R E V I E W

on a thesis for awarding the educational and scientific degree "Doctor"

Professional direction 4.3. "Biological Sciences"

Scientific specialty "Microbiology"

Thesis title: **Physiological and biochemical characteristics of the plant-microbial symbiosis of representatives of the genus *Pseudomonas***
Author: **Gloria Biserova Georgieva, PhD student**
Scientific supervisor: **Assoc. Prof. Dr. Trayana Nedeva**
Scientific consultant: **Prof. Dr. Petya Hristova**
Reviewer: **Prof. Dr. Svetlana Bratkova**
Department of "Engineering Geoecology"
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The preparation of this review was made in accordance with the normative documents - Law on the Development of the Academic Staff in the Republic of Bulgaria (LAD), the Regulations for its Implementation and the Recommendations of the Faculty Council of the Faculty of Biology on the criteria for acquiring scientific degrees and holding academic positions in the SU for the professional area "Biological Sciences".

1. General presentation of the procedure

The presented set of materials includes the following documents:
- a CV;
- diplomas for higher education (bachelor's and master's degrees) and their annexes;
- Rector's orders for enrollment in full-time doctoral studies;
– certificates of passed exams from the individual plan;
- a dissertation thesis;
- an abstract in Bulgarian and English;
- a list of scientific publications on the dissertation topic;
– copies of scientific publications;
– a list of participations in scientific forums;
- a report and an opinion of the plagiarism check;
- other documents related to the course of the procedure.

2. Biographical data

Gloria Georgieva graduated from the "Biotechnologies" specialty with a Bachelor's degree at the Faculty of Biology (BF) of the SU "St. Kliment Ohridski" in 2019. In 2021, she graduated from the Master's program in "Industrial Biotechnologies" at the Department of "Biotechnology", at the Faculty of Biology of SU "St. Kliment Ohridski". In the period 2021-2024, she was a full-time doctoral student at the Department of "General and Industrial Microbiology", BF of SU with scientific supervisor Assoc. Prof. Dr. Trayana Nedeva and scientific consultant Prof. Dr. Petya Hristova, during which time she fulfilled the tasks of a dissertation work on the topic "Physiological and biochemical characteristics of the plant-microbial symbiosis of representatives of the genus *Pseudomonas*". Since March 2024, she has been teaching part-time at the Department of General and Industrial Microbiology.

4. Current state of the scientific problem and relevance of the dissertation topic

The interaction of plants with symbiotic and beneficial rhizosphere microorganisms plays an important role in their development. The role of rhizosphere bacteria in plant nutrition, resistance to root pathogens and abiotic stress factors has long been known. Rhizosphere microorganisms stimulate plant growth through a number of direct and indirect mechanisms: nitrogen fixation of atmospheric nitrogen, transformation of difficult-to-dissolve phosphorus compounds into easily available for plants, by means of bacterial phosphatases, increasing the assimilation of nitrates, producing siderophores that chelate iron in a form that is bioavailable by plants, synthesis of physiologically active substances, changes in the permeability of the cell membrane of the roots, protection against stress factors of the environment, phytopathogens, etc.

Worldwide, research on the rhizosphere microflora has seen significant growth. A number of authors establish the high potential of representatives of the genus *Pseudomonas* to improve the mineral nutrition of plants through the production of phytohormones, organic acids and enzymes. Representatives of various species belonging to the genus *Pseudomonas* are known as Plant Growth-Promoting Microorganisms (PGPM). Bacteria belonging to the genus *Pseudomonas* have potential for application in a number of biotechnological productions, bioremediation and biological control. Therefore, I find the topic of the dissertation to be extremely relevant and of great practical importance, given the economic significance of the alternative for purposeful modeling of the rhizosphere habitat in the search for ecologically sustainable solutions for sustainable agricultural practices.

4. Knowledge on the subject

The literature review of the dissertation work is thorough and examines in detail the individual aspects of plant-microbial relationships. It covers 45 pages and contains 6 figures. The

role of microorganisms in the rhizosphere, the phyllosphere and the mechanisms of action of rhizosphere plant growth-promoting microorganisms have been discussed in detail. Special attention has been given to two representatives of the *Pseudomonas* genus: *Pseudomonas chlororaphis* and *Pseudomonas yamanorum*. The metabolic pathways of synthesis of phenazine pigments, their broad spectrum of antibiotic activity and role in virulence have been reviewed in detail. Information has been presented on the production of siderophores, their potential for bio-control of phytopathogenic microorganisms and their role in improving plant development through efficient iron uptake. The plant-microbe symbiosis, the mechanisms of plant-microorganism communication and the participation of phytohormones in the overall development of the plant have been thoroughly examined. Separate sections have been devoted to the plant immune response to biotic and abiotic stress and the role of microorganisms in it, and to the application of plant growth-promoting bacteria as biological control agents in conventional agriculture. A total of 347 literary sources has been cited in the dissertation, of which 343 in English and 4 in Bulgarian, and 70 of them from the last 5 years. I find that the doctoral student has done an extensive literature review on the subject of the dissertation work. The cited publications concern all aspects of its subject matter, its purpose and tasks, the methods used, and they have also been relevantly used in the reasoned discussion of the obtained results.

5. Purpose and tasks

The purpose of the dissertation work has been concisely and clearly formulated: selection and research of bacterial strains belonging to the genus *Pseudomonas* for studying the phenomenon of plant-microbial symbiosis and proving their PGP-potential. That has helped define eight formulated tasks, which have logically been united into three groups related to: a) screening of *Pseudomonas* isolates for the presence of PGP-potential based on complex biochemical characterization and analysis of key PGP-characteristics, b) proving the PGP-potential of the selected strains by optimizing the cultivation process in terms of C, N and P source, induction of indole-acetic acid biosynthesis, studying of the production of phenazine-1-carboxylic acid and siderophores, evaluation of the studied strains as biocontrol agents and optimization of preservation and stability of biologically active fermentation products of selected strains during lyophilization and spray drying and c) studying the plant-microbial symbiosis of the selected strains with model plant systems of cereal-cereal/cereal-legume crops and ornamental plants in different phases of the growing season.

6. Research methodology

In the dissertation, a wide range of methods, necessary to achieve the set tasks, has been used. A complete description of media for maintaining the strains and determining their biochemical characteristics, standard and modified media for conducting batch cultivation in flasks and bioreactor has been provided. The methods for monitoring the growth dynamics of bacterial cultures, the quantitative determination of soluble protein and residual sugars, the determination of key enzyme characteristics by standard methods and the ApiZYM system, and the assimilation characteristics by the API 20 NE system have been described in detail. Quantification of

the phytohormones produced has been performed via HPLC analysis. The genetic identification has been carried out on the basis of complete sequencing of 16S rRNA by the company Macrogen. The physiological effects of selected strains of the genus *Pseudomonas* on seeds have been studied by germination in rolls and agar, and on greenhouse plants by means of pot experiments.

7. Results and discussion

This is the main section of the dissertation and it covers 86 pages. The presented results in the dissertation follow the logically set goal and formulated tasks and subtasks, being well illustrated with 57 figures (graphs and photographic material) and 9 tables.

1. Screening of *Pseudomonas* isolates for plant growth-promoting potential (PGP)

An evaluation of the PGP potential of five *Pseudomonas* strains has been made based on their broad-spectrum biochemical characterization. As a result of biochemical and genetic characterization and proven PGP-characteristics, two strains – *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 – have been selected for further analyses. The selection of the two strains has been carried out on the basis of a complex assessment of their biochemical parameters, including key enzyme activities and, in particular, of their phytohormonal profile, according to which the two representatives have been ranked first in terms of variety and quantity of phytohormones produced.

2. Demonstration of the PGP-potential of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046

This section presents the results of the optimization of the cultivation process through a multifactorial experiment for testing twice higher and lower concentrations of carbon, nitrogen and phosphorus sources, selecting optimal ratios of biogenic elements for cultivation of the two strains. The efficiency of the selection has been confirmed by scaling up the cultivation in a 5 L bioreactor. An approach to optimize the biosynthesis of indole-acetic acid has been proposed and implemented, based on successively increasing the concentrations of the inducer: synthetic tryptophan or tryptophan of microbial origin from 0.01% to 0.04%. Induction of IOC production with tryptophan of microbial origin has been found to be 34 % more efficient compared to the synthetic variant. The production of phenazine-1-carboxylic acid and hydroxymate-type siderophores has been optimized, and the most suitable cultivation regimes for the producer strains have been specified. The antagonistic effect of *Ps chlororaphis* 1S4 and *Ps. yamanorum* 1046 against economically important pathogens of the genus Fusarium has been proven by *in vitro* study of their antifungal activity. The conditions for preserving the stability of the biologically active fermentation products from *Pseudomonas*

chlororaphis 1S4 and *Pseudomonas yamanorum* 1046 have been optimized through the selection of lyophilization and spray drying modes.

3. PGP-potential of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046: plant-microbial symbiosis with cereals and ornamental plants

The plant-microbe symbiosis of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 has been studied with model plant systems of cereals and ornamental plants at different phases of the growing season. Increased germination efficiency of maize, soybean and wheat seeds has been found, as assessed by biometric indicators of the root system of the model plants. The plant-microbial symbiosis of *Ps. chlororaphis* 1S4 and *Ps. yamanorum* 1046 has been studied through the use of the ornamental plants primrose, chrysanthemum and cyclamen. The positive effect of fresh and dried cultures of *Ps. chlororaphis* 1S4 and *Ps. yamanorum* 1046 (individually and in combination) applied to different vegetative parts of plants by different approaches (watering and foliar spraying) has been proven. A prototype series of effective preparations with potential for application as biofertilizers and biocontrol agents has been developed.

8. Conclusions and contributions

The eight conclusions summarize the obtained data very well. Conclusions and contributions are formulated clearly enough. They correspond to the scope of the conducted research and their significance in a scientific and applied aspect.

- The methodological approach is original, including a complex of biochemical characteristics for the selection of strains with the potential for their practical application in agricultural practice.

- Another original contribution is the proposed cost-effective approach to replace the inducer synthetic L-tryptophan with tryptophan of microbial origin to stimulate the production of the important plant hormone indole-acetic acid.

- The proven inhibitory effect of *Pseudomonas chlororaphis* 1S4 and *Pseudomonas yamanorum* 1046 strains against mycopathogenic species belonging to the genus *Fusarium* is also original, which is a prerequisite for their use as components of biological control preparations.

- Another important contribution of an applied nature is the constructed prototype series of fresh and dry cultures of the selected strains of the genus *Pseudomonas* as an ecologically appropriate alternative to conventional fertilizers.

- The developed scheme for evaluating the plant-microbial symbiosis between bacterial representatives and model plant systems through the proposed process monitoring indicators regarding the microbial component, the plant component and the application technology is original.

9. Evaluation of publications on the dissertation subject

The main results of the dissertation work are presented in 3 scientific publications: one published in *BioRisk* and two accepted for publication in *Acta Microbiologica Bulgarica*. The three articles are in English, with Gloria Georgieva as first author. The PhD student has participated with posters in three scientific forums, and she will participate with a fourth poster in the INTERNATIONAL SEMINAR OF ECOLOGY- 2024 in Bulgaria. Two citations were noted for the BioRisk article published in 2023: one in an impact factor journal publication and the other in a graduate student's thesis.

10. PhD thesis abstract

The PhD thesis abstract is written on 58 pages, accurately reflecting the main themes, results and achievements of the doctoral work. The most important results are presented and discussed. The abstract ends with the 8 conclusions, original and confirmatory contributions, a list of the 3 publications on the subject of the dissertation and participation in scientific forums. The PhD thesis abstract contains the most important figures and results, and fully meets the established requirements and standards.

11. Personal participation of the PhD student

I do not know Gloria Georgieva personally. My assessment of the PhD student's personal participation in the conducted research has been based entirely on the materials provided to me: the dissertation, the abstract, the publications and the participation in scientific forums. Gloria Georgieva is the first author in the publications on the dissertation, therefore, it can be concluded that the experimental work, as well as the activities on the processing and presentation of the obtained results, are largely her own merit.

12. Critical remarks and recommendations

I have neither critical remarks nor recommendations regarding the conducting of the studies, their interpretation, or the set of materials submitted to me for review. However, some corrections can be made in the PhD thesis:

Two graphs (Figures 10 and 11) are missing the abscissa and ordinate dimensions.

Several technical errors related to duplication of punctuation marks have been noted in the PhD thesis.

CONCLUSION

The PhD thesis of Gloria Georgieva reviewed by me with the title "Physiological and biochemical characteristics of plant-microbial symbiosis of representatives of the genus *Pseudomonas*" contains original scientific and scientific-applied results with a contribution to science. It meets the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (ZRASRB), the Regulations for the Implementation of ZRASRB and the relevant Regulations of BF-SU and SU. The presented results and publications fully correspond to the scientometric requirements for such a work.

The dissertation unequivocally shows that PhD student Gloria Georgieva possesses the theoretical knowledge and professional skills of a microbiologist and molecular biologist, demonstrating qualities and skills for independent conduct of scientific research, incl. solving scientific challenges.

Based on the above, I give my positive assessment of the dissertation work and propose to the honorable scientific jury to award the educational and scientific degree "Doctor" to Gloria Georgieva in field 4 "Natural sciences, mathematics and informatics; professional direction 4.3. "Biological Sciences" (Microbiology).

25/09/2024

Reviewer:
(Prof. Svetlana Bratkova, PhD)