media and with different, mostly rozette and sparse aerial mycelium. Sporangia were mostly formed terminally, regular ovoid to obpyriform with average dimensions of two measured isolates 43.14±7.08 × 28.64±5.23 µm, and range 30.2-64.4 × 18.0-43.3 µm. Empty sporangia exit pore averaged 11.05±1.48 µm, with range 7.7-14.5 µm. Length/breadth ratio averaged 1.52±0.24, with range 1.16-2.24. Minimum temperature for growth was at 5°C, optimum at 25°C and maximum around 35°C. Growth rate at optimum 25°C was 3,98±0,31 mm/day on CA. Based on all observed characteristics and on ITS sequence of selected isolate this species was identified as *P. gonapodyides* (Petersen) Buisman.

The remaining four isolates were similar to previous group of isolates, also with non-caduceus, non-papillate sporangia, with shapes ranging from regular ovoid, obpyriform to some irregular shapes with constrictions in the middle of sporangia-ampuliform shape. Sporangia formed mostly on simple, unbranched sporangiophores, sometimes with widening of both base of sporangia and mother hypha at the contact place. Nested and extended internal proliferation of sporangia was often recorded. Size of sporangia of two measured isolates averaged 39.28±7.64 × 26.8±4.34 µm, and ranged 23.91-54.47 × 17.63-33.85 µm. Empty sporangia exit pore averaged 12.39±1.83 µm, with range 11.4-15.14 µm. Length/breadth ratio averaged 1.46±0.15, with range 1.15-1.82. Minimum temperature for growth was at 5°C, optimum at 30°C and maximum between 35°C and 40°C. Growth rate at optimum 30°C was 4,45±0,28 mm/day on CA. Based on all observed features and ITS sequence of selected isolate, this species was identified as *P. lacustris* Brasier, Cacciola, Nechwatal, Jung and Bakonyi, sp. nov.

Molecular identification of isolates

After the morphological identification, molecular studies were performed according to described methodology with aim of confirmation of morphological data and final species identification. DNA was extracted from chosen representative isolates and PCR amplification was performed. Used ITS 4 and ITS 6 primers amplified ITS region of selected isolates and

amplicon size ranged between 800-1000 bp. In total seven isolates were sequenced, four from *P. plurivora*, and one isolate from each *P. cactorum, P. gonapodyides* and *P. lacustris*. After BLAST analyses of obtained sequences, identity of *P. cactorum* sequenced isolate with the closest sequence in the GenBank was 99% with 0% of gaps (table 6). For all the rest sequences identities with the closest sequences were 100%, also with 0% gaps, respectively. BLAST analyses of sequenced ITS region for selected isolates is shown in table 6. Sequences were submitted to the GenBank and assigned accession numbers are shown in table 5.

Table 6. BLAST analyses of sequenced ITS region for selected isolates

Isolats	Accession number	Identities (%)	Gaps (%)	Query Cover (%)	The closest sequence in GenBank	Species
18	JX276070	807/808 (99%)	0/808 (0%)	99%	EU109567	*P. cactorum*
5	JX276089	837/837 (100%)	0/837 (0%)	100%	KF444065	*P. gonapodyides*
20	JX276080	834/834 (100%)	0/834 (0%)	100%	EU240094	*P. lacustris*
11	JX276067	761/761 (100%)	0/761 (0%)	100%	HQ697237	*P. plurivora*
15	JX276066	761/761 (100%)	0/761 (0%)	100%	HQ697237	*P. plurivora*
22	JX276071	761/761 (100%)	0/761 (0%)	100%	HQ697237	*P. plurivora*
29	JX276075	761/761 (100%)	0/761 (0%)	100%	HQ697237	*P. plurivora*

DISCUSSION

Species from the *Acer* genus are important tree species for Serbian forestry as economically producing valuable wood products, as well as ecologically building different communities and forest types with beech, oaks and other noble hardwoods. Also, several species from this genus are often used as amenity and park trees in Serbia, including allochthonous species such as ash-leaved maple and sugar maple introduced from North America (JOVANOVIĆ 1961).

Several diseases were reported as aggressive to maple trees in Serbia causing significant damages to these hosts (KARADŽIĆ, 2010; KARADŽIĆ *et al*. 2011, 2014).

Susceptibility of maple trees to infections with the pathogens from the *Phytophthora* genus was previously reported in different studies (ERWIN and RIBEIRO, 1996; BRASIER and JUNG, 2006; JUNG and BURGESS, 2009), and the new species associated with decline of Sycamore maple, *P. acerina* sp. nov. was recently described (GINETTI *et al*. 2013).

Phytophthora species were often isolated from maple trees in this study, and they were recovered at nine out of ten studied localities (table 2). Also, from six tested hosts five were positive for the presence of these pathogens in different isolation tests.

Registered symptoms were different and depended from the sampling season and host species, as it was reported by MILENKOVIĆ *et al*. (2011 a, b).

Presences of bleeding cankers were rare, and the isolations from these necrotic tissues were successful only in early spring and two isolates were obtained from the active necrosis of *Acer pseudoplatanus* from Košutnjak (table 5). Moreover, during the repeated sampling on this locality in autumn, isolations were successful from soil using the baiting methods, but not from old cankers and necrosis. Isolation from necrotic tissues from *A. heldreichii* was successful in summer, but only from higher elevations on Jastrebac Mountain, where the average moisture rate

is certainly higher. Low average moisture rates and high average temperatures during the summer in Serbia could be the reason why the isolation was less successful in summer and autumn, but this requires additional surveys to be supported. Similar observations and the rare bark necrosis and bleeding cankers in the case of *Fagus sylvatica* L. in Serbia was reported by MILENKOVIĆ *et al*. (2012).

In opposite, most of the isolates in this study were obtained from the samples of soil and roots using the baiting methods. Symptoms on the roots and crown, as well as the frequency of pathogen isolations from soil samples and roots, suggested their significant role in decline of different woody hosts in forests, parks and amenity plantings, including maple trees, as reported before (ERWIN and RIBEIRO, 1996; JUNG *et al*. 1996; JUNG and BURGESS, 2009; GINETTI *et al*. 2013).

Also, role of the water as a source and in the distribution of *Phytophthora* species was previously reported (REESER *et al*. 2011; HULVEY *et al*. 2010; ORLIKOWSKI *et al*. 2007), and the isolations of these organisms from water samples in these study was expected. Previously, isolations from water samples in different ecosystems in Serbia were reported by MILENKOVIĆ *et al*. (2012, 2013).

Observed structures under the light microscope and the data about colony morphology and physiology of the isolates were compared with known identification keys for *Phytophthora* species (WATERHOUSE, 1963; STAMPS *et. al*. 1990; ERWIN and RIBEIRO, 1996), and with the data from original papers with species described recently (JUNG *et al*. 1999, 2002, 2011; JUNG and NECHWATAL, 2008; JUNG and BURGESS, 2009; HONG *et al*. 2011; NECHWATAL *et al*. 2012).

Due to high number of newly described species and taxa during the last 15 years (around 56 species until 1999 (ERWIN and RIBEIRO, 1996; ILIEVA *et al*. 1998; JUNG *et al*. 1999), and over 120 until 2014 (BRASIER, 2009, JUNG *et al*. 2011, NECHWATAL *et al*. 2012; KROON *et al*. 2012), whose characteristics are not included into previously listed keys, as well as that those keys are not following the natural classification of the species within this genus according to COOKE *et al*. (2000) and KROON *et al*. (2004), molecular studies seems to be very important and sometimes essential in final identification of the species and in natural phylogeny (COOKE *et al*. 2000; KROON *et al*. 2004). In particular, this is important in the species complexes that are morphologically and physiologically very similar but phylogenetically distinct, such as *P. citricola* and *P. gonapodyides*, and several different species were segregated from these complexes (BRASIER *et al*. 2003; JUNG and BURGESS, 2009; HONG *et al*. 2011; NECHWATAL *et al*. 2012; GINETTI *et al*. 2013).

The most common isolated species in this study was *P. plurivora*, previously known as *P. citricola* Sawada, with 22 out of 31 isolates, and this species was isolated from four out of six tested maple species (table 5). According to JUNG and BURGESS (2009), *P. plurivora* was isolated from both necrotic tissues and rhizosphere soils of *Acer* spp. and our findings correspond to these data. Also, this species was very often isolated from different other hosts in Serbia (MILENKOVIĆ and KEČA, 2012; MILENKOVIĆ *et al*. 2012, 2013), what suggests that this pathogen has established its population in different natural and semi-natural ecosystems in Serbia.

Two isolates of *P. cactorum* were obtained, and in the both cases they were isolated together with *P. plurivora* from soil samples from under the *Acer pseudoplatanus* trees.

Third identified species, *P. gonapodyides* with three isolates was isolated from wet stands in Kupinovo, from under single host trees of *Acer campestre*, and two isolates were obtained from under symptomatic and one from the tree without visible symptoms (table 5).

Interestingly, this species is connected and reported in wet and flooded stands (HANSEN and DELATOUR, 1999; BALCI and HALMSCHLAGER, 2003), but recently it was reported in xeric conditions and connected with Holm Oak decline in Spain (CORCOBADO *et al*. 2010).

Fourth identified species, *P. lacustris* was isolated from four different maple hosts, including *Acer heldreichii*, *Acer platanoides*, *Acer pseudoplatanus* and *Acer tataricum*. Three isolates were obtained from wet soil and one from water, collected under the symptomless trees, using the baiting technique. This species was previously known as *P.* taxon 'Salixsoil' (BRASIER *et al*. 2003) and was often reported in different wet and riparian stands in Europe, North America and Australia, as well as in some forests and nurseries (NECHWATAL and MENDGEN, 2006; JUNG *et al*. 2011; ORLIKOWSKI *et al*. 2011; REESER *et al*. 2011; NECHWATAL *et al*. 2012). Recently this species was connected with *Fraxinus angustifolia* Vahl. dieback in Turkey (AKILLI *et al*. 2013). Also, this species was consistently isolated from symptomatic, *Fraxinus angustifolia*, *Quercus robur* L. and other hygrophilic hosts in lowland forests and wet stands in Serbia (MILENKOVIĆ, unpublished data).

In this study we extended the distribution and host range of *Phytophthora* species in Serbia, and this is the first report of *P. plurivora* and *P. gonapodyides* on *A. campestre*, *P. plurivora* and *P. lacustris* on *Acer heldreichii* and first report of *P. lacustris* on *A. pseudoplatanus* and *A. tataricum* in Serbia.

However, after these preliminary studies, common isolation and morphological and molecular identification of *Phytophthora* species from different maples in Serbia, presence of these pathogens pose significant threat to forestry and biodiversity due to ecological value of maple trees in Serbia. Also, future field surveys on presence and diversity, as well as on the pathogenicity of obtained species are required for clarification of the role of these organisms in maple trees decline phenomenon.

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MORFOLOŠKA I MOLEKULARNA IDENTIFIKACIJA *Phytophthora* VRSTA NA JAVOROVIMA U SRBIJI

Ivan MILENKOVIĆ), Justyna A. NOWAKOWSKA[2], Tomasz OSZAKO[2], Katarina MLADENOVIĆ[1], Aleksandar LUČIĆ[1], Ljubinko RAKONJAC[1], Dragan KARADŽIĆ[3]

[1]Institut za Šumarstvo, Beograd, Srbija
[2]Univerzitet u Beogradu-Šumarski Fakultet, Beograd, Srbija
[3]Forest Research Institute-IBL, Sekocin Stary, Raszyn, Poland

Izvod

Phytophthora vrste inficiraju različite biljne delove, uključujući lišće, izbojke, koru, kambijum, fino i odrvenelo korenje i to velikog broja vrsta u poljoprivredi, šumskim i parkovskim ekosistemima, rasadnicima, staklarama i predstavljaju veliku pretnju biodiverzitetu. Javorovi su ekološki veoma važna vrsta i grade različite biljne zajednice sa tvrdim i plemenitim lišćarima, a u manjoj meri i sa četinarima. Vrste iz ovog roda važe za osetljive na infekcije vrstama iz roda *Phytophthora*. Uzimajući u obzir osetljivost ovih domaćina, registrovane simptome koji su mogli biti uzrokovani patogenima iz roda *Phytophthora*, kao i rizike koje prisustvo ovih vrsta nosi, sprovedeno je istraživanje sa ciljevima da se odredi prisustvo i diverzitet vrsta iz roda *Phytophthora* na javorovima u Srbiji. Nakon sakupljanja uzoraka i izolacije, ukupno je dobijen 31 izolat sa pet različitih vrsta iz roda javorova. Domaćin sa koga je dobijeno najviše izolata je bio gorski javor sa ukupno 18 izolata, zatim dolaze klen sa sedam izolata, planinski javor sa tri, mleč sa dva i žešlja sa jednim izolatom. Posle detaljno izvršene morfološke i fiziološke klasifikacije, izdvojene su četiri grupe izolata i izvršena je preliminarna identifikacija. Iz reprezentativnih izolata je izolovan DNK i izvedeno je sekvenciranje ITS regiona, pri čemu je potvrđena morfološka identifikacija. Dobijene sekvence su obrađene i prosleđene u banku gena i dobijeni su pristupni kodovi. Identifikovane su sledeće vrste *P. cactorum, P. gonapodyides, P. lacustris* i *P. plurivora.* Ovo je prvi nalaz *Phytophthora* vrsta na nekoliko vrsta iz roda javora u Srbiji.

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