

## REVIEW

**On dissertation work for awarding an educational and scientific degree "doctor" in: field of higher education 6: "Agrarian sciences and veterinary medicine" in professional field 6.2. "Plant protection", Scientific field "Plant protection" (Phytopathology).**

**Author of the dissertation:** Roshan Said Shaalan, distance doctoral student at the Department of Agronomy at the University of Forestry, Sofia.

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

**Member of scientific jury:** Prof. D.As Ivanka Lubenova Kamenova, professional field 6.2. Plant protection, scientific field Plant protection (Virology), designated as a member of the scientific jury by order ZPC No. 642/5.12.2022 г. from the Rector of Forestry University, Sofia

### **1. Brief introduction of the candidate.**

Roshan Said Shaalan was born on 06/23/1994 in Lebanon. In the period 2012-2015 she studied at the Faculty of Natural Sciences at the Lebanese University in Beirut, where was graduated in professional qualification "Biochemistry", and in the period 2015-2016 she received the professional qualification "agricultural engineer" from the faculty of Agronomy of the same university. In 2016-2018 she received the Master's degree in agricultural engineering in professional field Plant Protection. Since 2019 she has been a distance doctoral student at "Plant Protection" department of the Forestry University, Sofia. She has successively worked as: laboratory assistant - analyst of pollutants in water, soil and food at the American University of Beirut; agronomist at an agricultural pharmacy in Hasbaya; an agricultural engineer at the Lebanese Agricultural Research Institute, and at the American company "Agriseeds", Beirut, Lebanon. Presently she is a lecturer in plant sciences at Sin El Fiel School, Beirut, Lebanon. Roshan Said Shaalan has excellent computer skills and is proficient in Word, Excel and Power Point. She is also fluent in English.

Roshan Saeed Shaalan has successfully taken and passed her doctoral minimum exams according to the individual study plan.

### **2. Relevance of the problem.**

Cucumber is one of the most important, intensively cultivated vegetable crops susceptible to many pests and pathogens causing huge losses of agricultural production. The control of diseases and pests of cultivated plants is mainly based on the use of pesticides, which, however, has undesirable effects on the environment. This necessitates the search of new, alternative ways to

prevent and control plant diseases, therefore the efforts of many researchers in recent years have been focused on so-called "*biological control*" which is based on the use and application of natural and ecological resources.

The dissertation work of PhD student Roshan Shaalan includes studies of two specific, entomopathogenic endophytes in cucumber, following their influence on host development and the impact of both on the population size of *Aphis gossypii*, and on *Cucumber mosaic virus* known as one of the most widespread viral pathogens on this crop.

Following the above, I highly appreciate the relevance of presented by PhD student Roshan Shaalan dissertation work including a complex survey of the relationships among the host (cucumber) - *Aphis gossypii* as a cucumber pest and as a vector of *Cucumber mosaic virus*. This allowed to be traced both the hidden potential of entomopathogenic endophytes in their use as biopesticides against the most common aphid species on this crop, as well as to trace their possible defense mechanism against plant viruses transmitted by aphids.

Also, while some information on *Beauveria bassiana* endophytic activity against *A. gossypii* on various crop plants and viral pathogens is more or less available in the scientific literature, such information on *Metarhizium anisopliae* on this aphid species and CMV infection is completely absent.

The metabolic analyzes included in the study are also of particular relevance due to the fact that it is still not known how the plant endophytes can induce systemic plant resistance to viruses on a metabolic level.

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

Presented literature review is detailed and for its better comprehensibility and clarity, it is structured in subsections reflecting the scientific information for each of studied components, including the application of entomopathogens and their role in the absorption of nutrients and antimicrobial activity. The advantages of the application of endophytes over those of entomopathogenic fungi are clearly outlined. The use of *Beauveria bassiana* and *Metarhizium anisopliae* as a biocontrol approach is detailed with a description of their life cycle and their action as endophytes. A special attention is also given to the other components of the study, as that of the hosts of *A. gossypii* and *Cucumber mosaic virus*, the symptoms and the damages they cause to the attacked plants, the life cycle of *A. gossypii*, the genomic organization and epidemiological characteristics of CMV. The literature review presented in the way it is done reflects the good awareness and theoretical knowledge of the PhD student on the developed topic.

According to me the literature review is targeted and comprehensive and is based on 341 sources, of which 37.5% are from the last 10 years. It contains a well-explained and logical

argument for the main goal and tasks, therefore it is a successful transition to next described sections.

In general, PhD student Roshan Shaalan is well acquainted with the state of the problem and the developed thesis, interpreting and analyzing literature data correctly.

#### **4. Purpose, tasks, hypotheses and research methods. Correspondence of the chosen research methodology with the main goal and tasks of the dissertation work.**

The main aim of the dissertation work is clearly and precisely formulated - to study the possible use of the entomopathogenic endophytes *Beauveria bassiana* and *Metarhizium anisopliae*, as potential alternatives to synthetic, chemical insecticides against *Aphis gossypii* on cucumber for the direct reduction of its harmful effect as a pest and its indirect effect, as a vector of the *Cucumber mosaic virus*. The implementation of the goal is related to 8 specific tasks which are well defined and fully cover the goal achievement.

Section "Materials and Methods" is structured in subsections describing the used materials and methods for each studied the component. They are described precisely, and include the sources of *Beauveria bassiana* and *Metarhizium anisopliae* used, the methods of cultivation, sterilization of cucumber seeds and their inoculation. Both, a scanning microscope and a re-isolation method were used to prove the endophyte presence in the seeds. Methods for determination of the effect of endophytes on seed germination and the growth of the resulting seedlings, physiological and biochemical parameters with analysis of the content of phenol components, chlorophyll and carotenoids are described in detail. Regarding the experiments involving *Aphis gossypii* and its exact determination, both the classical morphological method and molecular PCR diagnostic with species-specific primers were applied. The method for determining the effect of endophytes on the population density of *Aphis gossypii* is described in detail. To prove the infection with *Cucumber mosaic virus*, a serological (DAS ELISA) and a molecular PCR test were applied. The methods and experimental setups are presented in detail and show not only a serious theoretical preparation of the PhD student, but also practical, entomological and phytopathological work skills.

In a conclusion, I think that the materials and methods used are suitable for the implementation of the tasks and allow the receiving of adequate results.

#### **5. Transparency and presentation of the obtained results.**

Presented dissertation work is structured correctly, according to a generally accepted model and meets the requirements for the educational and scientific degree "*doctor*". The total volume of the work is 160 standard, A4 format pages, divided into 7 sections, with the following ratios between them: Introduction (3 1/2 pages = 2.2%), Literature review (33 pages = 20.6%), Aim

and objectives (1 page = 0.6%), Materials and methods (15 pages= 9.4%), Results (33 pages.= 20.6%), Discussion (8 pages = 5.0%), Conclusions (1 page = 0.6%)), Contributions (1 page = 0.6%) and a Literary reference on 38 pages = 23.7% and contains 413 titles.

Illustrative material includes 7 tables and 44 figures that reflect and reliably support the survey data.

## **6. Discussion of results and used literature.**

Section "Results and discussion" covers 25.5% of the volume of presented work, summarizing study results of the three main components, as the impact of the endophytes on cucumber plants, on *Aphis gossypii* population and on cucumber mosaic virus infection. The results of each one of the components are arranged in subsections that follow the methodological setting, the determined features and the methods used. All this contributes to the very good layout and the tight, scholarly style of the dissertation.

The molecular identification of *Beauveria bassiana* and *Metharizum anisopliae* and the species *Aphis gossypii*, which confirms their phenotypically and morphologically determined affiliations, is a very good basis for the subsequent conduction of all experiments. Experiments with both entomopathogens were carried out both in sterile environment and in non-sterile substrates. The action of *Beuvaria bassiana* and *Metharizum anisopliae* as endophytes has been proven both by scanning electron microscope micrographs showing the direct penetration of the conidia of the entomopathogens into the epidermal cells of the treated cucumber seeds, and also by their re-isolation from different cells seed tissues. In both species, the most successful frequency of re-isolation was found from root tissues. At the same time, a difference was observed between *Beuvaria bassiana* and *Metharizum anisopliae*. While in case of *Metharizum anisopliae* re-isolation was carried out from all organs, including leaves and stems, it was absent from treated with *Beuvaria bassiana* plants.

A serious part of the dissertation work is related to studies on the effect of the endophytes *B. bassiana* and *M. anisopliae* on the germination of inoculated with them cucumber seeds, on the growth, development and physiological and biochemical parameters of treated cucumber plants. The results obtained show that the seeds inoculated with *B. bassiana* and *M. anisopliae*, not only did not have reduced germination, but on the contrary within the first 2 days it was increased by almost 26% compared to that of the non-inoculated controls. A greater increase in germination under natural, outdoor conditions was observed in seeds treated with *B. bassiana* (72.5%), while in seeds inoculated with *M. anisopliae*, germination was at 47.5%.

In addition, after a 10-day incubation period, the roots of the developed endophytes containing seedlings grew faster and were longer compared to those of the control plants. A greater number of roots was found in plants colonized with *B. bassiana*, in contrast to plants treated with *M. anisopliae*, where the number of roots was much lower. A positive effect of the action of the two endophytes was also found in relation to the physiological parameters. The

plants colonized with *B. bassiana*, formed twice more flowers and leaves, while plants colonized with *M. anisopliae* were almost 12 cm taller and had a greater number of cucumber fruits compared to the control plants. With the conducted analyses, PhD student proves that the colonization of entomopathogenic endophytes increases the phenol content in the experimental plants and does not change the content of chlorophylls and carotenoids.

Regarding the experiments with *Aphis gossypii*, a negative effect of endophytes on the population size was proven, which was reduced by 35% in plants colonized with *M. anisopliae* and by 32% in those colonized with *B. bassiana*.

The research on the impact of studied endophytes on *Cucumber mosaic virus* infection accompanied by metabolic analyzes is extremely interesting and promising, not only because in recent years metabolomics has established itself as one of the main scientific discoveries applied for accurate profiling of metabolites in microbes, plants and animals, but also due to still scarce scientific information regarding the endophytic impact on plant viral pathogens. Comparative metabolomic analyzes performed on untreated and endophyte-treated CMV-infected plants revealed differential expression of a total of 631 metabolites. The established increased content of basic phenol compounds (m-coumaric acid, p-coumaroyl quinic acid and cis-beta-D-glucosyl-2-hydroxycinnamic acid) and their activation in diseased plants treated with endophytes leads to an increase of cucumber resistance to CMV infection, indicating that most likely they are closely related to the plant defense mechanism. The results of the study in the dissertation reveal the positive effect of endophytes on the induced defense mechanism against CMV in cucumber plants. Additionally, the precise 12 full-color figures and 1 table support the performed metabolomic analyzes and greatly increase the quality of the research and the value of the dissertation.

In a conclusion, I would like to note that the obtained results were successfully analyzed, summarized and interpreted through the scope of the results of other authors worked on the problem. The competent discussion presented in a comparative aspect with the available data from the literature is of fundamental interest and is a good basis for further development in the search for new approaches for biological control of enemies and plant diseases.

## **7. Contributions of the dissertation work.**

As a result of the study, PhD student Roshan Said Shaalan presents 4 original contributions, which I support and approve. **Contributions 1, 2 and 3 have scientific value, and contribution 4 is with possible applied value.**

Studied relationships between *B. bassiana*, *M. anisopliae* and the experimental cucumber plants reflected in contributions 1, 2 and 3 greatly expand the scientific information and knowledge about the ability of entomopathogenic fungi to act as endophytes.

- Endophytic application of entomopathogenic fungi on cucumber seeds improves plant resistance against *A. gossypii*, who causes direct damage of affected plants and indirectly damage as a vector of *Cucumber mosaic virus* one of the most important viral pathogen on plants from family Cucurbitaceous.

- Metabolomic studies of endophytes-treated cucumber plants infected with CMV show an enhancement of the plant's defense mechanisms against the virus and are the basis for future gaining of deeper insight into defense mechanisms against CMV.

In general, the results of the dissertation work are encouraging and give a reason to assume that entomopathogenic endophytes fungi have the potential to be successful agents in the biological control of diseases caused by phytopathogens, and in that aspect the results have a practical contribution.

## **8. Evaluation of the degree of personal participation of the PhD student in the contributions.**

I do not know personally PhD student Roshan Said Shaalan but the presented list of publications and activities (1<sup>st</sup> author in three publications) gives me the reason to believe that the experimental work carried out is her personal merit. Most likely, the analysis, summation and the writing of the dissertation work are also a personal work of the PhD student, carried out without any doubt with the skillful and expert guidance of the scientific supervisor Assoc. Prof. Dr. Slavcho Slavov and the scientific consultants Prof. Dr. Lioudmilla Ibrahim и Asst. Prof. Dr. Walid El kayal.

## **9. Critical Notes and Questions.**

The remarks I made in my preliminary opinion on the abstract of the dissertation have been taken into account by the PhD student, **for which I thanks.**

I have the following **notes:**

1. In the literature review section in the *Cucumber mosaic virus* management section - on page 34 is stated that: "the application of chemical insecticides to control virus vectors can be effective". This is only partially true and is valid only for persistently transmissible viruses, whereas for non-persistent viruses such as CMV, insecticidal control is completely ineffective due to the fact that transmission of the virus takes place within seconds and minutes.

2. In section "Materials and Methods" in the part describing the *Cucumber mosaic virus* experiments, the 2 figures (Figs. 17 and 18) showing the results of proving CMV should be in section "Results".

3. Regarding conducted ELISA test, it should be indicated in how many repetitions the samples were tested, as well as the threshold value for positive and negative samples.

**and one question:**

1. Do you have data on the economic damage caused by *Cucumber mosaic virus* in Lebanon?

## **10. Published articles and citations**

The production related to the dissertation work includes 3 publications, of which 2 are in prestigious international journals with an impact factor (overall IF:3.3) and 1 in a Book of Proceedings from the International Scientific Symposium on Agriculture. In all 3 publications, PhD student Roshan Said Shaalan is the first author.

The PhD student participated with 1 poster and 2 oral presentations in 3 scientific forums, of which 2 were held in Lebanon and 1 in Bosnia and Herzegovin. For one of her presentations she received an award for the best presentation.

Six citations are presented.

The abstract is prepared according to an abbreviated model of the dissertation in a volume of 36 pages. It contains the main sections of the experimental work performed on the dissertation work, adequately reflects its content and complies with the requirements.

### **1. Evaluation of the publications on the dissertation work: number, nature of the editions in which they are printed. Reflections in Science - Use and Citation by Other Authors.**

In 3 out of 5 presented publications, Roshan Said Shaalan is the first author. In the separation protocol for author participation in 2 of these publications out of the maximum possible number of 30 points, PhD student receives 21 points each, equal to a 70% contribution. In the publication from the conference participation, out of a maximum 10 points she has 70% participation. The PhD student also participated in 2 papers included in book chapters, respectively with 6 and 9 authors, in which she received 8.33 points out of 30 maximum points.

The total number of points from the publication activity of Roshan Said Shaalan is equal to 57.33.

## **CONCLUSION**

Roshan Said Shaalan's dissertation is a well-thought-out, accurately conducted and presented study, which fully corresponds to the modern development of scientific research in the field. The developed topic is interesting, topical and dissertation-like. The research methods learned and applied, the experiments conducted methodically correctly, the summaries and conclusions drawn, and the and the circumstance that the dissertation work fully meets the requirements of the Law on the Development of Scientific Personnel in the Republic of Bulgaria and the Rules of the Forestry University for its application give me the reason to rate **POSITIVE**. The theoretical and practical contributions of the dissertation work are indisputable.

Based on the above, I would like to recommend to the honorable members of Scientific Jury, appointed by order of the Rector of the Forestry University, Sofia, to award the educational and scientific degree "*doctor*" to PhD student Roshan Said Shaalan in the scientific specialty "Phytopathology".

Date: 10.12.2022

Reviewer:

Sofia, Bulgaria

/ prof. D.As Ivanka Lubemova Kamenova/