REVIEW

on the dissertation work of Assoc. Prof. Dr. Lyuben Ivanov Zagorchev for awarding the scientific degree "Doctor of Sciences" in professional field 4.3. Biological Sciences (Molecular Biology)

on the topic: "Influence of biotic and abiotic factors on the parasitism of stem holoparasitic plants of the genus Cuscuta L. (family Convolvulaceae)"

by Prof. Rossitza Borissova Batchvarova - member of the scientific jury, according to Order No. RD 38-157 / 03.04. 2023 of the Rector of Sofia University "St. Kliment Ohridski".

As a member of the jury committed to consider the dissertation presented by Assoc.Prof. Dr. Lyuben Zagorchev for the award of the scientific degree "Doctor of Sciences", I confirm that I have been provided with all the documents and materials necessary for this procedure according to LDASRB and The regulations for the development of the academic staff of SU "St. Kliment Ohridski".

Brief information about the candidate

Lyuben Zagorchev graduated in 2006 with a master's degree in Molecular Biology at the Faculty of Biology of the Sofia University "St. Kliment Ohridski" with excellent success.

In the period 2007-2012, he was a doctoral student at the Department of Biochemistry, and in 2011 he defended his thesis on the topic "Biochemical changes in the composition and structure of the cell wall of embryogenic callus cultures of *Dactylis glomerata* L., treated with NaCl" for ESD "doctor" .

From 01.2010 to 03.2013 he held the position of assistant in the same department, and in the period 2013-2017 he was the main assistant. From 2017 to now, Lyuben Zagorchev is an associate professor in the Department of Biochemistry at the Faculty of Biology, Sofia University "St. Kliment Ohridski".

Since 2020, he is also the deputy dean for quality management, scientific and project activities and accreditations of BF. He is the supervisor of two doctoral students for ESD "Doctor".

Assoc.Prof. Zagorchev is a member of two international scientific organizations: International Parasitic Plant Society - since 2019 and Scandinavian Plant Physiology Society, part of FESPB - since 2011.

Information about the dissertation and the abstract

The dissertation work of Lyuben Zagorchev has a total volume of 276 pages, including an introduction and literature review - 48 pages, aim, tasks and material and methods - 18 pages,

results structured in 5 sections - 68 pages, discussion, conclusions and contributions - 30 pages and a bibliography of 337 titles, of which 335 are in foreign languages. The volume and especially the presented literary sources are proof of the serious and long-term studies on the research field of the dissertation work. Seven appendices are included, a reference to scientific contributions and a list of scientific publications. On the topic of the dissertation, Lyuben Zagorchev has published 20 specialized scientific publications, among them: with impact factor: 14 (11 Q1; 2 Q2; 1 Q3); with impact rank: 3 (1 Q1; 1 Q2; 1 Q3); book chapters: 3 (1 monograph co-authored in Chinese). In the publications, he is the first author in 13 of them and corresponding author in 11 with a total IF: 51.88. The established citations on Scopus of these publications are 351. On the subject of the dissertation, Assoc. Prof. Zagorchev participated with presentations in nine international and ten national scientific forums. He is the head of 7 scientific projects, 5 of which are international, and a participant in 12 scientific projects and one COST action. Lyuben Zagorchev is the scientific supervisor of 11 defended diploma theses of Bachelor's and Master's students on the subject of the dissertation work.

The abstract attached to the defense documentation has a volume of 53 pages and includes: table of contents, presentation of the main results of the dissertation work, bibliography, conclusions and a reference to the contributions and a list of scientific papers related to the dissertation work. The abstract reflects the main results of the dissertation.

The dissertation is written in a very good scientific style, and the results are illustrated with 86 figures, 15 tables and 4 appendices.

From the reference made for compliance with the minimum national requirements for the Doctor of Science degree in the relevant field, it is clear that Assoc. Prof. Zagorchev covers the required points according to groups of indicators A and B and significantly exceeds the minimum 100 points according to groups of indicators G and D, which are 405 and 556 respectively.

Timeliness of the problem

First of all, I want to note the originality and topicality of the chosen topic for Lyuben Zagorchev's dissertation work.

According to the IFIC Foundation, agricultural producers worldwide must contend with approximately 80,000 plant diseases, 10,000 insects and 30,000 weeds. Recent data indicate that losses from diseases, enemies and weeds achieve up to 45% of crop yields, including parasitic plants. About 2,500 parasitic flower plants belonging to 10 families have been identified. One of these families is the Convolvulaceae, genus Cuscuta, which includes about 200 species of stem holoparasitic plants that have a significant impact on natural and cultivated plants. They infect a large number of plant species, causing significant crop losses worldwide.

The present dissertation aims to deepen the knowledge about the distribution of the parasitic plants of the genus Cuscuta in the Republic of Bulgaria, their spectrum of hosts and genetic diversity through classical and modern molecular and biochemical methods.

Furthermore, to investigate the influence of biotic and abiotic factors on dodder-host plant interactions.

The influence of the abiotic stress factor - salinity on the parasitic plants and their hosts in the process of parasitism was studied. A wide range of biochemical and molecular approaches were used for this purpose. The impact of parasitism on soil microbial communities was also assessed through metagenomic analysis.

Analysis of the candidate's scientific and applied scientific achievements contained in the submitted dissertation

This is the first in-depth study on the parasitic plant dodder in our country. The results obtained in the dissertation have a significant contribution to the clarification of fundamental scientific problems concerning plant-parasite interactions.

In the period 2017-2021, plant material was collected from a total of 54 locations of four of the most widespread in Bulgaria Cuscuta species: *Cuscuta approximata* - 6 pieces, *C. campestris* - 35 pieces, *C. epithymum* - 9 pieces. and *C. europaea* – 4 pcs. All findings were determined morphologically and confirmed by DNA analysis by PCR amplification and sequencing of the ribosomal DNA region.

Host species (114) have been identified, belonging to 87 genera and 33 families, all from angiosperms (Magnoliophyta). The largest diversity of host species is among the representatives of the Fabaceae family - 19 species, followed by the Asteraceae family - 16 species, the Lamiaceae family and the Rosaceae family - 8 species.

The genetic diversity of the dodder was estimated by the ITS sequences and a phylogenetic tree was made by the maximum likelihood method, using the available sequences from the rDNA region of dodder. *C. campestris* was clearly distinguished from the other three studied species. In order to establish inter-population differences in Cuscuta, RAPD analysis was applied and on a database of RAPD markers of selected *Cuscuta campestris* populations revealed significant inter-species differences in the profiles, and a UPGMA phylogenetic tree was made.

The influence of various abiotic and biotic factors on the germination and developmental stages of the dodder before infection of the host plant was studied. The optimal temperature of 28^{o}C for the germination of the seeds of the parasite was established. The tendency to decrease their germination with the advancing age of the seeds and the influence of different concentrations of NaCl on the germination percentage were confirmed. Pre-germination under salinity was found to have a negative effect on the ability of dodder to infect plants and increase the period of infection and formation of secondary stems- longer period to attach to the host (3-4 day delay) and longer period for the formation of a secondary stem (a delay of 2-3 days).

The influence of host and salinity on haustoria formation was studied. Its formation has been found to be largely dependent on the host species, and microscopic photographs of sections have shown that in *Thymus vulgaris* (thyme), *Arabidopsis thaliana* and *Capsicum annuum* (pepper) haustoria successfully to penetrate host plants, while in non-host plants such as resistant

tomato *Lycopersicum esculentum* and *Zea mays* (maize) haustoria do not penetrate as a result of an active host defense response, turning brown and dying.

Enzymes likely involved in haustoria formation were determined by zymogram analyzes of cell wall proteins isolated at the contact site of *Cuscuta campestris* and *Arabidopsis thaliana* (host) treated with 0, 50 and 150 mM NaCl in phases: before contact with the plant host; when swirl around; in macroscopically visible haustoria and in the formation of a secondary stem. Proteases were investigated and it was found that the most pronounced isoforms associated with the interaction between parasite and host plant are observed in the stage of macroscopically visible haustoria. These changes have been shown to be more pronounced in doder than in *Arabidopsis thaliana* and depend on the salinity to which the host is subjected. Xylanases, cellulases, proteases, pectinases, pectinmethyl esterases and peroxidases were investigated by zymogram analysis. It was established that in all three studied plants - *Arabidopsis thaliana*, corn and tomato, there is the presence of new isoforms as a result of the infection with dodder.

Immunoblot analyzes of carbohydrate epitopes from hydroxyproline-rich proteoglycans at the infection site of the host (arabidopsis), resistant tomato and nonresistant (maize) at 0 and 150 mM NaCl were performed. The influence of the host plant on the metabolic profile of the parasitic plant was studied.

Changes in the proteome of the host *Arabidopsis thaliana* infected with *Cuscuta australis* were analyzed. The differential amount of proteins was assessed by two-dimensional polyacrylamide gel electrophoresis in the stem and leaves of the host plants. With the proteomic analysis at the protein level, an increase in the content of a number of proteins in the host, such as the glutathione S-transferase Atpm24.1, which is involved in the antioxidant response when the plants are attacked by pathogenic fungi, bacteria and viruses, was found when attacked by dodder.

Four soil enzymes were investigated to assess the influence of the parasitic plant on soil qualities - sulphorylases, glucosaminidases, acid phosphatases and beta-glucosidases in soil in which *C. campestris* was grown on an alfalfa host. A tendency was found to decrease the activity of all four enzymes, which leads to less availability of phosphates, sulfates and nitrogenous compounds for plants.

The interaction of parasite and host with other biotic factors such as epiparasitic gall-forming insects of the genus Smicronyx was studied, and the photosynthetic activity of the galls, following the attack of the host *Arabidopsis thaliana*, was studied.

The influence of parasitism on Cuscuta spp. on the hosts is significant and this has been demonstrated by studying the photosynthesis and transcriptome of the hosts. Transcriptome analysis of *Trifolium repens* plants infested with *Cuscuta australis* was performed. Differentially expressed genes (DEGs) were identified between infected and control host- 1,601, of which 945 DEGs were up-expressed and 656 DEGs were down-expressed. A similar analysis was performed in arabidopsis infected with *Cuscuta chinensis*, and 2,216 DEGs were identified, of which 1,411 were up-expressed and 805 were down-expressed.

The host response to salinity in parasitism by *C. campestris* was also analyzed as a function of distance to infection. Infected host plants showed an almost twofold decrease in L-Pro accumulation in both uninfected and infected leaves and a decrease in SOD and CAT activity, particularly at the site of infection.

C. australis parasitism was found to increase the effective operational taxonomic units OTUs of Acidobacteria Gp4 and Acidobacteria Gp9, bacteria from Xanthobacteraceae and decrease for bacteria from Nocardiaceae, Rhizocola, Pseudoxanthomonas and Craurococcus, while increasing OTUs of Rhodospirillales, Latescibacteria and other unidentified genera. A significant decrease in the amount of fungi from the genera Piriformospora, Orbiliaceae, Xylomyces and Devriesia was proven, while the number of representatives from Helotiales, Preussia and Davidiella was increased.

The interaction of the parasite and host with other biotic factors also has been studied. Several species of beetles of the genus Smicronyx have been found to express specific preferences for Cuscuta spp. Galls of Smicronyx sp. were found in about 10% of *C. campestris* populations but not on host plants. In *Cuscuta campestris*, the number and intensity of chitinase isoforms have been shown to increase when treated with herbivorous insects such as the cicada *Metcalfa pruinosa*.

An important result is the finding that *Cuscuta campestris* is a reservoir and vector of plant viruses. The four viruses tested TMV, AMV, TYLCV and CMV were selected based on their economic importance. By ELISA method, TMV and AMV were not detected in any of the tested samples, while TYLCV and CMV were detected among C. campestris populations. The possibility of transmission of CMV in the parasite-host direction has been demonstrated, demonstrating the potential of parasitic plants as viral vectors.

The following most significant contributions of the dissertation work are:

• For the first time in our country, mapping of the habitats of the parasitic plants of the genus Cuscuta has been done. Host species- 114 were found, assigned to 87 genera and 33 families belonging to the division of angiosperms (Magnoliophyta).

• The genetic diversity of the parasite was investigated and a phylogenetic tree was built using the maximum likelihood method, using sequences from the rDNA region of the dodder. *C. campestris* was clearly distinguished from the other three studied species: *Cuscuta approximate*, *C. epithymum* and *C. europaea*.

• Significant interspecific differences in the profiles of individual *Cuscuta campestris* populations were detected by RAPD analysis and a UPGMA phylogenetic tree was constructed.

• For the first time, the influence of abiotic stress on parasitic plants was studied, and significant effects of the host species on the adaptation to salinization of Cuscuta spp. were found.

• Infestation of dodder was found to negatively affect host photosynthetic activity in both light-dependent and CO2 assimilation reactions.

• A significant effect on the interactions between the root system and rhizospheric microbial communities in dodder parasitism has been demonstrated, altering root metabolism, relative

proportions of microbial taxa and soil enzyme activity, impairing the exchange of organic matter and mineral compounds.

• It has been established that parasitic plants of the genus Cuscuta can be carriers of viruses such as CMV (Cucumber Mosaic Virus), which can infect the host plants without symptoms of viral infection on the parasite.

• Enzymes and glycoproteins have been identified that are involved in the process of haustoria formation.

• The data on the changes in the photosynthetic apparatus of *Cuscuta campestris* during epiparasitism of gall-forming insects of the genus Smicronyx have been enriched.

• The negative influence of dodder on rhizospheric interactions and soil health has been confirmed.

Critical notes and recommendations

The text of the dissertation often lists "...compatible (arabidopsis), resistant (tomato), and irreversible (maize) host." The host in this case is only arabidopsis, which is attacked by the dodder - penetration of the parasite's haustoria and contact with the plant's vascular tissue-xylem is successfully carried out. Maize and resistant tomatoes are not hosts of the dodder.

I have only one recommendation: in the literature review, some papers by Bulgarian scientists related to the subject of the dissertation are not cited.

Conclusion: I confirm that the dissertation work presented by Assoc. Prof. Lyuben Zagorchev and the related scientific publications, as well as the quality and originality of the results and achievements presented in them, meet the requirements of LDASRB, the Regulations for its application and the relevant Regulations of SU "St. Kliment Ohridski" for the candidate's acquisition of the educational and scientific degree "Doctor of Sciences".

Proceeding from the significance of the investigated issues, the positive characteristics of the dissertation work, the undoubted contributions of the author, I confidently give my positive assessment of the considered dissertation work and vote "Yes" for awarding the scientific degree "Doctor of Sciences" to Assoc. Prof. Dr. Lyuben Ivanov Zagorchev in the professional field. 4.3. Biological sciences, scientific specialty "Molecular Biology".

05.06.2023

Prepared the review:

Prof. DSc. Rossitza Batchvarova