

## REVIEW

Of the member of the scientific jury: **Prof. Lilyana Rumenova Nacheva**, Ph.D., Fruitgrowing Institute - Plovdiv, Agricultural Academy (SSA), professional field 6.1. Horticulture, scientific specialty "Fruitgrowing" (Professor) and 4.3. Biological Sciences, scientific specialty Plant Physiology (Associate professor), appointed as a member of the Scientific Jury according to Order № 75/06.10.2023 of the Director of The Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences

regarding the PhD Thesis for awarding the educational and scientific degree "**Doctor**" (PhD) in field of higher education: 4. Natural sciences, mathematics and informatics; professional direction: 4.3. Biological Sciences, scientific specialty: Botany

**PhD Student: Asya Pavlova Kozhuharova**

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

**Research supervisor: Prof. Marina Stanilova, PhD - Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences**

### **I. General characteristics of the dissertation - volume and structure**

Excessive use and lack of control over natural habitats leads to their rapid disappearance. The application of biotechnological methods for the protection of biological diversity is a modern and relevant task. *Glycyrrhiza glabra* L. is one of the plant species with great commercial value, with numerous applications in the cosmetic, food and pharmaceutical industries, but it is endangered according to the Red List of higher plants.

The dissertation presents a large-scale interdisciplinary scientific research that can be a methodological model for working with the study of rare and endangered medicinal plants and their transformation into cultivated species.

The dissertation work is sufficient in volume, it contains all the necessary sections, which are well structured and balanced. It is written in 135 standard pages, with the literature review covering 24% of its volume, material and methods 11% and more than 64% being

devoted to results and their discussion. The work is well illustrated with 16 tables and 48 figures.

## **II. Literary awareness and theoretical preparation of the candidate**

The literature review covers the main literature sources and problems in the distribution, biology, methods of propagation and cultivation of bare licorice (*G. glabra* L.), as well as the content of biologically active substances (BAS) in it. The cited literature includes 204 sources, 188 in Latin and 15 in Cyrillic. More than 37% of cited sources are from the last 10 years.

## **III. Methodical approach**

The methodological approach is wide-ranging and at the same time focused on the main subject of the study. Materials and methods used are accurately described with relevant sources. I really liked the graphical presentation of the experimental work at the beginning of section 4.2. Methods. All experiments were carried out in a sufficient number of replicates so that the obtained results were subject to statistical processing. A clear goal of the study was set - experimental determination of the appropriate conditions for effective *in vitro* cultivation and *ex vitro* adaptation of *Glycyrrhiza glabra* L. and analysis of the main biologically active substances in the starting forms and in the *ex situ* adapted plants. To fulfill the set goal, 8 main and 4 additional tasks have been formulated. A number of important indicators related to seed germination and characterizing the influence of various factors on *in vitro* cultures and the content of biologically active substances were investigated.

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

The condition of the Bulgarian localities of licorice (*G. glabra* L.) was investigated for four years. A decrease in the deposits and the disappearance of the deposit near the town of Nikopol has been established, which once again confirms the need for this type of development and the relevance of the dissertation work. Low germination and uneven seed germination were reported, as well as zero germination of seeds without stratification. Attempts to increase seed germination with growth regulators have also been unsuccessful. The problems with seed germination are overcome after a combined physical and physiological seed dormancy is established. A huge amount of work has been done.

The conclusions drawn as a result of the study are logical, the most important of which are:

- ✓ Storing the seeds at room temperature for 6 months before introducing them into *in vitro* culture has a beneficial effect, significantly reducing microbial contamination (from 27% to 73% surviving at origin Dolni Vit) and increasing germination;
- ✓ Stratification by repeatedly submerging the seeds in ice and boiling water is much more effective under *in vitro* conditions than *in vivo* (respectively 66.0% and 13.3% germinated, in Beltsov origin).
- ✓ The seed viability of the three studied Bulgarian populations is similar and varies between 40-48%.
- ✓ *G. glabra* L. is a slow-growing species, difficult to cultivate *in vitro*, and the growth of the cultures is uneven even with the same origin and the same composition of the nutrient medium.
- ✓ Growth regulators added to nutrient media affect the growth and development of *in vitro* plants, with kinetin being the most relevant cytokinin to stimulate multiplication, and the auxin IBA favoring root formation. Appropriate explants and optimal nutrient media for accelerated *in vitro* propagation of naked licorice were selected. *Ex vitro* and acclimatized *in vitro* propagated plants were successfully adapted, thus working out the main parameters of a future technology for accelerated propagation of licorice.
- ✓ As a result of the phytochemical analysis of the main biologically active substances, it was established that the plants from the four tested Bulgarian licorice populations differ significantly in terms of glycyrrhizin content, and seasonal fluctuations were also noted. Also, the percentage content of total flavonoids expressed as hyperoside is much higher in the flowering phase than in the fruiting phase.
- ✓ Methanol extracts of licorice roots are richer in flavonoid aglycones than glycosides. The profiles of total flavonoids in the four known Bulgarian populations were similar with respect to glycosides, and flavonoid aglycones showed greater variability;
- ✓ Significant differences were found in the physical and chemical characteristics of the soil samples from the four Bulgarian deposits and from the *ex situ* collection at IBEI.
- ✓ The significant increase in the content of glycyrrhizin and total flavonoids in the *ex situ* cultivated plants originating from Dolni Vit compared to the donor plants from the site and equalizing their values with those in the *ex situ* cultivated plants originating from Beltsov and Koilovtsi confirms the possibilities for creating commercial

plantations from licorice, regardless of their origin, which would help to preserve their natural populations.

## V. Critical notes to the dissertation work

I have no significant remarks on the dissertation work. I have some technical notes:

1. In table. 3 (page 34) the third column with the stock solutions is unnecessary, it is sufficient to present the components of the nutrient medium in mg/l;
2. The concentration of the growth regulators (tables 3 and 4.) would also be good to give in micromoles ( $\mu\text{M}$ );
3. On page 37, line 5, it is written "on each medium in several repetitions". The exact number of replicates (n) in each experiment would be mentioned in the tables or elsewhere as appropriate.

I also have one recommendation - for greater visibility, it would be good to summarize the results of a population survey in a table.

These minor technical notes in no way detract from the value of the dissertation work, but are only intended to improve the text and future research of the PhD student.

### *Questions:*

1. Has the germination of seeds from plants from the ex situ collection been studied?
2. What is the role of activated carbon (used in the third passage) in the cultivation of plants?

## VI. Nature of scientific contributions

I accept the author reference for the contributions. I would classify them into the following two groups:

### *A. Contributions of an original nature:*

- For the first time, a comparative study of the vitality of the seeds originating from the known Bulgarian deposits of *G. glabra* L. was made;
- For the first time, comparative studies of the known Bulgarian populations have been carried out regarding the possibility of breeding *G. glabra* L. using biotechnological methods;
- An efficient protocol for *in vitro* micropropagation of licorice from seeds has been established;

- An *ex situ* collection was also created with plants obtained from cuttings of stolons from 3 Bulgarian populations and in vitro propagated and ex vitro adapted and acclimatized plants from 2 Bulgarian populations and one from Ukraine;
- Seeds of bare licorice originating from the site near the village of Koilovtsi have been handed over for storage in the National Seed Bank in the town of Sadovo;
- In the deposit next to the village of Dolni Vit, 12 plants obtained from cuttings of stolons and in vitro, obtained from the same deposit, were planted;
- For the first time, comparative studies of glycyrrhizin and total flavonoid contents in situ in roots from the four known Bulgarian localities of *G. glabra* L. were made and a comparison was made with reference origins of commercial importance from Ukraine and Uzbekistan.

*Contributions of a confirmatory nature:*

- Data on glycyrrhizin content in known Bulgarian populations have been updated thanks to more advanced chromatographic methods (HPLC);
- It has been confirmed that the richest Bulgarian population of licorice in glycyrrhizin is the one near the village of Beltsov;
- Improved soil nutrient status significantly improves licorice quality by increasing both yield and glycyrrhizin content.

## **VII. Evaluation of the quality of the scientific works reflecting the dissertation research**

In connection with the procedure, 3 publications in English are presented, which contain results of the dissertation work. They have undergone a peer review process and have been published in reputable scientific journals. One of them was published in a scientific journal, referenced in the worldwide recognized databases Web of Science (Q3) and Scopus (Q2). The other article was published in a journal also referenced by Web of Science (core collection), but without an index. This confirms their significance and scientific value. One of the articles already has 4 citations in quality scientific journals (Q3-Q4).

The doctoral student has presented the results of her research at 7 international scientific forums - 3 abroad and 4 in Bulgaria.

## **VIII. A motivated answer to the question to what extent the dissertation research is primarily the personal work of the doctoral student**

Doctoral student Asya Kojuharova has acquired the necessary knowledge in the relevant scientific field - research into the possibilities of conservation and cultivation of bare licorice (*Glycyrrhiza glabra* L.), Fabaceae through biotechnological approaches. The PhD student has conducted a large-scale interdisciplinary scientific study of the possibilities of conservation and cultivation of bare licorice (*Glycyrrhiza glabra* L.). She has mastered many and varied methods of research, with which she has fulfilled one of the main goals of the educational and scientific degree "Doctor".

Based on her educational program, the student has covered study material from specialized courses that correspond to the required 130 credits. The doctoral student meets the requirements set by the Training Center at BAS for the approval of the implementation of the scientific program, presentation of the results on the topic of the dissertation in publications and at scientific forums, and has collected a total of 462 credits, with a mandatory minimum of 250.

## CONCLUSION

On the basis of the various research methods learned and applied by the doctoral student, the correctly conducted experiments, the generalizations and conclusions made, I believe that the presented dissertation represents an original contribution to science, meets the requirements of the ZASRB and the Regulations for the conditions and procedures for acquiring scientific degrees and for occupying academic positions at the Institute of Biodiversity and Ecosystem Research at the Bulgarian Academy of Sciences, which gives me reason to rate him **POSITIVELY**.

I allow myself to propose to the honorable Scientific Jury to also vote positively and award **Asya Pavlova Kozhuharova** the educational and scientific degree "**Doctor**" (PhD) in the field of higher education 4. Natural sciences, mathematics and informatics, professional direction: 4.3. Biological Sciences, scientific specialty "Botany".

16.11.2023

Plovdiv

Reviewer: .....

(Prof. Lilyana Nacheva, PhD)