REVIEW

on a PhD thesis for obtaining the educational and scientific degree "doctor" in: field of higher education 6. "Agrarian sciences and veterinary medicine", professional field 6.2 Plant protection, scientific specialty "Plant protection (phytopathology)".

Author of the PhD thesis: **Part time PhD** student Roshan Said Shaalan at the Department of "Plant Protection" at the University of Forestry, Sofia

PhD thesis title: "Impact of *Beauveria bassiana* and *Metarhizium anisopliae* on the interactions between *Cucumis sativus* L., cotton aphid *Aphis gossypii* Glover and *Cucumber mosaic virus* (CMV)"

Member of the Scientific Jury: Prof. Dr. Rumen Ignatov Tomov, University of Forestry, professional field 6.2. "Plant protection", scientific specialty "Plant protection", appointed as a member of the scientific jury by order No. ZPS-642/5.12.2022 of the Rector of the University of Forestry.

1. Brief biographical data of the applicant

PhD student Roshan Shaalan graduated from Lebanese University, Faculty of Agronomy, Beirut, Lebanon in 2018, where she obtained the Master's Degree Agronomist-Engineer in Plant Protection

She started his work experience in 2015 after receiving a Biochemistry diploma. After receiving the Agronomist Engineer diploma in 2016, during the period Jun-Sep 2016 he worked as an Agronomist at Hasbaya Agricultural Pharmacy, Hasbaya, Lebanon, then in the period Oct 2016 – 2020 he was a Plant Science Teacher at Sin El Fiel Official School, Beirut, Lebanon. She worked as an Agriculture engineer at US Agriseeds Company, Beirut, Lebanon in 2018.

From 2019, by order of the Rector of University of forestry No. ZSD-38/04.02.2020, she was enrolled in part-time PhD studies at the Department of "Plant Protection" in professional direction 6.2. "Plant protection", scientific specialty "Plant protection (phytopathology)". PhD student Roshan Shaalan completed her individual study plan, as a result of which, by order of the Rector of the University of forestry ZSD-500/07.11.2022 г. she was dismissed with the right of defense.

2. Actuality of the problem.

Cucumber is one of the most important greenhouse crops, infested by pests such as cotton aphid *Aphis gossypii* whose main damage is the transmission of plant viruses such as cucumber mosaic virus. The cucumber mosaic virus has a wide host range and cannot be destroyed by chemical pesticides therefore the development of new plant virus management approaches is required.

In recent years, there has been increasing interest in the development and application of plant protection products based on microorganisms. Entomopathogenic fungi infecting non-

feeding stages such as eggs and pupae have great potential as low-risk plant protection products, but they are sensitive to environmental factors such as UV light, temperatures, and low humidity, which limit their wide application. This is the reason why research on these entomopathogenic fungi has been directed recently toward the possibilities of their application as endophytes, which would overcome the adverse conditions of the external environment, and which would facilitate their wider application in practice.

In recent years, plant metabolomics - technology for crop improvement has been developing rapidly. Metabolomics uses technological advances in analytical chemistry for accurate metabolite profiling in microbes, plants, and animals. At present, a metabolomics study is used to detect the metabolites produced by fungal endophytes of various host plants. In addition, it is used to study plant-endophyte interactions, entomopathogenic fungi-insect interactions, and the effect of fungal endophytes on plant-pest interactions. As a result, it was established that entomopathogenic fungi could be successfully explored as endophytes for the management of many insect pests.

The study is focused on the abilities of entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* to colonize cucumber seeds through artificial seed inoculation and to determine the endophytic activity of these fungi on growth and induction of defense mechanism against the *Cucumber mosaic virus* in cucumber plants.

The study contributes to understanding the endophyte-plant-pest interactions, mechanisms, the process of plant colonization, and the endophytic effects of *B. bassiana* and *M. anisopliae* on *A. gossypii* and *Cucumber mosaic virus* infection.

All this emphasizes the actuality of the present study, which is excellently presented in the introduction and literature review chapters.

3. Degree of knowledge of the state of the problem and creative interpretation of the literature review.

The list of referenced literature sources used for the development of the dissertation contains 416 publications, the main part of which is from the last 20 years. This shows that the study is based on almost all the major recent scientific publications related to the topic of the dissertation.

An extremely detailed review of the literary sources related to the two studied species of fungi was made, and a comparison was made of their capacity as entomopathogens and endophytes. The mechanisms for inducing resistance are presented - pathogenic microorganisms, herbivores, and beneficial microbiological agents.

The research on five species of organisms - two fungi, one plant, one insect, and one virus, is extremely well interpreted and brought together in this highly interdisciplinary work on the complex relationships between them.

The excellent knowledge of the scientific studies related to *B. bassiana* and *M. anisopliae* as endophytes on different crops, allows Roshan Shaalan to draw the conclusions that despite the fact that entomopathogenic fungi *B. bassiana* and *M. anisopliae* are ones of most studied biological control agents still mechanisms underlying the negative effect of endophytic *B. bassiana* and *M. anisopliae* on herbivores and pathogens remain unreachable and very few studies were achieved.

The literature review presents PhD student Roshan Shaalan as well-informed in investigations on the problem and fluent in scientific information interpretation. With the

literature review, she skillfully and thoroughly presents the research carried out on the main topics covered in the dissertation. The in-depth literature analysis successfully highlights the still unsolved problems related to the complex relationships between the complex plant, pest, pathogen, and microbiological agent. In addition, the literature review is illustrated with 9 figures.

All this contributes to making the dissertation a valuable reference source for the importance of microorganisms as plant protection products, as well as a tool for the induction of resistance in plants to crop pests.

4. Purpose, tasks, hypotheses and research methods. Relevance of the chosen research methodology with the set goal and tasks of the thesis.

The main objective of the thesis is to study the entomopathogenic endophytes *B. bassiana* and *M. anisopliae* as a potential alternative to synthetic chemical insecticides against *A. gossypii,* and their possible use to eliminate this enemy and its transmission of *Cucumber mosaic virus*, as well as to investigate the metabolomics of plants infected with the virus and the effects of applied endophytes on the induced defense response against him.

There are 8 tasks in the dissertation and they fully meet the set goal. They are clearly articulated and in an optimal way structurec this very interdisciplinary study, as follows:

1. Artificial inoculation of cucumber seeds with the biological control agents *B. bassiana* and *M. anisopliae* .
2. Study the endophytic ability of both applied entomopathogenic fungi through Re-isolation method and Scanning Electron Microscope (SEM).
3. Study the effect of inoculated entomopathogenic endophytes on the physiological and biochemical parameters of cucumber plants.
4. Study the effect of entomopathogenic endophytes on the population size of A. gossypii infesting cucumber plants.
5. Mechanical inoculation of non-resistant cucumber plants and plants endophytically colonized with entomopathogenic fungi with *Cucumber mosaic virus.*
6. Assessment of virus infection by Double-Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) and PCR test.
7. Preparation of freeze dried cucumber leave samples after methanol extraction.
8. Large-scale untargeted metabolome profiling of the plant samples using High-Performance Chemical Isotope Labeling LC-MS.

The methods used fully correspond to the goals and tasks of the dissertation. The implementation of all tasks is described in detail. Source of fungal inoculum as well as methods used for: molecular identification of *B. bassiana* and *M. anisopliae*, sterilization of cucumber seeds, preparation of fungal inoculum and seed inoculation, and assessment of fungal colonization are presented.

The proposed 3 groups of experiments are described in detail: (1) Growth-experiment: the approaches for determining the effect of entomopathogenic fungi on seed germination, the effect of entomopathogenic fungi on seedling's growth, effects of endophytic entomopathogenic fungi on physiological, and biochemical parameters of cucumber plants; (2) *Aphis gossypii* - experiment: effect of endophytic entomopathogenic

fungi on the population size of *A. gossypii* on cucumber plants; (3) *Cucumber mosaic virus* -experiment: *Cucumber mosaic virus* infection and plant sampling, effects of endophytic entomopathogenic fungi and *Cucumber mosaic virus* on the metabolites produced by *Cucumber mosaic virus* infected cucumber plants.

The statistical analysis of the results is described in detail and is supported by 13 annexes. The methods used are illustrated with 10 figures, which emphasizes the personal involvement of Roshan Shaalan student in the conducted experiments.

The morphological, molecular, and statistical methods used show that the work on the dissertation was carried out at a modern and extremely high technological level.

5. Visualisation and presentation of the obtained results.

Within 3 years, an extremely large-scale survey was carried out on the interaction between the host, fungi, and virus, which is presented in a well-structured form. Molecular identification of microorganisms and aphids is made, aimed at determining the taxonomic position of the studied species.

The colonization of *B. bassiana* and *M. anisopliae* in cucumber plants is documented by a scanning electron microscope. The results of the conducted Growth-Experiment, Aphis gossypii-Experiment, and Cucumber mosaic virus - Experiment are presented in an excellent and comprehensive manner.

Part of the results is presented in 7 tables, which contributes to its clarity and facilitates its use by readers. The text is excellently illustrated with 24 original figures, of which 5 photos and 19 diagrams. In addition, the statistical analysis of the obtained results are presented in 13 annexes.

5. Discussion of results and references used.

All planned tasks have been completed, as a result of which significant scientific results have been obtained in several aspects.

The microorganisms and aphids used have been identified by molecular diagnostics, which is extremely important for the precision of the experiments. The results regarding seed inoculation are significant in theory and practice

The artificial inoculation of cucumber seeds was observed under scanning electron micrographs. The process is well documented and it has been proven that applied entomopathogenic fungi became endophytic.

It is proven that entomopathogenic fungi, not only had endophytic capacity but could also act as plant growth promoters in cucumber. The germination test results showed that inoculating seeds with entomopathogenic fungi and incubating at 25 °C for 2 days would significantly improve the germination rate if compared to non-inoculated seeds.

The dissertation presents evidence that endophytic entomopathogenic fungi have effects on the physiological and biochemical parameters of cucumber plants. The results show that there was a significant difference in the number of flowers, leaves, and height of the plants colonized with tested fungal endophytes compared to uncolonized control plants. In addition, cucumber plants react to endophytic colonization by increased induction of soluble phenols which confirms that endophytes could indirectly influence the defense reaction in the host plant.

In the framework of the *Aphis gossypii*-Experiment it is evidenced that endophytic

application of entomopathogenic fungi to cucumber seeds can enhance plant resistance against A. gossypii. It is observed that the number of aphids in fungal-treated plants, was significantly lower than that in the control plants

The conducted *Cucumber mosaic virus* - Experiment contributes to clarifying the mechanisms involved in defense against cucumber mosaic virus in endophytic B. bassiana and M. anisopliae - treated diseased cucumber plants. The comparisons between non-fungal-treated and fungal-treated cucumber mosaic virus inoculated plants made by metabolomics analysis showed significant differences in metabolite expression. It is proved that endophytes can influence the production of specific metabolites which was evidenced by the fluctuating concentrations found in the study.

The obtained results are analyzed and interpreted within 7 pages. A critical comparison of the obtained results with previous studies by other authors is made. The skillfully selected literary sources and the presented discussion of the obtained results demonstrate the PhD student's in-depth knowledge of the issues discussed in the thesis and her excellent ability to handle scientific information.

This allows Roshan Shaalan to highlight the originality of her results regarding (1) interactions between *B. bassiana, M. anisopliae,* and cucumber plants, and (2) the discovery that the entomopathogenic endophytes can be useful in the management of cucumber mosaic virus disease by enhancing cucumber defense metabolites against the virus infection.

The results of the conducted studies and observations are summarized in 7 well-formulated conclusions.

7. Contributions of the thesis

The dissertation contains an extremely large amount of information and significant original scientific and scientific-applied contributions.

Scientific contributions: The more significant scientific contributions of PhD student Roshan Shaalan's work are the following:

1. It is proven that entomopathogenic fungi *B. bassiana* and *M. anisopliae* act as endophytes in cucumber plants and could also act as plant growth promoters in cucumber.
2. It is proven that the endophytic presence of entomopathogenic fungi in cucumber plant tissues can increase plant resistance against *A. gossypii.*
3. By metabolomics analysis has been shown that endophytes *B. bassiana* and *M. anisopliae* enhance cucumber defense metabolites against *cucumber mosaic virus* infection and could be used in the management of virus disease.
4. Observed changes in diseased cucumber plant metabolic pathways due to fungal endophyte treatment could give future direction for using these endophytes to gain in-depth insights into the defense response to *Cucumber mosaic virus* pathogen.

Scientific-applied contributions:

The results obtained could contribute to the development of new fungi-based Plant protection products for plant viral disease control.

The thesis contains an extremely detailed and well-structured literature review on a relatively new topic, making it a valuable source that can be used by teachers and students.

8. Assessment of the degree of personal involvement of the PhD student in the contributions.

It is indisputable that the conducted experiments and observations, as well as the analysis of the obtained results, are the personal work of PhD student Roshan Shaalan under the guidance of the scientific supervisor and the two scientific consultants.

The leading role of Roshan Shaalan is emphasized by the fact that she is the lead author of the three scientific publications prepared, as well as by the presented separation protocol, which states that her contribution is 70%.

9. Critical remarks and questions.

I have no critical remarks

Questions:

1. Do you have information on to what extent the microbiological plant protection products based on *B. bassiana* and *M. anisopliae* exhibit their endophytic character?

2. Are there microbiological products available on the market that you would recommend for control of *Cucumber mosaic virus*?

10. Published articles and citations.

Part of the obtained results is published in one publication in a refereed scientific journal, Journal of Plant Protection Research, and in one publication in the proceedings of a scientific conference.

One publication has been accepted for publication in the journal Horticulturae, Roshan Shaalan is the first author of all three publications. The scientific works of the PhD student are reflected in the scientific literature with 7 citations.

11. Assessment of publications based on thesis: number, nature of editions in which they are published. Reflections in science - use and citation by other authors.

The scientific publications concern the main results obtained regarding the relationships of the endophytes *B. bassiana* and *M. anisopliae* with *A. gossypii* and *Cucumber mosaic virus.*

Induced growth promotion effect and increase of the resistance of cucumber plants against Aphis gossypii by the colonization by endophytic *B. bassiana* and *M. anisopliae* is published in Journal of Plant Protection Research indexed in SCOPUS SJR 0,32 Q3. The iImpact of *B. bassiana* and *M. anisopliae* on the metabolic interactions between cucumber (CucumissativusL.) and C*ucumber mosaic virus* is accepted for publication in Horticulture,

indexed in Web of Science IF 2,923 Q1.

Participation in the AGROSYM 2018 scientific conference was also realized with a report published in the conference proceedings. Scientific works significantly exceed the minimum requirements of 30 points.

The presented abstract reflects objectively the structure and content of the thesis.

CONCLUSION:

Based on the studied and applied by the PhD student different methods of research, the correctly performed experiments, and the summaries and conclusions made, I believe that the presented dissertation work meets the requirements of the Law for the development of academic staff in the Republic of Bulgaria and the Regulations of the University of Forestry for its application, which gives me reason to evaluate it POSITIVE.

I allow myself to propose to the honorable Scientific Jury also to vote positively and award Roshan Said Shaalan the educational and scientific degree "doctor" in the scientific specialty "Plant protection (Phytopathology)".

Prepared by:

Prof. Rumen Tomov RhD

Review delivered on: 12/12/2022