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Review of Doctoral Dissertation by

Lakshmipriya Perincherry MSc.

”The role of *Fusarium* mycotoxins and lytic enzymes in fusariosis of pea (*Pisum sativum* L.)”, carried out in the Department of Plant-Pathogen Interaction, Institute of Plant Genetics, Polish Academy of Sciences under the supervision of Prof. dr. hab. Łukasz Stępień

The research presented in the reviewed doctoral dissertation was carried out in a research center known for significant achievements in investigations on plant pathogens and their genetics. These studies are related to the research topic of the Department of Plant-Pathogen Interaction and meet the current needs, as they try to solve a very important problem of the influence of plants on the course of infection caused by phytopathogenic fungi through the impact on the expression of phytopathogen genes responsible for the synthesis of secondary metabolites of fungi, which are crucial for pathogenesis: toxins and lytic enzymes degrading the cell wall. Extensive, in-depth knowledge of the fungus-plant interaction gives the opportunity not only to understand the nature of plant resistance to fungal diseases and the functioning of agricultural ecosystems but also to create modern strategies for protecting plants against phytopathogens.

The research objects, i.e. the plant-pathogen interaction partners, were very well selected. A very important crop, i.e. peas (*Pisum sativum*), was selected for the research as well as *Fusarium* spp., which are particularly harmful to both plants and animals. Numerous plant diseases caused by these fungi, collectively known as fusarioses, are a very serious phytopathological and economic problem in the cultivation of most plants, including representatives of the Fabaceae family. However, literature data on legume diseases caused by hemibiotrophic *Fusarium* spp. are relatively sparse, especially when compared to the number of publications presenting studies on cereal fusariosis.

The reviewed doctoral dissertation presents breakthrough results of research on the regulation of the expression of genes responsible for the synthesis of secondary metabolites of phytopathogens by plants resistant to infection with this phytopathogen. These results were obtained thanks to the excellent and, at the same time, very simple idea of comparing the effect of plant extracts from two plants that differ in their susceptibility to infection by *Fusarium* spp. For this purpose, two varieties of peas were selected: susceptible (Santana) and resistant (Sokolik) to infection by fungi of the genus *Fusarium*.

The second partner of the pathogen-plant interaction was also very accurately selected. In preliminary tests, four strains distinguished in terms of the ability to synthesize toxins were found. These strains represent two species of *Fusarium* spp. Two strains (PEA1 and PEA2) representing the *F. proliferetum* species showed high efficiency in the production of the polyketide fumonisin toxin FB1 (and much weaker fumonisins FB2 and FB3), while the other two strains (34OX and 1757OX) were *F. oxysporum* producing a toxin from the group of non-ribosomal peptides – the cyclodepsipeptide beauvercin.

The thematic consistency of the research presented in the dissertation with the subject addressed by the entire team of co-authors of the publications and the continuation of the previous research conducted by the team based on a very good workshop, equipment facilities, and thoroughly tested methodology are highly valuable.

The selected strains of *Fusarium* spp. caused fusarium wilt in both pea cultivars, but the effect on growth limitation and plant biomass was significantly lower in the resistant cultivar than in the susceptible one. Differences in the intensity of the pathogenic interaction between the *Fusarium* species were also shown - the *F. oxysporum* species turned out to be more pathogenic than *F. proliferetum*.

It was shown that aqueous extracts from pea leaves from phytotron cultivation in sterile soil influenced the expression of secondary metabolites of the *Fusarium* spp. strains, i.e. both toxins (beauvericin and fumonisin) and hydrolytic enzymes from the group of Cell Wall Degrading Enzymes (CDWE). Additionally, the activity of as many as seven enzymes (polygalacturonase, pectate lyase, xylanase, cellulase, chitinase, lipase, and protease was tested *in vitro* and *in vivo*) .

Pea extracts introduced into the culture medium as an additive in the form of 10 ml of extract per 250 ml of Ryes and Byrde medium were found to increase the activity of three of the seven enzymes studied in *in vitro* cultures: β-glucosidase, pectate lyase, and xylanase. The *in vitro* effect of the extracts in liquid cultures was compared not only with the control but also with cultures supplemented with other additives, i.e. glucose, citrus pectin, and oat bran introduced individually at a concentration of 0.05%. In contrast, the *in vivo* studies of enzymatic activity in soil and roots of plants infected with the tested strains showed that the activity of enzymes in plant tissues is higher than in the liquid after supplementation on a substrate with the extract and depends both on the strain and on the susceptibility of the pea variety to infection. In 14-day liquid cultures with the addition of the extract from the resistant Sokolik variety, a trend towards a significant reduction in the dry matter of mycelium was observed. According to Fig. 1 published in publication No. 2, a statistically significant approx. 4-fold decrease in the biomass was observed in the *F. proliferatum* PEA1 , and the approx. 2-fold decrease in the biomass of the *F. oxysporum* 34OX strain seems statistically insignificant.

The LC-MS metabolomic analysis allowed comparative analysis of metabolites present in the resistant and susceptible pea cultivars presented on a heat map. It clearly indicated the difference between the resistant and susceptible varieties. Based on the assignment of the mass to the results available in the databases connected to the program, it can be hypothesized that the differentiating metabolites in the Sokolik cultivar are mostly flavonoids or products from the phenylpropanoid pathway involved in the systemic acquired resistance of plants against pathogens. Determination of this profile allowed selection of compounds to be studied separately to identify their impact on the growth of the *Fusarium* strains and the synthesis of toxins.

It was shown that the leaf extracts of the resistant Sokolik cultivar contained higher concentrations and greater diversity of secondary metabolites from the group of flavonoids and phenolic compounds. The extracts from both the susceptible and resistant cultivars changed the level of produced toxins and limited the growth of *Fusarium* spp. mycelium in liquid cultures. In the plant extracts, compounds that may be responsible for resistance to fusariosis were selected, emphasizing the importance of flavonoids and phenolic compounds, and even the effectiveness of the compounds inhibiting the production of fumonisin was determined: coumaric acid> chlorogenic acid> spermidine> coumarin. It was found that these compounds present in the extracts limited the synthesis of beauvercin by the four tested strains.

It was very valuable to demonstrate that these extracts can act both directly on the growth of *Fusarium* spp. and indirectly on their pathogenic potential by inhibiting the expression of mycotoxin genes. It was extremely valuable to show that infection with *Fusarium* spp. affects the accumulation of toxins in a completely different way in the susceptible variety than in the resistant cultivar. Namely, in the resistant cultivar Sokolik, no toxins were found to be present after the infection (fumonisin and beauvercin or perhaps others), or their concentration was found to be much lower than in the susceptible Santana cultivar.

Assessment of the layout of the doctoral dissertation and the formal page:

Lakshmipriya Perincherra's doctoral dissertation was prepared as a series of four publications: one review and three original scientific publications published in 2019 (one publication), 2020 (one publication), and 2021 (two publications).

It should be emphasized that all the works that make up the series have been published in reputable and, importantly, in various journals from the *Journal Citation Reports* list with very high impact factors and high scores from the Ministry of Science and Higher Education: *Toxins* (IF2020 = 4.546, MNiSW = 100), *Pathogens* (IF2020 = 3.492, MNiSW = 100), *International Journal of Molecular Sciences* (IF2020 = 5.923, MNiSW = 140), and *Journal of Fungi* (IF2020 = 5.816, MNiSW = 20). The total IF value for these studies was 19,777 (average IF = 4,944) and 360 points of the Ministry of Science and Higher Education were scored (average 90). Bibliometric data was provided by the PhD student before individual publications and declarations of co-authors informing about their participation in these publications, and it would be good to include them in the list of publications along with a summary of the total Impact Factor values and scores from the Ministry of Science and Education list. The declarations of the PhD student and co-authors indicate that the PhD student was very committed to the creation of all the papers from the series, but it is a pity that the percentage contribution of the PhD student was not estimated.

In all the publications that make up the cycle, which is the basis of the dissertation, the PhD student is the first author. She is also a corresponding author in the three experimental papers. Lakshmipriya Perincherry planned the experiments, developed, analyzed, and discussed the obtained results, performed the infection tests, and determined enzymatic activity. Together with the co-authors, she studied the expression of the genes of *Fusarium* spp. strains and interpreted the chromatographic results, prepared the manuscripts for publication, and provided answers to the reviews, which proves her great commitment and independence.

The composition of the publications presented in the dissertation indicates the ability of the PhD student to work in a team as well as practical skills and knowledge of a variety of advanced research techniques. The high quality of the publications indicates her very vast knowledge and interest in the subject, as well as experience and high skills of the team of the authors in writing this type of work. It is clearly visible that the experiments were very well planned and reliably performed.

The publication of the review as the first paper provided the PhD student with a very good introduction to planning and carrying out experiments as well as writing subsequent publications. The very high quality of the review publication broadly describing the factors of plant-pathogen interaction proves that the PhD student has mastered the knowledge of the determinants of the plant-pathogen interaction and can properly formulate a research hypothesis and plan the research. The publication is not limited to the role of mycotoxins produced by *Fusarium* spp. It also refers to other secondary metabolites and primarily takes into account the lifestyle of fungal pathogens, metabolic pathways, and cytogenetic changes induced by mycotoxins. On the other hand, it discusses in detail plant metabolites influencing the biosynthesis of toxins produced by fungi from the genus *Fusarium*. The PhD student gives a thorough, comprehensive overview of the pathogenicity effectors in these fungi, signal transduction pathways, as well as host defense reactions. The author also emphasizes the great importance of using new molecular methods of next generation RNA-Seq technologies for detecting or verifying the expression of secondary metabolites, i.e. the possibility of unquestionable understanding of the influence of plants on the metabolism of a pathogenic fungus.

The publications that make up the series are very well and carefully prepared. They contain transparent graphic documentation, which was properly selected to present a specific type of results, including photographic documentation showing the effect of infection, as well as extensive supplementary material.

The short description of the results contained in the publications and the whole dissertation are of high quality. The Author presented all the assumptions in a very synthetic form, highlighted the goals and course of the research, and emphasized the importance of the obtained results, pointing to the contribution of the results to biological, phytopathological, and agricultural knowledge.

The documentation contained in the publications constituting the basis of the dissertation has rich graphic material: 5 tables and 25 drawings, including macroscopic photos (Publications: No 1 - 7 figs, including a very interesting diagram summarizing the pathogenicity effectors in *Fusarium* spp., No 2 - 3 figs , 3 tab; No 3 - 10 figs, including a macro photo comparing the effect of infection with individual strains of two pea cultivars, 1 tab; No 4 - 5 figs, including a macro photo and a heat map of metabolites, 1 tab). In addition, the PhD student introduced as many as 9 tables in some of the supplementary materials, in which she published the results of the impact of such compounds as isoorientin, chlorogenic acid, apiin, quercetin, coumarin, spermidine, and p-coumaric acid on the growth of the tested *Fusarium* strains and the synthesis of fumonisin and beauvericin toxins . This material showed very little or no effect of these compounds at doses of 1, 10, and 100 ng/ml on the growth of strains, but a very strong inhibitory effect on the synthesis of these two toxins. It was also noted (Table S8) that in the post-culture fluids of the *F. oxysporum* 34OX strain with the addition of apiin, isoorientin, and spermidine, which did not produce the above-mentioned toxins in the control culture, the presence of fumonisin was found.

The entire graphic material was carefully prepared. Only the legibility and size-related reservations are raised by graph 2 from the second publication, showing the dynamics of changes in enzymatic activity in cultures with various additives, which the PhD student improved in the additional material of the dissertation (Figures S1-S3) for activity the activity of endo β-1,4-glucanase, exo β-1,4 glucanase (Avicelase), and chitinase. The results were subjected to statistical analysis described in the subchapters Materials and methods. Standard deviations were marked on the graphs, and the significance of differences between the results determined in the One-Way ANOVA analysis of variance was marked with asterisks.

In the dissertation, the PhD student cited 42 scientific papers. In turn, the review of knowledge presented in the publications was based on 304 valuable well-selected literature items. In the subsequent experimental studies, the PhD student referred to 168, 49, 57, and 30 publications, respectively.

The layout of Ms Lakshmipriya Perincherry's doctoral dissertation complies with the standards adopted for this type of study, and it is worth emphasizing that it is synthetic, systematic, and transparent, which results from the properly adopted concept of the dissertation.

The doctoral dissertation contains, in addition to biometric data and authors' declarations, eight chapters: (I and II) summary in English and Polish; (III) introduction; (IV) research hypothesis and objectives; (V) materials and methods; (VI) the most important results and discussion divided into 3 very well-separated parts; (VII) summary and conclusions; (VIII) references.

The Doctoral student formulated the hypotheses in 5 points, which she verified in order to achieve three basic research goals concurrently answering two basic questions: (1) can plant extracts alter the growth and metabolism of *Fusarium* species? (2) can plant extracts alter the production of host cell wall degrading enzymes?

Achievement of the results described in the dissertation was possible thanks to the great idea and selection of the right research objects as well as matching them to the workshop, equipment, and research methods, which yielded spectacular results.

Striving to achieve these goals, the PhD student used a number of modern research tools and methods, which were very well selected and adapted to the needs of her research.

In the dissertation, the Doctoral student not only clearly presented the obtained results but also interpreted them very carefully and compared them with the results of other researchers. The Author synthetically summarized the research, emphasizing the greatest achievements of the reviewed work and formulating four conclusions.

To sum up, the reviewed doctoral dissertation is a comprehensive study.

Its undoubted achievement is the demonstration of ability of the resistant plant extract to inhibit the synthesis of toxins as well as flavonoid and phenolic compounds of key importance for the pathogenesis of *Fusarium* spp. Particular emphasis in the doctoral dissertation prepared by Lakshmipriya Perincherry MSc. should be placed on the innovative idea of comparing the impact of extracts from plants susceptible and resistant to infection with phytopathogens, the modern and diverse methods adequate to the assumed goal, and, the high quality of publications presenting the obtained results.

The results described in the peer-reviewed doctoral dissertation are a significant contribution to the knowledge of the fungal pathogen-plant interaction and plant protection technology. Therefore, they are extremely important from the theoretical and application point of view, as they open up practical prospects for the use of specific plant metabolites in the regulation of the synthesis of fungal metabolites responsible for the pathogenic impact of fungi.

The doctoral dissertation presented by Lakshmipriya Perincherry MSc gives an original solution to a very important research problem. The results described in the doctoral dissertation, in accordance with the hypothesis, confirmed the role of metabolites contained in the extract of plant and made us aware of the advisability of testing the impact of other plant compounds on the expression of genes of a number of fungal metabolites associated with pathogenesis.

The very well-chosen experimental models (peas as a host and toxin-forming *Fusarium* phytopathogens) and the experimental techniques that allowed achieving the assumed goal deserve special mention. The high quality of the published papers that are the basis for the doctoral dissertation indicates that the Doctoral Student has perfectly mastered microbiological, molecular, and biochemical techniques and, by using them, she fully completed the tasks that were set in the hypothesis and goals of the work.

The reviewed work was written in a way that indicates extensive theoretical and practical knowledge, an attempt to solve basic and application problems, the ability to interpret and present the results in a synthetic way, and insightful, creative discussion.

When reading Lakshmipriya Perincherry's doctoral dissertation, some minor remarks arise:

In the text of the dissertation, it would be good to clearly state the origin of pea cultivars, as well as to often indicate whether an aqueous plant extract or an extract in another solvent (e.g. alcohol) was used.

Although the graphs and tables show standard deviations from the presented mean values of the results or the significance of the differences marked with asterisks, the author should explain the meaning of the asterisks in the notes to the graphs. In addition, the titles of the graphic material presenting the results should contain references to the statistical analysis to which the results presented in tables or graphs were subjected.

In the titles of the graphs, there should also be a reference to the Anova analysis of variance. With a large number of presented results, e.g. enzymatic activities, it would be useful to use the Principal Component Analysis (PCA) to determine the dependence of the dynamics of the formation of lytic enzymes. In addition, in most of the graphs, it was possible to standardize the scale to facilitate comparison of the results, e.g. between the activity of individual enzymes in the cultures of the two *Fusarium* species or between the roots and soil.

Enzymatic activities should be converted into a unit of volume of post-culture fluids and then into the mass of mycelium, roots, and soil.

In cases where the publications refer to methodological details in other previous works by the co-authors, a better solution would be to describe the methods in detail in the dissertation. For instance, the description of rice culture toxins, important for determining the synthesis of rice culture toxins, should be described clearly in the methodology of the dissertation. It was only referred to the third cited work, although I admit that reading the earlier works of the co-authors was very pleasant.

The presentation of the composition of metabolites contained in the two plant cultivars by means of a heatmap metabolic analysis showed the quantitative differences between the two cultivars very well, but did not allow recognition of the qualitative differences. This is somewhat unsatisfactory due to the lack of information on components that dominated quantitatively in the extracts of the resistant plants and in the extracts of the susceptible plants. There is also no information about whether the extract of both plants was characterized by the presence of components characteristic of only one cultivar.

These comments are of a marginal nature and do not diminish the value of the dissertation in any way.

I would like to ask the PhD student for answers to the questions related to her research and results:

Flavonoids and phenolic compounds found in pea extracts have been intensively tested. To the PhD student's knowledge, could other metabolites contained in the extracts have a similar effect on phytopathogens?

How were the cultivation period, conditions, and the time of plant harvesting after infection selected for the study of the toxin content and enzymatic activity? Why were these two toxins from two separate groups selected? Would the effect of extracts be similar on the synthesis of other toxins?

I ask the PhD student for an opinion: is the resistance of the Sokolik cultivar based on five known types of resistance, or is it based only on the third (III^{rd}) type of resistance to the accumulation of mycotoxins or also on the first two types?

Did the analyzed extracts of the two cultivars of peas come from one crop? Were they always obtained from 20-day-old plants or from plants with different degrees of maturity? Were all leaves taken for the extraction or were they taken from a special areas, e.g. the lower or upper part of the plant? Is a study of pea root extract planned?

The extraction of toxins from post-culture fluids was very aptly preceded by a freeze-drying stage, and this process was also used to estimate the dry matter of the mycelium. Was the mass of the pea extracts also determined in this way and was the mass of an extract equivalent to the other supplemented ingredients: glucose, citrus pectin, and oat bran used in the substrates?

Was the dry matter of this extract equivalent to 0.05% of the addition of the other additives used: glucose, citrus pectin, oat bran? Why were these additives chosen at relatively low concentrations in relation to the 0.1% yeast extract, 0.1% peptone, and 0.05% malt extract. What decided that they were added on the 5th day of culture?

It was very important to determine the profile of metabolites in the two pea cultivars in the extracts obtained from these plants and then to use pure metabolites (reagents) in the research to check their influence on the metabolism of *Fusarium* spp. How much would extracts from other parts of plants differ in their composition? Harvested at a different time or cultivated in non-sterile or stressful conditions?

For stimulation of the synthesis of toxins by *Fusarium* spp. strains, a substrate with the addition of 2% fructose was used. What components or other factors stimulating this synthesis could be used to increase the efficiency of toxin production? What role does temperature, substrate reaction, and osmotic pressure, i.e. factors that create stress conditions conducive to the production of toxins, play in this process at extreme values? Were more production-conducive culture conditions used?

Are both strains of *F. oxysporum* more pathogenic to peas than both strains of *F. proliferatum*? Does beauvercin exert a stronger effect and does it accumulate in pea tissues more intensively than fumonisin? Do other toxins from the group of non-ribosomal peptides act on peas more strongly than polyketide toxins, or is the more intensive colonization of the plant vascular system by *F. oxysporum* in comparison with *F. proliferatum* responsible for the stronger induction of *Fusarium*-caused wilt.

Has the enzymatic activity in the above-ground parts of the plants been tested or has the PhD student knowledge on this subject from the research of other authors?

Can particular species or strain of *Fusarium* spp. and particular pea cultivars be attributed a preference for the production of particularly high activity of a given CDWE?

Have the compounds contained in the pea leaf extracts been presented in the decreasing order of beauvercin synthesis inhibition as in the case of fumonisin?

How can the stimulating effect of apiin, isoorientin, and spermidine on the production of fumonisin by the *F. oxysporum* 34OX strain in liquid cultures, in which the control cultures did not contain this toxin, be explained?

Lakshmipriya Perincherry's doctoral dissertation is a satisfactory study meeting all conditions required by the relevant act for doctoral dissertations.

Final conclusion

In conclusion, I state that the doctoral dissertation presented by Ms Lakshmipriya Perincherry entitled "The role of *Fusarium* mycotoxins and lytic enzymes in fusariosis of pea (*Pisum sativum* L.)" for review is an original solution to a significant scientific problem and meets the requirements of the Act of 14 March 2003 on academic degrees and academic title as well as degrees and titles in art (Journal of Laws of 2016, item 882, as amended) and the Regulation of the Minister of Science and Higher Education of September 26, 2016 on the detailed procedure for carrying out activities in doctoral and postdoctoral dissertations and in the procedure for granting an academic title (Journal of Laws of 2016, item 1586). It was carried out in compliance with the existing rules in the field of agricultural sciences, discipline of agriculture and horticulture specified by Art. 179 paragraph 1 of the Act of July 3, 2018 - provisions introducing the Law on Higher Education and Science (Journal of Laws of August 30, 2018, item 1669).

In connection with the above, I recommend that the High Scientific Discipline Council of the Institute of Plant Genetics- Polish Academy of Sciences admit Ms Lakshmipriya Perincherry to the further stages of the doctoral proceedings.

Recommendation to award the Doctoral Dissertation by Lakshmipriya Perincherry MSc.

"The role of *Fusarium* mycotoxins and lytic enzymes in fusariosis of pea (*Pisum sativum* L.)"

At the same time, taking into account the very high level of the reviewed dissertation, the scientific value of the conducted research, the great contribution of the obtained results to the extension of basic and practical knowledge in the field of agricultural sciences: phytopathology, agriculture and horticulture, and the scientific achievements of the Author, **I recommend that the doctoral dissertation "The role of *Fusarium* mycotoxins and lytic enzymes in fusariosis of pea (*Pisum sativum* L.)" should be awarded appropriately**.

dr hab. Jolanta Jaroszuk-Ściseł, prof. UMCS