

**OPINION**  
**on the PhD Thesis**  
**of Asya Asya Pavlova Kozhuharova**

entitled

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

for obtaining the educational and scientific PhD degree

Scientific field: 4.3. Biological sciences

Scientific topic: 01. 06. 03 Botany

Section „Applied Botany“

Department „Plant biodiversity and resources“

Institute of Biodiversity and Ecosystem Research

Bulgarian Academy of Sciences

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Prepared by

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The present opinion has been prepared based on the Order № 75/06.10.2023 r. of the Director of IBER (BAS) - Sofia, Assoc. prof. Vladimir Vladimirov, PhD.

The author of the PhD thesis is a PhD student at the Department „Plant biodiversity and resources“, Section Applied Botany, IBER-BAS.

## **1. Actuality of the topic**

Medicinal and aromatic plants are becoming increasingly important for the production of biologically active substances, as well as other raw materials and substances for the needs of the pharmaceutical and food industry, cosmetics, etc. The object of work of the present PhD thesis is *Glycyrrhiza glabra* L., Fabaceae. The utilization of its roots in the form of tea, as well as standardized extracts and products thereof, is widely spread in the world's traditional and conventional medical practices. The plant is also found in our country in the Danube Plain, but its status is of an endangered species according to the Red List of higher plants, and the licorice is under strict protection as it is included in Appendix 3 of the Law on Biological Diversity. That is why the work included in the presented thesis is of extreme importance both for its scientific study and for the preparation of strategies for its preservation, propagation with the aim of re-introduction into natural habitats and its field cultivation with the aim of resource supply of plant material for pharmaceutical, food, cosmetic and other industries.

## **2. Awareness with the topic**

The bibliographical survey presents a comprehensive summary of the botanical description, distribution, habitat status and conservation status of the species, its phytochemical composition and biological activity. Data are summarized with respect to information available *in vitro* initiation and propagation of both the studied species and other representatives of the genus *Glycyrrhiza*. The theoretical principles regarding methods for callus formation, indirect organogenesis and somatic embryogenesis, for carrying out genetic transformations, *ex vitro* adaptation and acclimatization to field conditions in *Glycyrrhiza* species and in other medicinal and aromatic plants are also thoroughly examined. Based on the comprehensive bibliographic reference, the need for a systematic study of the phytochemical composition and the development of effective biotechnological approaches for the protection of licorice in the conditions of the natural deposits in Bulgaria have been motivated.

## **3. General impression of the PhD thesis**

The work has been written on 130 pages, the material - illustrated with 16 tables, 48 figures and 204 references have been cited. A total of three papers have been published on the subject of the dissertation, in the Journal of BioScience and Biotechnology, Proceedings of the Seminar of Ecology - 2017 and Comptes rendus de l'Académie bulgare des Sciences (SJR, Scopus - 0.205, JCR-IF, Web of Science - 0.321). In all of the three publications, the candidate is the first author. Four citations of one of the papers (in J BioSci Biotechnol) in international journals were noted, with three of the citing papers being Q-ranked. The results of the dissertation work have been reported in seven scientific events (four of them – international). The doctoral student is a participant in seven scientific projects and a leader in three youth projects under the "Program for supporting young scientists and postdoctoral students" - BAS. The complete scientific biography of Asya Kozhuharova includes participation in a total of six scientific publications and ten scientific forums. The candidate significantly exceeds the required points according to the BAS credit system (a total of 462 points with a required minimum of 250, distributed in the relevant categories: Implementation of the educational program - 130 points of the required 130 points, Approbation of the implementation of the scientific program - 208 points of required 40 points and Publications of scientific results on the topic of the dissertation - 124 points out of required 80 points). Thus, the dissertation meets the requirements for structure, qualitative and quantitative criteria, as the candidate exceeds the minimum national requirements for obtaining the educational and scientific degree "doctor", set by the Law of the Development of the Academic Staff in the Republic of Bulgaria (ZRASRB), as well as The regulations for the terms and conditions for acquiring scientific degrees and holding academic positions at the Institute of Biodiversity and Ecosystem Research at the BAS. The dissertation work, abstract and set of documents presented by Master Asya Kozhuharova are in accordance with the above requirements in order for her to be admitted to the defense of her dissertation.

## **4. Significance and persuasiveness of the obtained results, interpretations and conclusions**

**The doctoral student has mastered and applied a wide range of research methods to obtain the scientific results of the conducted experiments.**

- A comparative botanical survey and collection of plant material from four Bulgarian localities of the species were carried out. Plant material from Ukraine and Uzbekistan has also been supplied.

- Analyzes and experiments were carried out to determine the germination and vitality of the seeds. After sterilization, *in vitro* cultures were successfully started. A wide array of biotechnological approaches have been successfully mastered and experimented - direct organogenesis, callusogenesis, somatic organogenesis, root formation, experiments on induction of genetic transformation. For successful rooting and *ex vitro* adaptation, the direct

organogenesis approach was chosen. Extensive culture media testing has been conducted and efficient *in vitro* multiplication protocols have been developed. *Ex vitro* adaptation of obtained plants was successfully carried out in the collection of the Institute of Biodiversity and Ecosystem Research - BAS, as well as in the native localities of the plant.

- A comparative analysis of the soil composition from the different localities of the species in the Danube plain was carried out.

- Extraction of root material and phytochemical analyzes were performed for quantitative HPLC (high performance liquid chromatography) determination of glycyrrhizic acid, as well as TLC (thin layer chromatography) and spectrophotometric determination of total flavonoids.

- Methods of statistical processing, systematization and presentation of data have been mastered.

## **5. Characterization and evaluation of the dissertation work and contributions**

The results of the dissertation work have both scientific and scientific-applied value and would be useful in the future development of licorice plantations for economic purposes. This refers, on one hand, to the established protocol for rapid propagation of the species using biotechnological methods, and on the other hand, to the established features related to the content of glycyrrhizin and total flavonoids in the natural habitats and in the adapted *ex situ* plants of the different origins.

### ***Contributions of conservation value***

- For the first time, the Bulgarian populations of the species were compared in terms of the vitality and germination of the seeds and the different types of explants for their potential for biotechnological propagation. In addition, a comparison was made with a reference origin of commercial importance from Ukraine.

- An effective protocol for *in vitro* propagation of licorice was created, achieving a high multiplication rate and rhizogenesis, leading to optimization of its *in vitro* rooting.

- Two *ex situ* collections were developed in the experimental field of IBER: from plants obtained from stolon cuttings from three Bulgarian populations of *G. glabra*: Dolni Vit, Koilovtsi and Beltsov, as well as from *in vitro* propagated and *ex vitro* adapted and acclimatized plants originating from Koilovtsi, Beltsov and Ukraine.

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

- Seeds from the village of Koilovtsi were deposited for storage in the National Seed Bank in the town of Sadovo, in accordance with the recommendations for the protection of the species formulated in the Red Book of the Republic of Bulgaria.

- In the site next to the village of Dolni Vit, at the protected area "Cherveniya Bryag", 12 individuals were planted: 5 obtained from stolon cuttings and 7 *in vitro* propagated and *ex vitro* adapted from source plant material taken from the same site.

### ***Contributions regarding the content of biologically active substances***

- The results regarding the content of glycyrrhizin in the known Bulgarian populations of the species were compared and evaluated, and it was confirmed that the Bulgarian licorice population richest in glycyrrhizin is the one near the village of Beltsov.

- For the first time, comparative studies of the content of glycyrrhizin and total flavonoids in roots from the four known Bulgarian localities of the species in different phenological phases were carried out, and the seasonal fluctuations regarding the content of these substances were confirmed.

In addition, a comparison was made with reference origins of commercial importance from Ukraine and Uzbekistan in the fruiting phase.

- It was established that the content of glycyrrhizin and total flavonoids in plants propagated vegetatively from cuttings of stolons of different origin (Dolni Vit, Koilovtsi and Beltsov) leveled off after three 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.

### **Conclusion**

The presented work shows that the candidate has obtained the required skills for summarizing and critical analysis of bibliographic data, as well as diligence and persistence in learning and applying the learned set of experimental biotechnological and phytochemical methods. All this enables me to recommend to the members of the respected Scientific Jury to award the doctoral student a M.Sc. Asya Pavlova Kozhuharova the educational and scientific degree "Doctor" in Scientific field: 4.3. Biological Sciences, Scientific topic: 01. 06. 03 Botany

Sofia

November 20<sup>th</sup> 2023

/Assoc. prof. Kalina Danova, IOCCP-BAS/