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Original scientific paper

MORPHOLOGICAL AND MOLECULAR IDENTIFICATION OF *Phytophthora* SPECIES ISOLATED FROM THE RHIZOSPHERE OF DECLINING OAK TREES IN KROTOSZYN PLATEAU

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The following paper presents the results on the determination of the diversity of species from the *Phytophthora* genus occurring in the declining oak stands in Krotoszyn Plateau in Poland. From the 50s of the last century, significant deterioration of oak health was observed in these stands, and *Phytophthora* species were suggested as one of the factors of the decline. In order to determine the presence of pathogenic organisms from the *Phytophthora* genus in these stands, 180 rhizosphere soil samples from three forest districts throughout the Krotoszyn Plateau were collected and subjected to the isolation method. *Phytophthora* species were consistently isolated from all the sampled stands, and 194 isolates from 111 positive samples were obtained. However, 150 (77%) and 44 (23%) isolates originated from the samples taken under the symptomatic and asymptomatic trees, respectively. All the obtained isolates were morphologically classified using the light and scanning electron microscopy and divided into morphological groups. Genomic DNA was isolated from selected isolates representing each group, ITS regions were amplified and sequence analyses were performed. In total, four different *Phytophthora* species were detected, including *P. cactorum*, *P. plurivora*,

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P. quercina and *P. europaea*. The most often isolated species were *P. cactorum* and *P. plurivora*. This is the first report of *P. europaea* in oak stands in Poland.

Key words: morphology, PCR, *Phytophthora*, *Quercus robur*, Sequencing, SEM

INTRODUCTION

The *Quercus* genus is representing important forest tree species, valuable both economically (wood products), and ecologically (ANDRZEJCZYK, 2009). The species from this genus which naturally grow in our climatic conditions are *Quercus robur* L. and *Q. petraea* (Mattuschka) Liebl. Additionally, *Q. rubra,* as an alien species was planted on the poorest forest sites in order to increase their biodiversity (TOKARSKA-GUZIK *et al.*, 2012). In the 80s and 90s of the last century several waves of oak decline phenomena were observed, and no clear single factor was found to be responsible for mass mortality of oak trees in Poland (OSZAKO *et al.*, 2009) or in many other countries (MONTECCHIO *et al.*, 2004; DENMAN and WEBBER, 2009; MCCONNELL and BALCI, 2014). One of the first descriptions of severe damage to oak stands in Poland came from the 50s of the last century from the region of the Krotoszyn Forest District (OSZAKO *et al.*, 2009, 2013).

However, oak stands growing on the Krotoszyn Plateau (West - Central part of Poland) are among Europe's largest compact complexes of oak forests. This area is characterized by high biodiversity, nevertheless oak stands showed symptoms of their poor health status, including high defoliation and low vitality, consequently leading to decline. Several intensive studies and observations were performed, with aims to find the causes of oak decline (SIWECKI, 1987, 1989; SIWECKI and RATAJCZAK, 1991; WAŻNY *et al.* 1991; WOŹNY and SIWECKI, 1991).

There are numerous described harmful factors that may cause the syndrome of oak decline (OSZAKO, 2007; MARÇAIS and DESPREZ-LOUSTAU, 2014). For instance, in Poland, several diseases were reported being aggressive to different oak organs, causing significant damages to acorns (STOCKA, 1997; KOWALSKI, 1999), leaves (MAŃKA, 1998), shoots and stems (KOWALSKI, 1996), roots (PRZYBYŁ, 1999) or young plants (GRZYWACZ, 1990; MAŃKA, 1998). Currently, among the other species, oaks exhibit the worst health conditions in Poland (LASY W POLSCE, 2015). According to the same source, the symptoms of decline in the year 2015 were recorded in around 35% of all the oak stands in Poland.

Among different factors, *Phytophthora* infections were recognized as important part in oak decline phenomenon (JUNG *et al.*, 1996, 1999, 2000; HANSEN and DELATOUR, 1999; VETTRAINO *et al.*, 2002; BALCI and HALMSCHLAGER, 2003a, b; JUNG and NECHWATAL, 2008; JANKOWIAK *et al.*, 2014). Species from the *Phytophthora* genus are fungi like organisms and belongs to SAR super group (ADL *et al.*, 2012). They are responsible for different damages of the roots, stems, shoots and fruits of many plants in both agriculture and forest ecosystems (ERWIN and RIBEIRO, 1996). Detailed relationships between the presence of these pathogens in rhizosphere soil and their damages to fine roots, and in consequence damages to crowns of oaks and other hosts, was previously well studied (JUNG *et al.*, 1996, 2000, 2005; JUNG, 2009; VETTRAINO *et al.*, 2002; JANKOWIAK *et al.*, 2014).

During the studies of oak decline phenomenon in Krotoszyn Plateau, different symptoms that could be indicative for *Phytophthora* infections were recorded, including increased crown transparency, yellowing of leaves, dieback of shoots, branches and tops of the crowns, necrosis on

mother roots, and loss of fine feeder roots. Additionally, several trees with appearance of bleeding cankers (usually around the stem base and up to two meters on the trunks) were recorded.

The aims of this study were to: (i) determine the presence and diversity of pathogenic *Phytophthora* species in declining oak stands in Krotoszyn Plateau; (ii) identify obtained *Phytophthora* isolates based on morphological characteristics and document typical sexual and asexual structures of this genus; and (iii) confirm the presence of the isolated species using the sequencing of the ITS region as one of the basic in *Phytophthora* identification.

MATERIALS AND METHODS

Studied localities

The sampling was performed in *Quercus robur* L. stands in three forests districts (FD) in Poland, geographically belonging to the area of Krotoszyn Plateau, including Krotoszyn, Karczma Borowa and Piaski FDs. The ages of the two first stands were similar (around 120 years) and the third stand was younger, aged around 60 years. In total, 180 samples were collected (Table 1), and sampling was performed between 2013 and 2014. Most of the samples were collected in April and May.

Table 1. Isolation of Phytophthora species under the oak trees in Krotoszyn Plateau

Forest District	Sampled trees (No.)		Positive samples (%)		Obtained isolates (No.)	
	Symptomatic	Healthy	Symptomatic	Healthy	Symptomatic	Healthy
Krotoszyn	50	10	88	70	78	27
Karczma Borowa	30	30	90	3.33	40	3
Piaski	44	16	59	37.5	32	14
Total	124	56	78.2	25	150	44

Sampling and isolation methods

Sampling was performed according to methodology of JUNG (2009) and JUNG *et al.* (1996, 2000). Soil samples were collected as soil monoliths, (around 25 × 25 × 25 cm) from four sides of each tree in the distance of ca. 1m from the stem base. Isolation tests were performed using the baiting method NECHWATAL *et al.*, 2013; JUNG, 2009; JUNG *et al.*, 1996, 2000). Collected soil samples around each oak were mixed and portions of ca. 400-500 g were placed in the 1 liter plastic containers, flooded with distilled water and left at 22-25°C for incubation. Both, symptomatic and asymptomatic oak trees were sampled (Table 1). Seven to ten-days-old *Quercus robur* and *Fagus sylvatica* L. leaves were used as baits, and plated on the surface of the cleaned

water over the flooded soil (JUNG *et al.*, 1996). Ten to 12 leaves per vessel were placed on the water surface in order to cover the whole surface. Brownish spots which appeared after two to seven days on the surface of the leaves were cut into small pieces (approx. 5 × 5 mm) and transferred on the selective V8 agar media (V8A-PARPNH) (JUNG *et al.*, 1996). The incubation lasted for three to five days at 22-25°C in the dark. After appearance of first *Phytophthora* hyphae they were subcultured on the unclarified V8A media, prepared with 900 ml l^{-1} of distilled water, 100 ml l^{-1} of V8 juice (Tymbark, Poland), 18 g l^{-1} of agar (BTL, Poland) and 3 g l^{-1} of $CaCO_3$.

All the obtained isolates are stored at 8-10°C in the Forest Research Institute-IBL laboratory of phytopathology, and are transferred after every 6-8 months on the fresh V8A media.

Identification of obtained isolates

Light microscopy of the obtained isolates

For the morphological identification, typical sexual (antheridia, oogonia and oospores) and asexual (e.g. sporangia, sporangiophores, chlamydospores and hypha) *Phytophthora* structures were developed and observed. Non-sterile soil extract was prepared (ERWIN and RIBEIRO, 1996), and the obtained isolates were transferred on clarified V8A media, and incubated in the dark at 22-25ºC. After 3 to 5 days, the pieces measuring ca. 1 × 1 cm from the edges of young colonies were transferred onto sterile Petri plates and flooded with non-sterile soil extract. The pieces of agar with fresh colony were washed with distilled water after 6 h. Distilled water was replaced twice after 6 and 12 h (JUNG and BURGESS, 2009). In parallel, all the isolates were incubated at 22-25ºC on V8A media for four weeks in order to stimulate formation of sexual and asexual resting structures. Different sexual and asexual structures were observed under the light microscope (ZEISS Axioskop 2, equipped with Nikon Ds-fi1 camera, and NIS Elements AR4® software), at ×400 magnification. The recorded structures were compared with known *Phytophthora* identification keys, with the species listed in ERWIN and RIBEIRO (1996), and from original papers describing particular species, e.g. JUNG *et al.*, 1999, 2002, 2011; JUNG and NECHWATAL, 2008; JUNG and BURGESS, 2009; HONG *et al.*, 2011; NECHWATAL *et al.*, 2013).

Observation of *Phytophthora* structures using the scanning electron microscope (SEM)

For each representative isolate, 5 pieces of homothallic mycelium (ca. 5 × 5 mm in size) with previously developed sexual and asexual structures were prepared. The samples for SEM observations were fixed overnight at 5°C in a solution of 2.5% paraformaldehyde and 2.5% glutaraldehyde (in a volume ratio 1:1) in 0.1 M sodium cacodylate buffer at pH 7.2. The samples were then washed three times with a 0.05 M buffer solution for 15 min. Post-fixation was carried out in 1% osmium tetroxide in the same buffer for two hours. After fixation the samples were rinsed in distilled water three times (5 min.) and serially dehydrated in ethanol (10, 30, 50, 70, 80, 90, 96 and 100%). Such dehydrated specimens were dried at critical point in CO_2 (Leica EM CPD300), transferred onto SEM stub covered with double-sided adhesive carbon disc. The samples were coated with gold/palladium and viewed under high vacuum in a Hitachi S3000N SEM equipped with a secondary electron detector (DEHPOUR *et al.*, 2007).

Molecular identification

The genomic DNA was extracted from fresh mycelium from the representative isolates of all the recorded morphotypes, using Gen EluteTM Plant Genomic DNA miniprep Kit (Sigma – Aldrich http://www.sigmaaldrich.com), according to the producer's instructions. PCR mixture

(Taq PCR Core Kit, Qiagen https://www.qiagen.com) contained 1 x Q buffer, 1 x PCR buffer, 1.5 mM $MgCl_2$ (Qiagen), 0.2 mM of dNTPs, 0.15 µM of each primer (ITS4: Reverse TCCTCCGCT TATTGATATGC and ITS6: Forward GAAGGTGAAGTCGTAACAAGG, according to COOKE and DUNCAN, 1997), 1 U Taq polymerase (Qiagen), 25 - 50 ng of genomic DNA adjusted with water (MilliQ) up to the total volume of 25 µl. The PCR was performed in PTC-200™ Programmable Thermal Controller (Bio-Rad http://www.bio-rad.com) machine. The PCR master cycler program parameters were as follows: 3 min of initial DNA denaturation at 94°C and 35 cycles of amplification (30 sec of denaturation at 94°C, 30 sec of annealing at 55°C, 60 sec of elongation at 72°C), and 5 min of final elongation at 72°C. The PCR products were cleaned using Clean-up kit (A&A Biotechnology, Poland http://www.aabiot.com) according to the producer's instruction, and sequenced using ABI 3500 Genetic Analyzer (Applied Biosystems™, USA https://www.thermofisher.com). The obtained sequences were compared with online sequence database GenBank using BLAST (http://www.ncbi.nlm.nih.gov) and assembled using the Clustal algorithm of the BioEdit software (http://www.clustal.org/).

RESULTS

Isolation of *Phytophthora* species from oak trees in Krotoszyn Plateau

In total, 194 isolates of different *Phytophthora* species were obtained from soil samples (Table 1). From 124 samples taken under the declining, symptomatic trees, 97 samples (78%) were positive, while from 56 samples taken under the non-symptomatic trees, only 14 (25%) were positive on *Phytophthora* presence after the isolation tests. Most isolates originated from oak stand in Krotoszyn FD, and from 60 soil samples total of 105 isolates were obtained. In two other forest districts Piaski and Karczma Borowa, a number of obtained *Phytophthora* isolates was quite similar, with 46 and 43 obtained isolates, respectively. Most of the obtained isolates (77%) were isolated under the symptomatic oak trees, while 44 isolates (23%) were obtained under the apparently healthy oak trees (Table 1). After the detailed morphological and molecular analyses, four different species were identified, including *P. cactorum*, *P. europaea*, *P. plurivora* and *P. quercina* (Tables 2 and 3).

Table 2. Obtained species and number of isolates from different studied localities

Obtained species	Localities			
	Krotoszyn	Karczma Borowa	Piaski	Total
Phytophthora cactorum	38	16	10	64
Phytophthora europaea	13	9	21	43
Phytophthora plurivora	36	8	15	59
Phytophthora quercina	18	3	7	28
Total number of isolates	105	43	46	194

Table 3. Morphological identification of isolated Phytophthora species

Species	*Phytophthora quercina*	*Phytophthora plurivora*	*Phytophthora europaea*	*Phytophthora cactorum*
Sporangia	Papillate	Semipapillate	Nonpapillate	Papillate
Length range (μm)	36.40 – 63.88	40.66 – 62.83	35.62 – 57.38	33.04 – 47.88
Length mean (μm±SD)	46.22 ± 7.24	50.40 ± 5.50	45.09 ± 11.42	40.96 ± 3.89
Breadth range (μm)	27.87 – 45.53	28.98 – 45.68	12.32 - 38.11	25.14 – 38.66
Breadth mean (μm±SD)	35.37 ± 4.75	35.78 ± 4.22	32.38 ± 6,10	31.82 ± 2.70
Length to breadth ratio (L/B) mean	1.31	1.41	1.39	1.29
Length to breadth range	1.31 – 1.40	1.40 – 1.38	1.01 – 0.32	1.31-1.24
Oogonia	Homothallic	Homothallic	Homothallic	Homothallic
Diameter range (μm)	18.5 – 43.6	17.6-32.4	28.4-43.9	25.8- 35.9
Range mean (μm±SD)	28.5 ± 4.5	26.9 ± 5.6	35.8 ± 5.4	29.8 ± 2.5
Oospores	Plerotic to irregular, thick walled	Plerotic to slightly aplerotic	Plerotic	Plerotic
Diameter range (μm)	18.2 – 36.8	22.2 – 29.5	24.5-39.7	19.2 – 26.8
Range mean (μm±SD)	26.8 ± 2.5	26.2 ± 1.9	33.0 ± 5.1	22.8 ± 3.3
Antheridia	Paragynous	Paragynous	Paragynous	Paragynous
Length range (μm)	8.4 – 15.2	7.0-13.7	7.3 – 14.2	8.5-14.2
Length mean (μm±SD)	13.4 ± 8.5	10.5 ± 2.0	10.2 ± 2.4	11.3 ± 2.0

Scanning electron and light microscopy observation

After the detailed observation under the microscopes, mostly typical *Phytophthora* structures were recorded among the observed isolates while *Chlamydospores* were observed only in 20% of the *P. cactorum* isolates.

Isolates of *P. cactorum* were homothallic, with globose oogonia and paragynous antheridia (Fig. 1 d). Oospores were mostly plerotic. Sporangia were papillate, ovoid, obovoid to lemoniform, often caduceus, formed terminally in clusters on short pedicels, and on sympodially branched sporangiophores (Fig. 2 d).

Isolates of *P. europaea* were also homothallic with tapered based oogonia (Fig. 1 b), and plerotic oospores that almost filled the oogonia. Antheridia were paragynous. Sporangia were persistent, nonpapillate, ovoid, and ellipsoid, elongated and formed terminally (Fig. 2 b).

Isolates of *P. plurivora* were homothallic with globose to slightly subglobose oogonia (Fig. 1 c) and with mostly plerotic oospores. Antheridia were paragynous. Sporangia were formed terminally on sympodially branched sporangiophores (Fig. 2 c) and very variable in shape, from regular ovoid, obovoid, ellipsoid, to elongated irregular, and with two or three papilla. Also, intercalary formed sporangia were recorded.

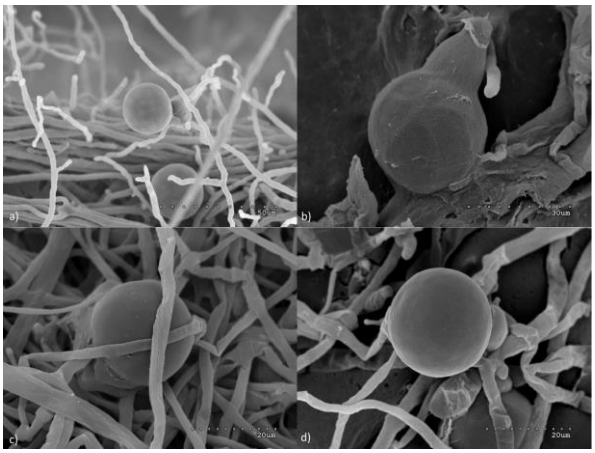

Fig. 1. Electron micrographs of oogonia and antheridia structures formed by *Phytophthora* species: a) *Phytophthora quercina* oogonia and antheridia; b) *Phytophthora europaea* oogonia; c) *Phytophthora plurivora* oogonia; and d) *Phytophthora cactorum* oogonia and antheridia

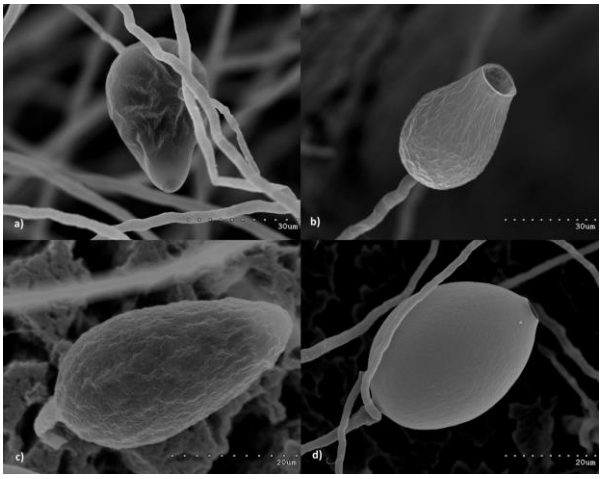

Fig. 2. Electron micrographs of sporangia structures formed by *Phytophthora* species: a) *Phytophthora quercina*; b) *Phytophthora europaea*; c) *Phytophthora plurivora*; and d) *Phytophthora cactorum*

Isolates of *P. quercina* were also homothallic, with globose, subglobose, ellipsoid to irregular shapes of oogonia. Oospores were with thick walls and with similar shapes of the mother oogonia. Antheridia were paragynous (Fig. 1 a). Sporangia were papillate (Figure 2 a), persistent, ovoid, obovoid to lemoniform, often with irregular shapes, also formed intercalary and with two or even three papilla.

Molecular identification

Primers used in this study successfully amplified ITS region of analyzed isolates, and total of four isolates were sequenced, including one isolate of *P. cactorum*, *P. europaea*, *P. plurivora* and *P. quercina* (Table 2). The BLAST analyses allowed to identify sequenced isolates with the closest sequences in the GenBank was 99-100% with 0% of gaps (Table 4). BLAST analyses of sequenced ITS region for selected isolates and assigned GenBank accession numbers are shown in table 4.

Table 4. BLAST analyses of sequenced ITS region for selected isolates

Code of isolate	Accession number	Identities (%)	Gaps (%)	Query Cover (%)	The closest sequence in GenBank	Species
OAK89	KX242303	604/604 (100%)	0/604 (0%)	100%	KU053237	*P. cactorum*
OAK60	KX242302	608/610 (99%)	0/610 (0%)	100%	KJ755092	*P. europaea*
OAK12	KX242300	585/586 (99%)	1/586 (0%)	100%	KF963048	*P. quercina*
OAK163	KX242301	576/577 (99%)	0/577 (0%)	100%	KU221328	*P. plurivora*

DISCUSION

Phytophthora species were frequently isolated in declining oak stands in Krotoszyn Plateau in these studies. However, 77% of obtained isolates originated from the symptomatic oak trees. Moreover, 78% of samples collected under symptomatic trees gave positive result to the isolation test, contributing to 77% of obtained isolates. All of the obtained *Phytophthora* isolates were isolated from rhizosphere soil of sampled oak trees.

In our inventory *Phytophthora cactorum* was the most frequently isolated species from oak stands in Krotoszyn Plateau, with 64 isolates obtained. Our results confirm previous data on prevalence of this species in the soil rhizosphere of different plant species, including the apples (JEFFERS and ALDWINCKLE, 1987), and other hosts (ERWIN and RIBEIRO, 1996). It is also worth to mention that, this species is also abundant in Polish nurseries, from where it could be likely transferred to plantations and stands, threatening their biodiversity or durability (sustainability) (ORLIKOWSKI *et al.*, 2006; OSZAKO *et al.*, 2007; JUNG *et al.*, 2016).

Phytophthora plurivora was the second most frequently isolated species, and 59 isolates of this species were obtained. According to JUNG and BURGESS (2009), *P. plurivora* was isolated from rhizosphere soils of *Quercus* spp. and numerous other hosts, and our results correspond to this data. In Poland *P. plurivora* is also known from rhizosphere soil of oaks in Southern Poland

(JANKOWIAK *et al.*, 2014), and other declining hosts including ash (ORLIKOWSKI *et al.*, 2011) or alder (TRZEWIK *et al.*, 2015).

In this study we recorded presence of *Phytophthora europaea* for the first time in the investigated oak stands in Krotoszyn Plateau. The taxonomical belonging to this species was proved by morphological and molecular analysis. This species was reported from oak trees and soil in forests in Austria, France, Germany and USA (HANSEN and DELATOUR, 1999; HARTMANN and BLANK, 2002; JUNG *et al.*, 2002; BALCI and HALMSCHLAGER, 2003a; BALCI *et al.*, 2006). Although this species is consider not being strongly pathogenic to oak trees (JUNG *et al.*, 2002), its role in oak decline phenomenon should be more carefully investigated in the future, due to abundant isolates of this species found under the declining oak trees in this studies.

Phytophthora quercina was isolated from 18 rhizosphere soil samples. This species is considered to be the main cause of oak stands decline (JUNG *et al.* 1999). According to JUNG *et al.* (2000), this species is mainly responsible for the damage to fine roots, which in turn leads to the severe weakening of trees, especially during the alternation of wet and drought periods.

Photo documentation by SEM, allowed observation of sexual and asexual structures from a new perspective. Although that quality photos were obtained during the SEM, the method has its limitations, since after the scanning of the surfaces of structures, observations of oospores is almost impossible. Additionally, the preparation of sporangia could cause artifacts due to their very thin walls and location at the ends of young hyphae that exposes them to chemicals during the SEM preparations so they could be vulnerable to damages.

In conclusion: our preliminary studies on the presence and diversity of species from the *Phytophthora* genus in the declining oak stands in Krotoszyn Plateau, showed abundance and high diversity of pathogenic organisms. Taking into account progressive loss and declining health conditions of important oak stands, we argue that further surveys of the isolated species are urgent. Furthermore, soil infestation tests with the obtained species are also required in order to determine the role of *Phytophthora* species in oak decline in Krotoszyn Plateau.

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MORFOLOŠKA I MOLEKULARNA IDENTIFIKACIJA *Phytophthora* VRSTA IZOLOVANIH IZ ZEMLJIŠTA RIZOSFERE PROPADAJUĆIH STABALA HRASTA NA PODRUČJU KROTOSZYN PLATOA

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Izvod

U radu su prikazani rezultati istraživanja diverziteta vrsta iz roda *Phytophthora* prisutnih u propadajućim sastojinama hrasta na području Krotoszyn Platoa u Poljskoj. Značajno narušavanje zdravstvenog stanja hrasta je zabeleženo u ovim sastojinama od 50-ih godina prošlog veka, a *Phytophthora* vrste su označavane kao jedan od faktora propadanja. Radi utvrđivanja prisustva patogenih organizama iz roda *Phytophthora* u ovim sastojinama, sakupljeno je 180 uzoraka zemljišta rizosfere iz tri šumske uprave na području Krotoszyn Platoa i sprovedeni su testovi izolacije. *Phytophthora* vrste su konstantno izolovane iz svih uzorkovanih sastojina i ukupno je dobijeno 194 izolata iz 111 pozitivnih uzoraka. Naime, 150 (77%) izolata je dobijeno iz uzoraka sakupljenih ispod simptomatičnih stabala, dok je 44 (23%) izolata dobijeno iz uzoraka sakupljenih ispod stabala bez simptoma. Svi dobijeni izolati su analizirani pomoću svetlosnog i skening elektronskog mikroskopa i razvrstani u morfološke grupe. Iz odabranog reprezentativnog izolata svake grupe izolovana je genomska DNK i sprovedeno je sekvenciranje ITS regiona. Ukupno su zabeležene četiri različite vrste roda *Phytophthora*, uključujući *P. cactorum*, *P. plurivora*, *P. quercina* i *P. europaea*. Najčešće izolovane vrste su bile *P. cactorum* i *P. plurivora*. Ovo je prvi nalaz *P. europaea* u hrastovim sastojinama u Poljskoj.

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