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

Asya Pavlova Kozhuharova

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Summary

Glycyrrhiza glabra L. (Licorice) is a perennial plant species from the family Fabaceae used in both traditional and official medicine for treatment of many diseases, among them cough, chronic hepatitis, ulcers, psoriasis, and as sweetener of many drugs. Its healing properties are due to the roots (Radix Glycyrrhizae) of 3-year or older plants. Licorice is distributed in South and East Europe and South-West Asia, and it is naturalized in some places in South-West Europe. In Bulgaria the area of *G. glabra* localities has decreased significantly due to their overexploitation in the past. The species has been assessed and categorized as "endangered" in the Red List of Vascular Plants in Bulgaria, it is under strict protection by the Biodiversity Act (2002), and was included in the Red Data Book of Bulgaria (2015).

The objective of the present PhD thesis was the experimental determination of the appropriate conditions for effective *in vitro* cultivation and *ex vitro* adaptation of *G. glabra* and analysis of its main biologically active substances: glycyrrhizin and total flavonoids, in the wild-growing plants from the Bulgarian populations and in 3-year-old *ex situ* cultivated plants originating from the wild ones.

The recent survey together with the regional authorities of the Ministry of environment and waters (MEW) confirmed the existence of only 3 licorice populations, situated in protected areas near to the villages Dolni Vit and Koilovtsi (Pleven district) and Beltsov (Russe district). In 2018, the locality of *G. glabra* near the village of Baikal, considered extinct, was also confirmed. The plant material for research was collected from all the localities in limited quantities with a special permission of MEW. *In vitro* cultures were initiated from seeds; stolon cuttings were used for vegetative reproduction of plants and establishment of an *ex situ* collection at the field plot of IBER, while root segments were used for phytochemical analyses. In addition, root segments originating from commercial plantations in Ukraine and Uzbekistan were used as referent samples for comparative analyses.

The seed viability of the three studied Bulgarian populations was similar: 40% for Beltsov, 46% for Koilovtsi and 48% for Dolni Vit. Seed dormancy was found to be combined: physical and physiological (PY + PD). Physiological dormancy was overcome by storing seeds at room temperature for several months, and physical dormancy was overcome by 10 times successive immersion in ice and boiling water for 5 s. with stratification being much more efficient under *in vitro* than *in vivo* conditions (66.0% and 13.3% germinated seeds, respectively, for Beltsov origin).

G. glabra is a slow-growing species *in vitro*. It was established that the biological feature of the species, related to the drying of the old shoots when new ones appear the following year, is preserved in *in vitro* conditions. In this regard, the most effective method for its cultivation was clonal micropropagation by sub-culturing the *in vitro* plant stem and its branches and

using the internodes as secondary explants. The most suitable of the nutrient media tested, selected on the base of qualitative and quantitative indicators (stem branching, rooting, presence of callus, number of new shoots per explant, etc.) was MS agar-solidified medium containing 1 mg/l active charcoal and supplemented with 1 mg/l Kin and 0.5 mg/l IBA (between 4 and 6 shoots per explant over a period of 4 months for the different origins).

In vitro obtained licorice plants were successfully *ex vitro* adapted first in a growth chamber (42.2% survival from 185 plants), then in a room phytotron (62.8% survival), and acclimated in an unheated greenhouse rates (87.8% survival). Plants transferred to the experimental field plot are growing forming underground stolons and new shoots.

In 2017, an *ex situ* collection of licorice has been created at the experimental field plot consisting of plants propagated from stolon cuttings originating from 3 Bulgarian populations of the species: Dolni Vit, Koilovtsi and Beltsov. Rapid growth and development of numerous stolons and shoots around the initially propagated plants was noted, especially for Dolni Vit origin where the shoots appear at a distance of more than 5 m from the first plant.

Data on glycyrrhizin content in known Bulgarian populations have been updated thanks to modern chromatographic methods (HPLC). The wild-growing plants from the four Bulgarian localities of licorice differed significantly in glycyrrhizin content ($P < 0.001$). The richest Bulgarian population in glycyrrhizin is the one near Beltsov village (29.6 ± 2.3 mg/g DW during fruiting and 39.4 ± 7.5 mg/g DW in the flowering phase), which is comparable to the reference origin from Uzbekistan and richer than the one from Ukraine (both with commercial importance). The content of total flavonoids was similar in the four Bulgarian populations during flowering, while in the fruiting phase there were significant differences ($P < 0.001$). An interaction of the factors origin and phenophase was found for both glycyrrhizin and total flavonoid content in plants growing in the wild localities ($P < 0.001$).

It was established that the content of glycyrrhizin and total flavonoids in plants propagated vegetatively from cuttings of stolons of different origins (Dolni Vit, Koilovtsi and Beltsov) 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 under *ex situ* conditions in plants of Dolni Vit origin compared to that under *in situ* conditions ($P < 0.01$). The analysis of soil samples from the four Bulgarian localities and from the field plot showed significant differences in the physical and chemical characteristics of the sample from Dolni Vit compared to all other samples: predominance of larger soil particles; lower content of total nitrogen, humus and absorbable potassium, etc. This could explain 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 the leveling of their values with those in the *ex situ* cultivated plants from Beltsov and Koilovtsi. In all three origins the glycyrrhizin content meets the requirements of the Japanese Pharmacopoeia for a minimum of 2.5% in the dry mass. In order to select the most appropriate starting plant material when establishing a licorice plantation, it is necessary to clarify the relative influence of plant origin and soil characteristics on the accumulation of glycyrrhizin and total flavonoids in roots. For this purpose, both genetic researches on the Bulgarian licorice populations and cultivation of plants with the same genotype on soils of different types are necessary.