

OPINION

by Dr. Antoaneta Borissova Trendafilova-Savkova, Professor at the Institute of Organic Chemistry with Centre of Phytochemistry – Bulgarian Academy of Sciences

on the PhD Thesis for awarding the educational and scientific degree "doctor" in higher education field: 4. Natural sciences, mathematics and informatics, professional field: 4.3. Biological Sciences, PhD Program: Botany

Author: Asya Pavlova Kozhuharova

Title: Biotechnological approach for conservation and cultivation of licorice (*Glycyrrhiza glabra* L.), Fabaceae

Research supervisor: Prof. Dr. Marina Stanilova - Institute of Biodiversity and Ecosystem Research – BAS (IBER-BAS)

Research consultant: Prof. Dr. Strahil Berkov- Institute of Biodiversity and Ecosystem Research – BAS

1. General presentation of the procedure and the PhD student

According to order ПД 75/06.10.2023 г. of the Director of IBER-BAS, I have been appointed as a member of the scientific jury in the procedure for the defense of a dissertation on the topic " Biotechnological approach for conservation and cultivation of licorice (*Glycyrrhiza glabra* L.), Fabaceae " for the acquisition of the educational and scientific degree "doctor" with author Asya Pavlova Kozhuharova.

The set of materials presented by Asya Kozhuharova on paper and electronic media is in accordance with the Regulations for the Development of the Academic Staff of IBER - BAS, meets the criteria and includes all necessary documents. The PhD student submitted a report on the accumulated credits as follows: implementation of the educational program - 130 points (mandatory minimum 130 points), approval of the implementation of the scientific program - 208 points (mandatory minimum 40 points), published scientific results -124 (mandatory minimum 80 points). The total number of credits for the doctoral period (462 credits) significantly exceeds the mandatory minimum of 250 credits. The PhD student has also attached 3 publications on the topic of the dissertation, a list of noted citations to the scientific works included in the dissertation, a list of participation in scientific events, a list of participation in the implementation of scientific projects, and the relevant evidentiary material. The presented

abstract is prepared according to the requirements and it fully presents the results, discussions and conclusions included in the dissertation work.

2. Brief biographical data of the PhD candidate

Asya Kozhuharova graduated from the Bachelor's program "Biology" at the Faculty of Biology of the "St. Kl. Ohridski" in 2013 and master's program "Medicinal and Aromatic Plants" at PU "Paisii Hilendarski" in 2015. She was enrolled as a full-time PhD student at IBER-BAS, scientific specialty "Botany" and was awarded with the right of defense in 2018. In 2015, she was appointed as a biologist at IBER - BAS, and in the period 2019-2023 as an assistant at the same institute. Currently, she is again in the position of "biologist" at IBER - BAS. In the framework of her scientific research activities so far, she participated in a total of 10 projects and in 10 scientific forums. Asya Kozhuharova is a co-author of 6 scientific publications, 1 of which has 4 citations.

3. Relevance of the topic and appropriateness of the set goals and tasks

Plants have been used as food and medicine since ancient times. Plant products, including extracts, can be found in the pharmacopoeias of many countries. Despite the impressive development of synthetic drugs, plants remain the source of more than a quarter of all known drugs, and scientists continue to discover new drugs based on the structural diversity of secondary metabolites. In addition, the indiscriminate collection of medicinal plants leads to a significant decrease in their natural populations and even to their extinction. This requires not only the introduction of strict measures for their collection, but also the search for ways to cultivate them. This is the case with the licorice (*Glycyrrhiza glabra* L.), the subject of this dissertation, from which the roots of 3-year-old or older plants are used. In our country, the species is found only in the area of the Danube plain and is categorized as endangered according to the Red List of higher plants in Bulgaria and the Red Book of the Republic of Bulgaria, and its collection from natural habitats is prohibited. The topic is extremely relevant and interesting, both from a scientific and a practical point of view, as it proposes the development of a method for *in vitro* micropropagation, allowing the production of many plants in a short period, which can later be used for planting material. The goal and tasks are clearly formulated and fully correspond to the topic of the dissertation.

4. Knowledge of the problem

A total of 204 sources are cited in the PhD dissertation, a significant part of which in the last 5 years. The literature review includes detailed information on the botanical characteristics of the species, its distribution and conservation status, application in folk medicine, the chemical composition of the roots, the propagation of licorice *in vitro* and its *ex vitro* adaptation and

acclimatization to outdoor conditions. The PhD student has mastered the specific terminology and shows skills in systematization and critical analysis of sources from several scientific fields - botany, biotechnology and phytochemistry. The presented material shows the candidate's good literary awareness and theoretical preparation.

5. Research methodology

Plant material from 4 Bulgarian populations of *Glycyrrhiza glabra*, as well as roots and seeds of economically important plants from Ukraine and roots from Uzbekistan were used to fulfill the tasks. The seeds were used to initiate *in vitro* cultures and to determine their germination and viability. Roots were used for phytochemical analyses, and stolon cuttings were used to obtain plants for the IBER *ex situ* collection and to obtain vegetative buds to initiate *in vitro* cultures. For the introduction of *G. glabra* seeds into *in vitro* culture, MS basic medium was used without and with growth regulators in different combinations and concentrations, as well as the addition of activated carbon in the tested media. The *ex vitro* adaptation to non-sterile conditions and soil was carried out in 2 stages: in a climate cabinet and in a phytotron room. Acclimatization of the plants was carried out in an unheated greenhouse and outdoors, in the experimental areas of IBER. A soil analysis was also carried out from the four known localities, as well as the soil of the experimental area of IBER-BAS according to ISO 10390. For the phytochemical analysis, extracts from the plant material were obtained, used for the determination of glycyrrhizic acid by HPLC method, TLC comparison and spectrophotometric determination of total flavonoids. Statistical methods were used to analyze the obtained results. The research methodology used fully meets the goals and objectives set in the dissertation work.

6. Characterization and evaluation of PhD thesis and its contributions

The dissertation work, written on 130 pages, is constructed in a classic style with a good balance between the individual parts: introduction (2 pages), literature review (25 pages), aims and objectives (2 pages), experimental part (13 pages), results and discussion (58 pages), conclusion (9 pages), contributions (2 pages), and a 19-page bibliography with 204 titles. There are 16 tables and 48 figures in the dissertation. In the "Results and Discussion" section, the obtained results are described, analyzed and interpreted in detail. The conclusions are clearly stated. The contributions of the dissertation can be systematized as follows:

In relation to the reproduction of the licorice with biotechnological methods and the conservation of the species:

For the first time, comparative studies of the known Bulgarian populations were performed regarding the possibility of breeding *G. glabra* using biotechnological methods and a comparison was made with a reference origin of commercial importance from Ukraine.

Comparative study of the vitality of the seeds originating from the known Bulgarian deposits of *G. glabra* was performed for the first time.

An efficient protocol for *in vitro* micropropagation of *G. glabra* has been established. Seeds have been found to have a combined physical and physiological dormancy, and suitable conditions to overcome it have been experimentally determined. A suitable nutrient medium was selected for clonal *in vitro* propagation of *G. glabra*, which provides both a high multiplication factor and rhizogenesis and shortens the *in vitro* rooting step.

Two *ex situ* collections were created in the experimental areas of IBER: from plants obtained from stolon cuttings from 3 Bulgarian populations of *G. glabra*, as well as from *in vitro* propagated and *ex vitro* adapted and acclimatized plants.

The locality of *G. glabra* near the village of Baikal, considered as extinct, has been confirmed.

Five hundred seeds of *G. glabra* have been handed over for storage in the National Seed Bank in Sadovo, thus fulfilling one of the recommendations for the protection of the species formulated in the Red Book of the Republic of Bulgaria.

Plants obtained from stolon cuttings and *in vitro* propagated and *ex vitro* adapted plants were planted in the site next to the village of Dolni Vit at the protected area "Cherveniyat bryag", all propagated from source plant material taken from the same site.

In relation to the phytochemical analysis of the main biologically active compounds:

The data on the content of glycyrrhizin in the known Bulgarian populations have been updated and it has been confirmed that the population of *G. glabra* near the village of Beltsov is the richest in glycyrrhizin.

For the first time, comparative studies of the content of glycyrrhizin and total flavonoids *in situ* in roots from the four known Bulgarian localities of *G. glabra*, in the flowering and fruiting phases, were made and statistically significant differences between them were established. These results confirm the seasonal fluctuations in glycyrrhizin and total flavonoid content in the cultivation of licorice of foreign origin under Polish conditions. In addition, a comparison was made with reference origins of commercial importance from Ukraine and Uzbekistan in the fruiting phase.

An interaction between origin and phenophase was found regarding both the content of glycyrrhizin and that of total flavonoids in plants growing under natural conditions in the deposits.

It was found that the content of glycyrrhizin and total flavonoids in plants propagated vegetative from stolon cuttings of different origins leveled off after 3 years of cultivation in the controlled conditions of the IBER *ex situ* collection. This is due to the significant increase in

their content in *ex situ* conditions in plants of Dolni Vit origin compared to that in *in situ* conditions.

The results of the conducted research have fundamental scientific contributions, which can be formulated as a study of the state of natural populations of *G. glabra*, creation of a protocol for its *in vitro* propagation and *ex vitro* adaptation, obtaining data on the chemical content and on the accumulation in the different phases of plant development, as well as providing seeds for storage in the National Seed Bank. The obtained results also have a scientific-applied contribution with the potential for practical application, namely the possibility of obtaining planting material for the purpose of increasing populations, as well as of economic importance. I accept the statement of scientific contributions formulated by the PhD student.

7. Evaluation of the publications and personal contribution of the PhD student

I believe that the experimental results, as well as their presentation and analysis, are to a large extent the personal work of the PhD student. The dissertation research is summarized in 3 publications in scientific journals, of which 1 in quartile Q3. Asya Kozhuharova is the first author in these publications, which confirms her personal contribution to achieving the published results. Four citations from foreign authors are noted.

CONCLUSION

The PhD thesis contains scientific and scientific-applied results that represent an original contribution to science and meet all the requirements of the Law on the Development of the Academic Staff in the Republic of Bulgaria (LDASRB), the Regulations for the Implementation of the LDASRB and the Regulations for the Implementation of the LDASRB of the Bulgarian Academy of Sciences. The presented materials and results fully comply with the specific requirements of the Rules of the IBER-BAS for the application of LDASRB. The PhD thesis shows that the PhD student Asya Kozhuharova possesses in-depth theoretical knowledge and professional skills in the scientific specialty "Botany" by demonstrating qualities and skills for independent scientific research.

Due to the above, I confidently give my positive assessment of the conducted research and propose to the honorable scientific jury to award the educational and scientific degree "doctor" to Asya Kozhuharova in higher education field: 4. Natural sciences, mathematics and informatics, professional field: 4.3. Biological Sciences, PhD Program: Botany.

17.11.2023 г.

Reviewer:

(Prof. Dr. Antoaneta Trendafilova)