Annual of Sofia University "St. Kliment Ohridski"
Faculty of Biology
Book 4 - Scientific Sessions of the Faculty of Biology
2019, volume 104, pp. 90-99
International Scientific Conference "Kliment's Days", Sofia 2018

DIVERSITY OF OOMYCETE SPECIES IN THE RIPARIAN ECOSYSTEM OF ISKAR GORGE

KALOYAN KOSTOV*, PETYA CHRISTOVA, ANETA LYUBENOVA, SLAVTCHO SLAVOV

AgroBioInstitute, 8 Dragan Tsankov Blvd., 1164 Sofia, Bulgaria
**Corresponding author: kkostov@abi.bg*

Keywords: Oomycete, diversity*, Phytophthora, Phytopythium, Pythium*

Abstract: The fungus-like oomycete organisms are often associated with inland waters and riparian ecosystems. Some of them are primary saprophytes utilizing plant debris. Others are able to infect different plant species causing diseases, occasionally with devastating intensity. Rivers are major environment and distribution route of these organisms and as such their presence and composition in the inland waters is a subject of intensive research in the last years. In order to study the diversity of the oomycete species, with an accent to those which belong to genus Phytophthora, in the longest river in Bulgaria - Iskar, we have used a "baiting" method to catch swimming zoospores. "Baits" were placed in the river at 7 locations along the Iskar Gorge. The collected isolates were grown on nutrient media for their morphological and genetic characterization. As a result, we have found species of 3 genera - *Phytophthora, Phytopythium* and *Pythium* with respectively 3 (*Phytophthora lacustris, Phytophthora gonapodyides, Phytophthora chlamydospora*), 2 (*Phytopythium litorale, Phytopythium montanum*) and 1 (*Pythium* sp.) representatives. All the above *Phytophthora* species are phylogenetically related as members of clade 6, the representatives of which are commonly isolated from rivers. Some of these species had demonstrated weak to moderate pathogenicity against several plant species in controlled experiments. Most of the oomycete species we found are probably natural inhabitants of the studied riparian ecosystem. No quarantine species have been found.

INTRODUCTION

The species which belong to class Oomycetes are fungus-like organisms, which however are much closer to diatoms and brown algae than to the kingdom of Fungi (Beakes *et al.,* 2012). Because they are related to aquatic organisms, their dependence of water is strong as all or some stages of their lifecycles are

reliant on water (Walker & van West, 2007). Such stage is the production of motile flagellated zoospores, which are able to swim in water until reaching a suitable environment (Walker & van West, 2007). Some of these species are saprophytes, however others are able to colonize plant tissues and thus be pathogenic and cause diseases (Thines, 2014). Indeed, one of the most destructive plant pathogens belong to oomycetes and researchers have studied them intensively including many species within genus *Phytophthora* and *Pythium*. The most notorious example is *Phytophthora infestans* (Mont.) de Bary, the cause of potato late blight, which had great impact to the human society, before it was able to control it (Yoshida *et al.,* 2013).

The presence and diversity of plant pathogenic oomycete species in running waters and riparian environments have been studied extensively in the last years and it is evident that they are much more diverse and widespread than previously thought (Nechwatal *et al.,* 2008, Hansen *et al.,* 2012, Jung *et al.,* 2017, Marano *et al.,* 2016). This development is triggered by the appearance of new highly virulent species such as *Phytophthora ramorum* Werres, De Cock & Man in 't Veld, which causes forest declines in North America and Europe (Rizzo *et al.,* 2002) and *Phytophthora alni* Brasier & S.A. Kirk, causing severe damage in alder trees (Érsek & Nagy, 2008). Thus the need of studying the distribution roads and to monitor the appearance of these species in the natural habitats becomes evident.

Global trade is one of the main factors for the distribution of invasive species around the world (Meyerson & Mooney, 2007). The transportation of wood and plant materials from continent to continent can bring living nonnative species (including plants, insects and microorganisms) to a new environment where, in some cases, they can survive, reproduce and behave as invasive species. The harbors, airports and transportation hubs are points of entry for the alien species, and can serve as a source for the further spread to the natural areas which eventually can have a negative impact on the local biodiversity (Banks *et al.,* 2015)

The Iskar is the longest river in Bulgaria. It runs from Rila Mountain, passes in close proximity to the capital city Sofia and flow into the Danube River further north. The Iskar Gorge encompasses the passage of the river through the Balkan Mountain. It starts from the edge of the Sofia plain and stretches approximately 60 km north. The close proximity of the river to the biggest airport in the country and local gardening centers makes the risk of the appearance of invasive species in this area substantial.

The aim of the present study was to assess the diversity of oomycete species in the riparian ecosystem of Iskar Gorge with an accent to those which belong to the genus *Phytophthora*.

MATERIALS AND METHODS

Collection and isolation of oomycete species

The collection of oomycete isolates took place in October, 2017. The locations (near the following settlements: Vlado Trichkov, Svoge, Bov, Lakatnik, Opletnya, Zverino, Rebarkovo) were chosen to be evenly distributed along the Iskar Gorge (approx. 8 to 10 km distance) and to have good access to the river bank. The baiting method was used for our purpose. The "baits" were made with three rhododendron (*Rhododendron catawbiense* Michaux) leaves places in a square sized mosquito net (20 x 20 cm) and fixed with staples. Rhododendron leaves are the most commonly used baits to detect a wide range of *Phytophthora* species in water with proven efficiency and sensitivity over other plant species (Themann *et al.,* 2002, Orlikowski *et al.,* 2011). The baits were set to float on the surface of the water for 7 days. Water temperature was measured at the time of placing and collecting of the baits. Afterwards the baits were taken into the lab were the leaves with developed necrotic lesions were surface sterilized with 70 % ethanol and rinsed twice with sterile water. The oomycetes were isolated from the border of the necrotic areas by cutting small pieces and placing them on selective for *Phytophthora* species PARNHB media (Carrot Agar (CA) with 10 mg Pimaricin, 250 mg Ampicillin, 10 mg Rifampicin, 50 mg Nystatin, 50 mg Hymexazol and 15 mg Benomyl/1l). They were incubated at 20-23ºC until the appearance of growing mycelia. An agar blocks with mycelia were transferred to water agar and incubated for 3-4 days. The tips of the newly formed hyphae were transferred to a Carrot Agar (CA) media (100 ml HiPP Organic Pure Carrot Juice, 3 g $CaCO_3$, 8 g Duchefa Biochemie Plant agar and 900 ml distilled water).

Morphological characterization

The characterization of the colony morphology of the oomycete isolates was done by growing the cultures in Petri dishes (9 cm diameter) containing CA. Agar pieces (10 mm) with growing mycelia were taken from the edge of 7-10 days old cultures and were placed upside down in the center of the dish and incubated for 7 days at 25ºC in dark. The colony morphology (growth pattern, formation and structure of aerial mycelia) of *Phytophthora* species was described as previously done (Erwin & Ribeiro, 1996). We followed the same procedure for *Phytopythium* and *Pythium* isolates.

DNA isolation, amplification, sequencing and analysis

DNA was isolated from frozen mycelium using DNeasy Plant Mini Kit (QIAGEN GmbH) according to the manufacturer instructions. Primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') were used for PCR amplification of the internal transcribed spacer (ITS) region with the following thermal program:

96ºC – 2 min, followed by 35 cycles of 96ºC – 1 min, 55ºC – 1 min, 72ºC – 2 min and final elongation at 72ºC – 10 min. The PCR products were purified using the following procedure: in each well of 96-wells microtiter plates filled with approximately 34 mg per well of Sephadex (Sephadex G-50 Fine, Little Chalfont, UK) 300 µL of dH_2O was added, and remained for 4 hours at room temperature. The plates were placed on top of 96-wells plate and were centrifuged at 750 x g for 90 seconds. After spinning the waste plate was replaced by a clean PCR 96-wells plate and 25 µL of PCR reaction was loaded in the wells and centrifuged again at the same speed and duration with a benchtop centrifuge. Five µL from the purified PCR product was mixed with 5 µL of primer (5 pmol of ITS4) and sent for sequencing in the GATC Biotech AG (Germany). The sequence quality checks, alignments and phylogenic trees were made using CLC Workbench software. The DNA sequences were compared to the deposited sequences in the Gene Bank database at the National Center for Biotechnology Information (NCBI) using Basic Local Alignment Search Tool (BLAST). Neighbor Joining (NJ) tree was build using ITS sequence data of approximate length of 400 bp. A bootstrap test with 1000 replications was performed. Since some of the sequences were with insufficient quality they were not included, thus not all isolates are represented in this analysis.

RESULTS AND DISCUSSION

A total of 16 oomycete isolates were obtained from the baits in the Iskar Gorge. The water temperature in the different locations varied between 7 to 9.5°C at the date when baits were placed and in the time of pick up from 2 to 3°C. It is known that cool temperatures and environmental conditions in October favoured formation and development of zoospores (Porter and Johnson, 2004).

DNA was extracted from all the isolates and the ITS region was amplified and sequenced. The BLAST analysis showed high sequence similarity of 9 isolates with species within the genus *Phytophthora* of which 5 isolates - RBov2017/106a, RBov2017/106b, RZve2017/109a, RZve2017/109b and RReb2017/111a with 99.88 to 100% similarity with *Phytophthora lacustris* Brasier, Cacciola, Nechw., Jung & Bakonyi; 1 isolate (RSvo2017/105a) with 99.65% identity with *Phytophthora gonapodyides* (H.E. Petersen) Buisman and 1 Isolate (RReb2017/111b) with 99.88% identity to *Phytophthora chlamydospora* Brasier, C. and E. Hansen. Another 2 isolates (RSvo2017/105b and RVlTr2017/104b) showed similarity with *P. chlamydospora*, however the quality of the sequences received for them was not satisfying. Since after a certain number of nucleotides the histograms were unclear, with many double and triple signals. Therefore, only the parts with high quality reads were used for the identification of these

species. Thus isolate RSvo2017/105b had 99.29% similarity within 141 base pairs (bp) and isolate RVlTr2017/104b showed 100% similarity within 138 bp to *P. chlamydospora.*

In addition, 6 isolates showed high sequence similarity with two *Phytopythium* species. Likewise, with the two sequences of *P. chlamydospora* the quality of the reads for these isolates was not as desired, thus only parts of the sequences were used in the analysis. The following 4 isolates were identified as *Phytopythium litorale* (Nechw.) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque - RVlTr2017/104a (172 bp with 98.51% similarity), RVlTr2017/104d (306 bp with 99.02% similarity), RSvo2017/105c (374 bp with 97.06% similarity) and RBov2017/106c (374 bp with 95.45% similarity). The isolates ROple2017/108 (279 bp with 96.77% similarity) and RZve2017/109c (581 bp with 97.76% similarity) were identified as *Phytopythium montanum* (Nechw.) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque.

Even though we have used the fungicide hymexazol in the media for isolation to suppress the development of *Pythium* species, the ITS sequence analysis of the isolate named RLak2017/107a showed similarity with several *Pythium* species. An excellent quality sequence for the whole ITS region did not exceed 94% similarity with the deposited sequences of known species in the NCBI database, thus RLak2017/107a was referred as *Pythium* sp. The *Pythium* species with which RLak2017/107a showed the highest similarity are *Pythium coloratum* Vaartajaa and *Pythium inflatum* V.D. Matthews. The genetic distance is evident and could be an indication that isolate RLak2017/107a belong to undescribed species of *Pythium*, which however needs additional conformation including morphological and physiological characterisation and the inclusion of additional DNA regions for proper identification.

Phytophthora lacustris, P. chlamydospora and *P. litorale* were the most widely distributed species, each found at 3 different locations in the Iskar Gorge, followed by *P. montanum* which was found at two locations and the one location respectively for each of the single isolates of *P. gonapodyides* and *Pythium* sp. (**Fig. 1**)

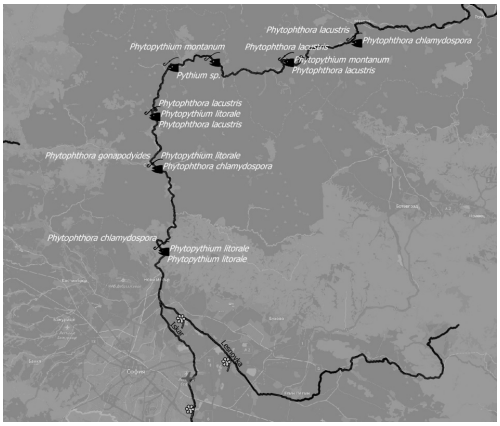

Fig. 1 Distribution map of oomycete species in the Iskar Gorge. The location of Sofia airport and plant nurseries are noted with appropriate symbols.

One representative from each group of isolates was chosen for morphological characterisation (**Fig. 2**). *Phytophthora gonapodyides* isolate RSvo2017/105a produced mostly submerged stellate colony with very limited aerial mycelia. *Phytophthora lacustris* isolate RBov2017/106a produced submerged petaloid to stellate colony and woolly aerial mycelia. *Phytophthora chlamydospora* isolate RReb2017/111b produced semi-petaloid and sparse aerial mycelia. Isolate RLak2017/107a referred as *Pythium* sp. produced fully submerged stellate colonies with no aerial mycelia. *Phytopythium litorale* isolate RVlTr2017/104a produced dense aerial mycelium fully covering the surface of the Petri dish and *P. montanum* isolate ROple2017/108 produced partially submerged partially grainy aerial mycelia (**Fig 2**).

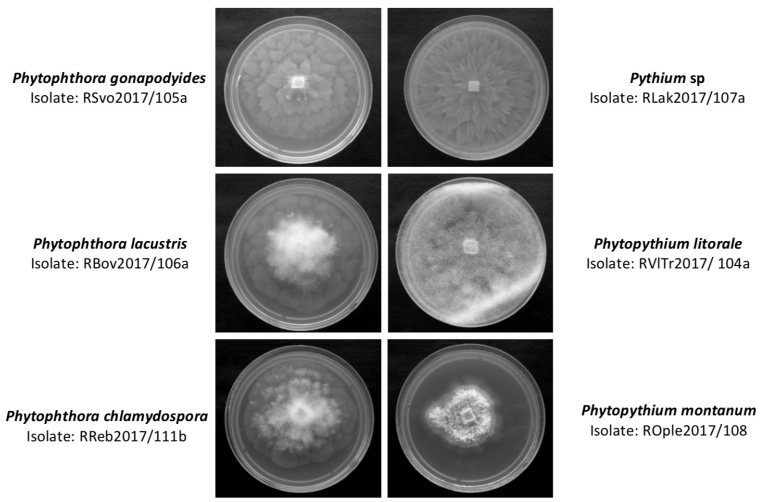

Fig. 2 Colony morphology of oomycete species in the Iskar Gorge

All the *Pythophthora* species found in the Iskar Gorge are phylogenetically close and belong to clade 6. The genetic relation between the isolates is shown on Fig 3. The specific characteristic of the species in this clade is that they are predominantly isolated from aquatic and riparian environments (Jung *et al.,* 2011, Meyerson & Mooney, 2007, Nagel *et al.,* 2013). All of them are self-sterile, a trait that is believed to be connected with the adaptation to water environments (Brasier *et al.,* 2003). The optimum growth temperature for *P. gonapodyides* is estimated between 25-28°C, for *P. chlamydospora* - 25-30°C and for *P. lacustris* - 28-33°C, which makes it the most high temperature tolerant from the three (Brasier *et al.,* 2003, Jung *et al.,* 2011, Nechwatal *et al.,* 2013).

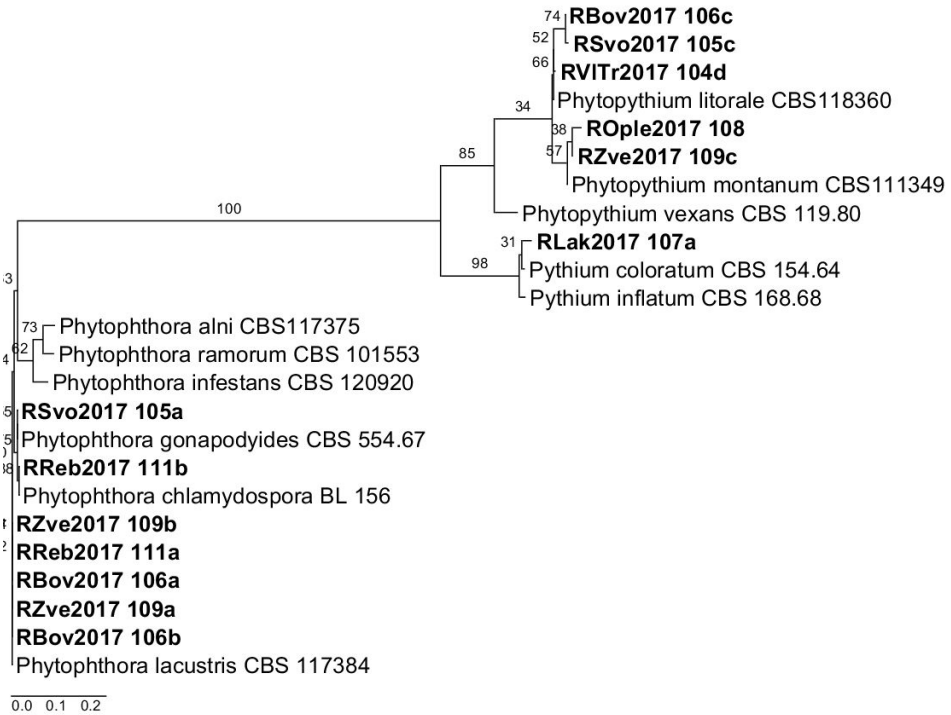

Fig. 3 Neighbor joining phylogenetic tree of the oomycete isolates in the Iskar Gorge and chosen representative species. The scale at the bottom depicts the genetic distance.

The most represented species (5 isolates) in our study *P. lacustris* is widespread in Europe and was also detected in New Zealand, Australia and the USA (Nechwatal *et al.,* 2013). In controlled conditions it is found to be weakly to moderately aggressive to some trees such as *Alnus, Prunus* and *Salix*. It has been suggested that *P. lacustris* may behave also as a saprotroph on plant debris

(Nechwatal *et al.,* 2013). The artificial inoculation of several plant species with *P. chlamydospora* have demonstrated that it can cause an infection of stems and roots. It is also reported to cause root and crown rot on almond (*Prunus dulcis* (Mill.) D.A.Webb)(Türkölmez *et al.,* 2016). Few studies identified the presence of *P. gonapodyides,* along with other *Phytophthora* species in declining oak and beech stands in Europe (Corcobado *et al.,* 2010, Jung, 2009, Jung *et al.,* 1996). In experimental conditions individual isolates demonstrated aggressiveness towards some varieties of poplar (Milenković *et al.,* 2018). The three species have been described mostly as an opportunistic pathogens and are more often associated with natural forest soils and riparian ecosystems than agricultural lands (Hansen *et al.,* 2015).

The genus of *Phytopythium* is relatively new and is composed of former *Pythium* species which share several common characteristics with *Phytophthora*. Such is the case with *P. litorale* (reassigned from *Pythium litorale*, in 2013), the second most abundant species in our study, which is previously isolated from littoral soils. It was found to be non-pathogenic to *Phragmites australis* (Cav.) Trin. ex Steud. and was suggested that it may be a saprophytic species able to feed on plant litter (Nechwatal & Mendgen, 2006). The other species from this genus which we found is *P. montanum.* It was isolated from the rhizosphere soil of Norway spruce (Picea abies (L.) H. Karst) in Bavarian Alps at 1000 m above sea level. In experimental conditions it has demonstrated weak aggressiveness against lupine (*Lupinus angustifolius* L.) and spruce (*P. abies*) (Nechwatal & Oßwald, 2003).

The *Phytophthora* species presented in the Iskar Gorge have been found previously in another Bulgarian river - Osam (Christova *et al.,* 2018) and also in other European countries (Jung *et al.,* 1996, Orlikowski *et al.,* 2011, Corcobado et al., 2010, Milenković *et al.,* 2018, Jung *et al.,* 2019). Most probably they are representatives of the natural oomycete community in the studied riparian ecosystem. Both *Phytophthora* and *Phytopythium* species in our survey have not been reported as extremely aggressive pathogens of crop or tree species. Nevertheless, some of them have demonstrated the ability to attack susceptible trees in favourable conditions (e.g. flooding), thus consciousness and regular monitoring of the surrounding vegetation is necessary.

CONCLUSION

We have found 6 oomycete species in the river along the Iskar Gorge. According to their morphological characteristics and DNA specifics they were identified as *Phytophthora gonapodyides, P. lacustris, P. chlamydospora, Pythium sp., Phytopythium litorale* and *P. montanum.* Most of them are known as common inhabitants of aquatic and riparian ecosystems. They are characterized as low to moderate pathogens. The pathogenicity of the collected isolates in this study

needs to be proved in separate experiments. No quarantine *Phytophthora* species such as *Phytophthora ramorum* and *Phytophthora alni* were detected.

Acknowledgements: This study was supported by the National Science Fund within the project „Responses of European Forests and Society to Invasive Pathogens (RESIPATH)" under the frame of the European science programme BiodivERsA 2012-2013 Joint call.

DECLARATION OF CONFLICT OF INTEREST

The authors declare that they have no financial interest in the subject matter discussed in this manuscript.

AUTHOR CONTRIBUTION STATEMENT

All authors contributed to the collection and characterisation of isolates, and to the analysis of the results. K.K. took the lead in writing the manuscript. P. Ch., A. L., and S. S. provided critical feedback and helped shape the manuscript.

REFERENCES

1. Banks NC, Paini DR, Bayliss KL, Hodda M, 2015. The role of global trade and transport network topology in the human-mediated dispersal of alien species. *Ecology letters 1*8, 188-99.
2. Beakes GW, Glockling SL, Sekimoto S, 2012. The evolutionary phylogeny of the oomycete "fungi". *Protoplasma* 249, 3-19.
3. Brasier CM, Cooke DE, Duncan JM, Hansen EM, 2003. Multiple new phenotypic taxa from trees and riparian ecosystems in Phytophthora gonapodyides–P. megasperma ITS Clade 6, which tend to be high-temperature tolerant and either inbreeding or sterile. *Mycological research* 107, 277-90.
4. Christova P, Lyubenova A, Kostov K, Slavov S, 2018. Diversity and pathogenicity of Phytophthora species, isolated from Osam River. *Journal of Mountain Agriculture on the Balkans* 21, 179-91.
5. Corcobado T, Cubera E, Pérez-Sierra A, Jung T, Solla A, 2010. First report of Phytophthora gonapodyides involved in the decline of Quercus ilex in xeric conditions in Spain. *New Disease Reports* 22, 2044-0588.2010.
6. Érsek T, Nagy ZÁ, 2008. Species hybrids in the genus Phytophthora with emphasis on the alder pathogen Phytophthora alni: a review. *European Journal of Plant Pathology* 122, 31.
7. Erwin DC, Ribeiro OK, 1996. Phytophthora diseases worldwide. American Phytopathological Society (APS Press).
8. Hansen EM, Reeser P, Sutton W, Brasier CM, 2015. Redesignation of Phytophthora taxon Pgchlamydo as Phytophthora chlamydospora sp. nov. *North American Fungi* 10, 1-14.
9. Hansen EM, Reeser PW, Sutton W, 2012. Phytophthora beyond agriculture. *Annual review of phytopathology* 50, 359-78.
10. Jung T, 2009. Beech decline in Central Europe driven by the interaction between Phytophthora infections and climatic extremes. *Forest pathology* 39, 73-94.
11. Jung T, Blaschke H, Neumann P, 1996. Isolation, identification and pathogenicity of Phytophthora species from declining oak stands. *European Journal of Forest Pathology* 26, 253-72.

12. Jung T, Jung MH, Scanu B, et al., 2017. Six new Phytophthora species from ITS Clade 7a including two sexually functional heterothallic hybrid species detected in natural ecosystems in Taiwan. *Persoonia* 38, 100-35.
13. Jung T, La Spada F, Pane A, et al., 2019. Diversity and Distribution of Phytophthora Species in Protected Natural Areas in Sicily. *Forests* 10, 259.
14. Jung T, Stukely M, Hardy GSJ, et al., 2011. Multiple new Phytophthora species from ITS Clade 6 associated with natural ecosystems in Australia: evolutionary and ecological implications. Persoonia: *Molecular Phylogeny and Evolution of Fungi* 26, 13.
15. Marano A, Jesus A, De Souza J, et al., 2016. Ecological roles of saprotrophic Peronosporales (Oomycetes, Straminipila) in natural environments. *Fungal Ecology* 19, 77-88.
16. Meyerson LA, Mooney HA, 2007. Invasive alien species in an era of globalization. *Frontiers in Ecology and the Environment* 5, 199-208.
17. Milenković I, Keča N, Karadžić D, et al., 2018. Isolation and Pathogenicity of Phytophthora Species from Poplar Plantations in Serbia. *Forests* 9, 330.
18. Nagel JH, Gryzenhout M, Slippers B, et al., 2013. Characterization of Phytophthora hybrids from ITS clade 6 associated with riparian ecosystems in South Africa and Australia. *Fungal Biology* 117, 329-47.
19. Nechwatal J, Bakonyi J, Cacciola S, et al., 2013. The morphology, behaviour and molecular phylogeny of Phytophthora taxon Salixsoil and its redesignation as Phytophthora lacustris sp. nov. *Plant Pathology* 62, 355-69.
20. Nechwatal J, Mendgen K, 2006. Pythium litorale sp. nov., a new species from the littoral of Lake Constance, Germany. *FEMS microbiology letters* 255, 96-101.
21. Nechwatal J, Oßwald WF, 2003. Pythium montanum sp. nov., a new species from a spruce stand in the Bavarian Alps. *Mycological Progress* 2, 73-80.
22. Nechwatal J, Wielgoss A, Mendgen K, 2008. Diversity, host, and habitat specificity of oomycete communities in declining reed stands (Phragmites australis) of a large freshwater lake. *Mycological research* 112, 689-96.
23. Orlikowski LB, Ptaszek M, Trzewik A, Orlikowska T, 2011. Usefulness of plant leaf baits for detection of Phytophthora spp. from water. *Sylwan* 155, 493-9.
24. Themann K, Werres S, Diener H-A, Lüttmann R, 2002. Comparison of different methods to detect Phytophthora spp. in recycling water from nurseries. *Journal of Plant Pathology,* 41-51.
25. Thines M, 2014. Phylogeny and evolution of plant pathogenic oomycetes—a global overview. *European Journal of Plant Pathology* 138, 431-47.
26. Türkölmez Ş, Derviş S, Çiftçi O, Ulubaş Serçe Ç, 2016. First Report of Phytophthora chlamydospora Causing Root and Crown Rot on Almond (Prunus dulcis) Trees in Turkey. *Plant Disease* 100, 1796-1796.
27. Walker CA, Van West P, 2007. Zoospore development in the oomycetes. *Fungal Biology Reviews* 21, 10-8.
28. Yoshida K, Schuenemann VJ, Cano LM, et al., 2013. The rise and fall of the Phytophthora infestans lineage that triggered the Irish potato famine. *Elife* 2, e00731.