REVIEW

FOR ASSESSMENT OF THE DOCTORAL THESIS "DISTRIBUTION AND DIVERSITY OF PATHOGENIC SPECIES OF THE CENUS *PHYTOPHTHORA* IN THE REPUBLIC OF MACEDONIA" BY MIHAJLO RISTESKI, SUBMITTED AT THE FACULTY OF FORESTRY IN SKOPJE

During the assembly of the educational and scientific council of the Faculty of forestry in Skopje, held on 25.12.2017, according to the Act numbered 02-896/3, an examining body was constituted for correction and examination of the submitted PhD thesis **"Distribution and diversity of pathogenic species of the genus *Phytophthora* in the Republic of Macedonia"**, written by MSc Mihajlo Risteski, composed by prof. Kiril Sotirovski, PhD, full professor at UKIM - Faculty of forestry Skopje; prof. Irena Papazova - Anakieva, PhD, full professor at UKIM - Faculty of forestry Skopje; prof. Nikola Nikolov, PhD, full professor at UKIM - Faculty of forestry Skopje; prof. Rade Rusevski, PhD, full professor at UKIM - Faculty for agricultural sciences and food - Skopje; and prof. Biljana Kuzmanovska, PhD, associate professor at UKIM - Faculty for agricultural sciences and food - Skopje. The examining body has reviewed the submitted PhD thesis, and submits to the educational and scientific council the following

R E P O R T

The PhD thesis "**Distribution and diversity of pathogenic species of the genus *Phytophthora* in the Republic of Macedonia**" written and submitted by MSc Mihajlo Risteski is an integral text which fully meets the requirements and the criteria for an original scientific work.

The PhD thesis is presented on 143 pages with the usual spacing and size of letters. The thesis includes 17 tables, 3 charts, 3 dendrograms, 3 maps and 46 individual and group figures.

The PhD thesis is presented in 5 basic chapters as follows: 1. Introduction, 2. Materials and methods of work, 3. Results and discussion, 4. Conclusions and 5. Literature cited (references), and all chapters are further developed in appropriate subchapters.

Individual description of the chapters:

1. INTRODUCTION

In this chapter, the candidate represents the significance and the importance of species belonging to the genus *Phytophthora*. The morphological and molecular characteristics of *Phytophthora* species are presented, and historical facts are also reviewed, related to the most known and most dangerous species of this taxon of plant pathogenic organisms. Using relevant literature, the candidate MSc Mihajlo Risteski describes the taxonomy, and consequently, the systematics of this species, which have arisen since their first discovery until the present. The candidate describes the essential similarities and differences between the class Oomycetes (taxon of which the investigated species are representatives) and representatives of the kingdom Fungi, the true fungi, respectively. Regarding life cycles of *Phytophthora* species, the candidate explains in detail the sexual and asexual phases of reproduction, with particular emphasis on the fertile structures produced by these organisms.

In continuation, quoting relevant authors, the candidate Risteski presents the development of the systematics of the genus *Phytophthora* from the moment of separation from the kingdom Fungi, up to current systematics. The candidate presents the changes in the systematics made over time to the rank of the lower systematic categories, as a result of using morphological phylogenetic

research, and recently these changes are pointed out due to molecular (genetic) methods of analysis. This review concludes with presenting the current systematics, according to the database on http:/www.speciesfungorum.org/. Furthermore, the candidate chronologically lists the main historical features of species of the genus *Phytophthora* from the moment of the first description of *Phytophthora* species to the "explosion" in the number of the newly discovered and/or newly described species in the last decade. MSc Risteski emphasizes the reasons for the recent dramatical increase in number of described *Phytophthora* species, and correctly concludes that further increase of newly described species is most probable. The candidate referred appropriately the recent research of the *Phytophthora* species on the territory of the Rep. of Macedonia, and he remarks that the research has been poor, both in quantity and in quality. A brief general conclusion is that there is lack of research and relevant data for the genus *Phytophthora* for the Rep. of Macedonia, both for agricultural crops and species important for forestry. Due to the proven devastating potential of species belonging to the genus *Phytophthora*, difficulties in locating or dealing with potentially present *Phytophthora* species, as well as difficulties in the determination of these pathogens, the candidate has set clear goals for his research:

- Observation of the health condition of forests, forest plantations and nurseries, as well as agricultural (fruit) plantations in respect to these organisms on the territory of the Republic of Macedonia;

- Collecting soil samples and plant material with symptoms specific for *Phytophthora* species;

- Isolation of the species belonging to the genus *Phytophthora* in pure cultures;

- Identification of cultures by means of morphological and molecular methods;

- Compiling a collection of live cultures of the genus *Phytophthora*, and their proper storage and maintenance;

- Pathogenicity assessment of the determined *Phytophthora* cultures/isolates;

- Map of distribution and diversity of *Phytophthora* species on the territory of the Republic of Macedonia;

- Proposition of preventive measures, as well as control measures for the organisms belonging to the genus *Phytophthora*.

2. MATERIALS AND METHODS USED

In the first subchapter of this chapter, the candidate presents the method by which sites have been selected for field research, both in forest and agricultural ecosystems, the methodology of collecting samples, its transportation and storage, prior to, and after isolation. For the process of culture isolation, both from soil samples and from necrotic tissue and roots, the candidate uses and describes in detail 4 methods, as follows: method with setting inocula of collected soil samples on fruits of apples or pears; method with using apples or pears as baits; method with direct isolation from soil samples and plant materials on selective nutritious media (PARPNH or CARP+); and method using leafs of different plant species as bait for zoospores. For morphological analyses of cultures, the candidate used Erwin & Ribeiro's (1996) determination key, which is among the most referred for identification of *Phytophthora* species.

In the subchapter for molecular analysis, MSc Risteski describes in detail and uses 3 methods for DNA isolation, as follows: method using SEVAG; method using Guanidine thiocyanate; and method according to the protocol of PureLinkTM Plant, Total DNA Purification

Kit (kit for isolation of DNA). Also described in details is the method and the molecular markers used for the polymerase chain reaction of ribosomal DNA, as well as preparation of DNA samples for sequencing, which was performed at an international laboratory per order. For the phylogenetic analysis, the candidate uses 2 types of software for editing and alignment of DNA sequences and an online software for composition of a phylogenetic tree.

Representative isolates of the detected *Phytophthora* species have been tested for pathogenicity according to the well-known and widely used method by Jung & Nechwatal (2008). For the tests, dormant sweet chestnut (*Castanea sativa*) sticks were used, collected in May from one-year-old shoots. The tests were carried out on sticks in two categories of thickness, i.e. diameters of 5-10 mm and of 10-15 mm. These two types of sticks were inoculated with 20 replicates (sticks) for each of the tested isolates. The pathogenicity tests were carried out for 7 isolates, i.e. for 1 representative isolate per the 7 *Phytophthora* species for which live culture were available (presented in the chapter Results).

For all above mentioned activities (isolation of pure cultures, morphological and molecular identification of the cultures, DNA isolation, polymerase chain reaction and pathogenicity tests), the PhD candidate uses a wide range of the methods, both classical or contemporary. The dedication of the candidate must be emphasized, as the fact that parts of his research have been realized during several scientific visits in foreign laboratories, in a period of several years. The methods used are described in detail and successfully implemented, which guarantees repetition of the results.

3. RESULTS AND DISCUSSION

The PhD candidate presented the obtained results very precisely and clearly, using coherent language. As with the previous one, this chapter is developed in several subchapters.

The first subchapter lists the data of the number of sites observed in agricultural and forest ecosystems. The number of symptomatic and asymptomatic samples, as well as the samples which resulted with isolates are indicated. The PhD candidate states that although the total number of collected samples from forest ecosystems is drastically higher than the total number of samples collected from agricultural ecosystems, the percentage of the symptomatic samples is of the same trend, which implies to properly performed observations and collection of samples. Regarding isolation in pure cultures, the candidate indicates and therefore uses the most the leaf-bait method on flooded soil samples and root fragments from symptomatic plants. In brief, for morphological and molecular analysis, the PhD candidate references 49 detected isolates belonging to 10 *Phytophthora* species. The following *Phytophthora* species have been detected:

1. P. cinnamomi, from a soil sample collected from micro-depression with plants of the species *Thuja ivone* (Northern white cedar) from a nursery in Tetovo;

2. P. colocasiae, from soil samples collected from pots with *Berberis thunbergii Golden rocket* (Japanese barberry) from a nursery in Tetovo;

3. P. megasperma, from soil samples collected from apple orchard (*Malus domestica*) from the Resen region;

4. P. rosacearum, from soil samples collected from a private apple orchard (*M. domestica*), v. Pretor;

5. P. cactorum, from soil samples collected from a sweet chestnut plantation (*C. sativa*), v. Skudrinje, Debar, and from apple (*M. domestica*), v. Mislesevo, Struga;

6. P. plurivora, from soil samples collected from *Salix alba* (poplar) near the v. Bunec and from *Chamaecyparis stricta* (chamaecyparis) from a nursery in Tetovo;

7. P. gonapodyides, from a soil sample collected from wild boar mud in mixed forest of fir (*Abies alba*) and beech (*Fagus moesiaca*) in the NP "Mavrovo";

8. P. inundata, from soil sample from *Trachicarpus forunei* (fan palm), from a nursery in Ohrid;

9. P. cambivora, from a soil sample of sweet chestnut (*C. sativa*) from v. Skudrinje;

10. P. taxon Walnut, from micro depression with thuja (*Thuja ivone*), nursery in Tetovo.

For each of the detected species, the PhD candidate documented and described in detail the morphology and the growth rate of the cultures in conditions *in vitro*, fertile structures from sexual and vegetative origin, typical surviving spores (for cultures producing such spores), as well as typical hyphal ornaments.

From the aspect of phylogenetic analysis, using the ClastalW Multiple alignment tool in the framework of BioEdit Sequence alignment editor software, the PhD candidate aligned the sequences to note the degree of variability between the DNA sequences within the ITS1 and ITS2 regions, as well as 5.8S gene. In addition, the variability between the sequences is attributed to the differences between each of the *Phytophthora* species. On contrary, lowest variation is registered at the 5.8S gene region. This variation and evolutionary distance or difference is presented in a graph and a table, and for the purpose of better visibility, the produced phylogenetic tree is presented in three different ways in order to underline the differences between sequences belonging to different species as well as the differences between the sequences belonging to same species.

One of the subchapters provides a detailed description of the morphological structures for each of the detected *Phytophthora* species, illustrated with original individual and group figures. The sites where the isolated cultures originate from, are presented on a map of the Republic of Macedonia for a better overview of the distribution.

The PhD candidate examined the results of the pathogenicity test by two-way ANOVA, where the two dimensions for diameter of the used sweet chestnut sticks were taken as factors, while the representatives for the *Phytophthora* species for which the pathogenicity was tested were taken as parameters for the two-way ANOVA tool. The results pointed out *P. cactorum* as most pathogenic and *P. inundata* as least pathogenic, of all *Phytophthora* species tested on sweet chestnut sticks.

At the end of this subchapter the PhD candidate suggests draft measures which are important for future protection in relation of detected *Phytophthora* species, as well as for other *Phytophthora* species. We mention the following: the necessity of permanent similar research in order to detect and identify new potentially pathogenic *Phytophthora* species in Macedonia; more frequent and rigorous phytosanitary inspections of the inspection services especially related to imports of plant materials; and implementation of pathogenicity tests for *Phytophthora* species on economically important plant species (forest and agricultural) of Macedonia.

4. CONCLUSIONS

In this chapter the PhD candidate clearly and precisely provides sustained conclusions, relevant for each of the outlined chapters. We emphasize the most important ones among them:

- The total number of observed sites in forest ecosystems is 36, of which 21 are natural or artificially raised plantations and 15 are nurseries, covering 41 host-plant species.

- A collection of 205 soil samples, roots and plant tissue, was made from forest ecosystems, of which 162 (79%) from symptomatic host plants, and 43 (21%) from host plants without manifestation of any symptoms of disease.

- The total number of observed plantations in agricultural ecosystems is 17, covering 3 host plant species, with numerous varieties within species.

- A total of 93 soil samples, roots and plant tissue were collected, out of which 74 (80%) samples were collected from symptomatic host plants, and 19 (20%) samples from host plants with no symptoms of disease manifested.

- The total number of isolates subjected to morphological analysis was close to 400, while 10 groups of isolates with similar characteristics were excluded.

- DNA was extracted and subjected to molecular analysis from 240 cultures. In addition, 49 sequences were detected, belonging to 10 *Phytophthora* species: *P. cactorum* (18 isolates), *P. plurivora* (12 isolates), *P. rosacearum* (1 isolate), *P. megasperma* (2 isolates), *P. colocasiae* (7 isolates), *P. taxon Walnut* (4 isolates), *P. cinnamomi* (1 isolate), *P. cambivora* (1 isolate), *P. gonapodyides* (1 isolate) and *P. inundata* (2 isolates). Additionally, nearly 30 isolates were detected belonging to several *Pythium* species, such as: *P. vexans, P. litorale, P. nigrum*, but also *Mortiela* spp. and *Phytopythium* spp.

- Detected isolates belonging to a particular *Phytophthora* species, originate from 18 collected soil samples with symptoms on the host plant, and 1 single soil sample from asymptomatic origin.

- This is the first report for all detected *Phytophthora* species for the Republic of Macedonia.

- Pathogenicity tests were implemented for isolates of 7 *Phytophthora* species (of the 10 detected), on sweet chestnut sticks. Pathogenicity tests pointed out *P. cactorum* as the most pathogenic towards sweet chestnut sticks, while the species *P. inundata* as least pathogenic towards sweet chestnut sticks, regardless of the diameter of the sticks.

5. LITERATURE CITED

In this chapter 115 references from authors relevant for this topic are listed, and for its citation and processing the PhD candidate used EndNote X7 software and available references from foreign and native authors.

CONCLUSION AND PROPOSAL

The PhD thesis entitled “Distribution and diversity of pathogenic species *Phytophthora* in the Republic of Macedonia” prepared by MSc Mihajlo Risteski, represents an original and independent scientific paper containing all elements of a PhD thesis. During its preparation all criteria for quality scientific paper are respected. The PhD candidate MSc Risteski decided to dedicate himself to these phytopathogenic species which are among most complicated for research for many reasons, on various sites throughout Macedonia and on a large number of plant species. The candidate successfully uses several methods - from classical to the most modern molecular techniques, and the results have not only practical value, but are also of fundamental value. Apart from being of interest for local science and practice, the determined *Phytophthora* species and

their characteristics are subject to a wider academic interest, due to their specificity, which further emphasizes the value of the thesis and the competence of the candidate. The literature and methodology used are relevant for the respected research aspects and the candidate MSc Risteski shows that he has wide and thorough knowledge of his research area.

The Examination body assesses positively the PhD thesis and is honored to propose it to educational and scientific council of the Faculty of forestry in Skopje to accept this thesis and to invite the candidate for a public defense.

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